final report

Project code: P.PSH.1351

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Date published: 19-Apr-2023

PUBLISHED BY

Meat and Livestock Australia Limited

Locked Bag 1961

NORTH SYDNEY NSW 2059

Efficacy and safety of Asparagopsis extract in a canola oil carrier for feedlot cattle

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

P.PSH.1351 - Efficacy and safety of Asparagopsis extract in a canola oil carrier for feedlot cattle

Abstract

Red seaweed, *Asparagopsis taxiformis* (*Asparagopsis*), has been shown to be highly effective at inhibiting the production of methane (CH_4) in ruminants. An alternative to feeding whole, freeze-dried *Asparagopsis* is extracting the bioactive compounds in vegetable oil, and feeding this oil directly to ruminants. This project measured the CH_4 reduction potential and safety of *Asparagopsis* extract in oil (Asp-Oil) in a trial with 20 Angus heifers, fed feedlot diets containing one of 3 levels of Asp-Oil to deliver the corresponding bromoform inclusion levels or a control oil with no bromoform. Compared to the control, the bromoform inclusion levels of 17, 34, and 51 mg/kg (Low, Med, High) reduced CH_4 yield (g CH_4/kg DMI) by 54.5, 85.2, and 95.0%, respectively. There were no effects on animal production or carcase characteristics. There were no impacts on animal health, welfare or rumen function, when the whole feeding period was considered. Carcase samples contained levels of iodine and bromide that were either not different compared to the control or were safe for human consumption, and there was no bromoform detected in any carcase samples. Overall, Asp-Oil was found to be effective for reduction of CH4 emissions and safe for animals and consumers of meat and edible offal.

Executive Summary

Asparagopsis taxiformis (*Asparagopsis*) has been shown to be highly efficacious at inhibiting the production of methane (CH_4) in ruminants. To date, *Asparagopsis* has been produced as a dietary supplement by freeze-drying to retain the volatile bioactive compound bromoform ($CHBr_3$) in the product. Extraction of *Asparagopsis* bioactive compounds in a vegetable oil solvent (Asp-Oil) is an alternative method of stabilising *Asparagopsis* as a ruminant feed additive. For Asp-Oil to be further commercialised and incorporated into National Greenhouse Gas Inventory methods, it must be demonstrated to be safe (for animals and consumers of product) and efficacious at inhibiting CH_4 production.

The aim of this project was to provide critical knowledge on the CH_4 reduction efficacy and safety of Asp-Oil, and elucidate the minimum effective inclusion level in feedlot diets. The objectives of this project were to determine the effect of increasing inclusion levels of an *Asparagopsis* extract in a canola oil carrier (Asp-Oil) on:

(1) Enteric CH_4 production of feedlot cattle (using respiration chambers as the emissions monitoring technique).
(2) Rumen temperature, rumen pH and redox potential, rumen volatile fatty acids and ammonia, thyroid hormone production (T_3 & T_4), blood haematology, blood iodine & bromine, haptoglobin and cortisol.
(3) Gross rumen pathology at slaughter as assessed by trained veterinary pathologists. Histopathology on all trial animals.
(4) Concentrations of bromoform, iodide and bromide in carcase and offal depots.
(5) Shear force and MSA sensory evaluation of striploins.
(6) Inform industry partners regarding the food safety aspects of feeding Asparagopsis oil to cattle.

The dose-response experimental design used 4 Asp-Oils of increasing levels of bromoform ($CHBr_3$) content (including zero $CHBr_3$ canola oil control) in a tempered-barley based feedlot ration, fed for 81 days to 20 Angus heifers (5 replicates per treatment). The diet and feeding regime were based on commercial practice in the Australian grain-fed cattle industry. On 7 occasions, liveweight and CH_4 emissions were measured, and blood, rumen fluid and faecal samples were collected. At the end of the experiment, all animals were slaughtered, with carcases graded for MSA, and samples of meat and edible offal collected for testing of shear force, shelf life, consumer sensory and residues of bromoform, bromide and iodine.

All Asp-Oil treatments reduced CH_4 yield (g CH_4/kg DMI) from Control levels, with the Low, Medium and High Asp-Oils (which provided 17, 34 and 51 mg $CHBr_3$/kg DMI, respectively) achieving 54.5, 85.2 and 95.0 % reduction , respectively. There was no effect of Asp-Oil treatment on rumen temperature, pH, redox potential, VFA and ammonia production, rumen pathology and histopathology over the whole feeding period. There were no differences in animal production and carcase parameters. There was no detectable $CHBr_3$ in faeces or any carcase samples, and iodine and bromide residues in kidneys were at levels unlikely to lead to consumers exceeding recommended maximum intakes.

Overall, Asp-Oil was found to be safe for animals and consumers of meat, and effective at reducing CH_4 emissions and yield by up to 99% within the inclusion levels tested.

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1 Background

Asparagopsis taxiformis (*Asparagopsis*) has been shown to be highly efficacious at inhibiting the production of methane (CH_4) in ruminants. To date, Asparagopsis has been produced as a dietary supplement by freeze-drying (FD-Asp) to retain the volatile bioactive compound bromoform ($CHBr_3$) in the product. Freeze drying is an option, however is energy-intensive and logistically challenging to scale-up (Magnusson et al. 2020). Magnusson *et al.* (2020) developed a method to extract and stabilise $CHBr_3$ from *Asparagopsis* biomass in a vegetable oil solvent (Asp-Oil). $CHBr_3$ concentration of this product was stable at room temperature for at least 12 weeks. Since vegetable oils are already widely used in feedlot diets in Australia, this development has potential for ready adoption of *Asparagopsis* in grain-fed beef production. For Asp-Oil to be further commercialised and incorporated into National Greenhouse Gas Inventory methods, it must be demonstrated to be safe (for animals and consumers of product) and efficacious at inhibiting CH_4 production.

Volatility of $CHBr_3$ may be a concern over time with FD-Asp and recent work has been done on the shelf-life of both FD-asp and Asp-Oil products under a variety of storage conditions (Tan *et al*. 2022). The study indicates that Asp-Oil demonstrates improved stability under challenging storage conditions, and typical of degradable products, the key to longevity is to keep the products in a sealed container. Accordingly, *Asparagopsis* researchers and producers continue to explore alternative processing methods that result in a stable, high $CHBr_3$ product, that is readily incorporated into ruminant diets.

A recent study where freeze-dried Asparagopsis was fed to dairy cows (Muizelaar et al. 2021) raised concerns about the safety of this product when the rumen walls of two low-Asparagopsis-intake animals showed abnormalities. Unfortunately, the experimental design of Muizelaar et al. (2021) was based on small numbers of animals, unbalanced across treatments and did not include the provision of a negative control. Given previous research has reported nodular proliferation, discolouration and papillae shape changes in sheep fed Asparagopsis (Li et al. 2018), there is a need to establish whether Asp-Oil causes damage to the rumen, or to other biological functions as a result of primary ($CHBr_3$) or secondary bioactive compounds in the product when fed at effective levels for CH4 abatement. The US EPA (2017) has suggested a reference dose for bromoform, an estimated level of daily oral exposure without negative effects, to be 0.02 mg/kg BW/day for human consumption. Previous research where freeze-dried Asparagopsis has been fed to ruminants has shown no accumulation of $CHBr_3$ in animal tissue or edible offal (Kinley et al. 2020; Muizelaar et al. 2021; Roque et al. 2021), and it is essential that this is confirmed for Asp-Oil also, before it is commercialised for beef production.

Guidelines for safe human consumption of $CHBr_3$ estimate 17.9 (WHO 2004) – 20 µg $CHBr_3$/ kg body weight (BW, US EPA (2017)) as the a safe level of oral exposure (oral reference dose). Although the US Centre for Disease Control and Prevention consider that $CHBr_3$ is not found in food, when feeding $CHBr_3$-containing feedstuffs to animals, it is important to demonstrate whether or not there is potential to transfer $CHBr_3$ into the human food chain (Vaskoska 2021).

The aim of this project was to provide critical knowledge on the CH_4 reduction efficacy and safety of Asp-Oil, and elucidate effective inclusion levels in feedlot diets. A fully controlled study with 20 Angus heifers in individual pens allowed for precision measurement of dry

matter intake (DMI) and employed gold-standard respiration chamber measurement of emissions from individual animals. Detailed assessment of meat quality, residues in meat and edible offal, and rumen necropsy contributed to information on the safety of Asp-Oil in feedlot diets for animals and consumers.

2 Project Objectives

The objectives of this project were to determine the effect of an *Asparagopsis* extract in a canola oil carrier (Asp-Oil) on:

(1) Enteric CH_4 production of feedlot cattle (using respiration chambers as the emissions monitoring technique).
(2) Rumen temperature, rumen pH and redox potential, rumen volatile fatty acids and ammonia, thyroid hormone production (T_3 & T_4), blood haematology, blood iodine & bromine, haptoglobin and cortisol.
(3) Gross rumen pathology at slaughter as assessed by trained veterinary pathologists. Histopathology on all trial animals.
(4) Concentrations of bromoform, iodide and bromide in carcase and offal depots.
(5) Shear force and MSA sensory evaluation of striploins.
(6) Inform industry partners regarding the food safety aspects of feeding Asp-Oil to cattle.

3 Methodology

3.1 Experimental design, diets and animals

Canola oil containing increasing levels of *Asparagopsis* extract (Asp-Oil) was tested in a dose-response experimental design, using Asp-Oils of 4 levels of $CHBr_3$ content (including zero $CHBr_3$ canola oil control) in a tempered-barley based feedlot ration, fed for 81 days. Each oil was tested in 5 *Bos taurus* Angus heifers from a single sire (total 20 animals, in 2 blocks of 10 (high and low initial liveweight), Figure 1). All procedures were approved by the Animal Ethics Committee of the University of New England (Animal Research Authority number ARA21-106).

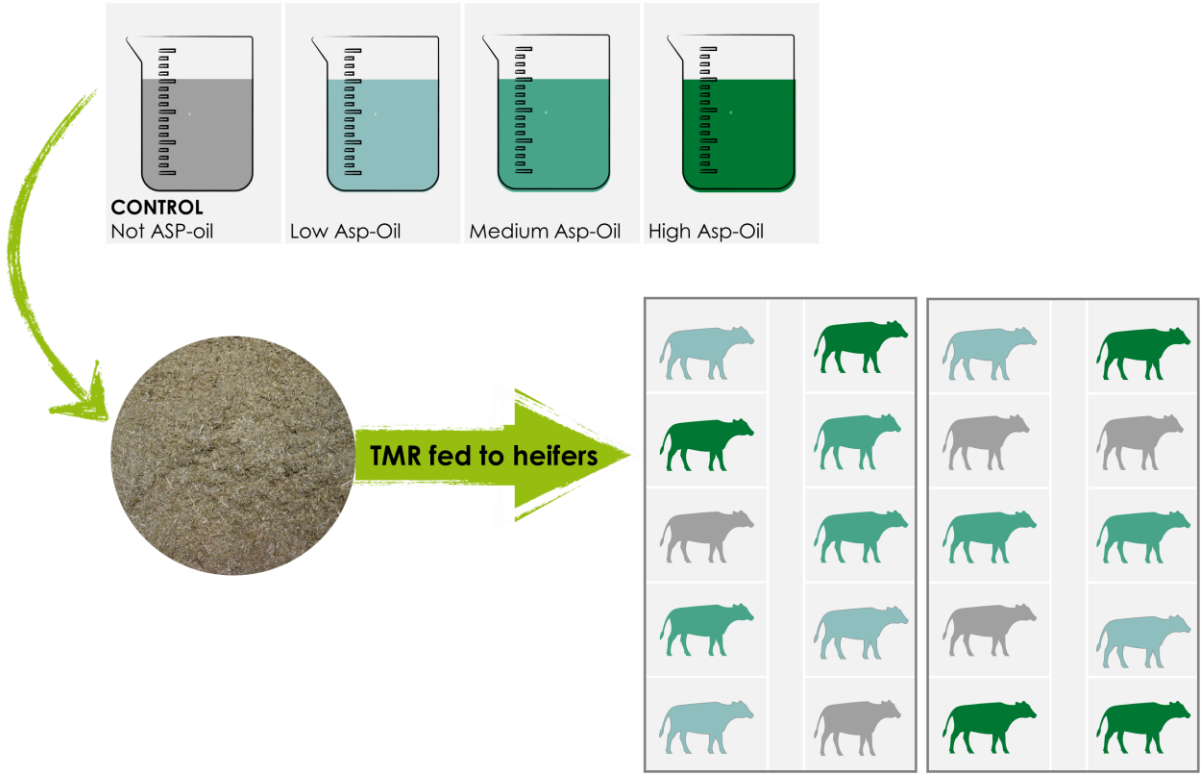


Figure 1. Experimental design. Treatments: 4 blends of Asp-Oil with increasing levels of bromoform; Blocks: n=2 (high and low initial weight); replicates = 5 Angus heifers/Treatment.

The treatments were 4 blends of oil containing a stock Asp-Oil of *Asparagopsis* extract in a canola oil carrier (Sea Feed, 3.58 mg $CHBr_3$ /kg oil DM, supplied by Sea Forest Ltd, Triabunna, Tasmania) and a blank canola oil mixer, blended to achieve formulated $CHBr_3$ contents of 2.4 mg/kg oil DM (High Asp-oil), 1.6 mg/kg oil DM (Medium Asp-oil), 0.8 mg/kg oil DM (Low Asp-oil), and 0 mg/kg oil DM (Control). These oils were stored at 4°C until mixing into the ration (3 times per week).

The diet and feeding regime were based on commercial practice in the Australian grain-fed cattle industry. A 22-day transition period increased barley content to 80 % DM and total dietary fat to 5.0 % DM in the finisher diet (Table 1) in 4 equal increments, to achieve a target finisher $CHBr_3$ content of 0, 17, 34, and 51 mg/kg diet DM in Control, Low Asp-Oil, Medium Asp-Oil, and High Asp-Oil treatments, respectively (Table 2). The diet ingredients were initially mixed as a single batch without any oil, and then the treatment oils were added separately to the diet for each treatment group. Mixing time required for homogenisation of the treatment oils through the diet was established by testing the mixing time required to achieve a CV < 5 % for dietary ether extract content for 10 grab-samples.

Throughout the experimental period, the heifers were housed indoors in individual pens, and fed *ad libitum* once daily (at 0900 h for Block 1 and 0930 h for Block 2). The Block 2 heifers commenced the experiment 1 day after Block 1, to enable sampling to occur on the same experimental day in both blocks. Feed offered and refusals were recorded daily, and sampled for DM, and feed on offer adjusted to target a 5% refusal rate. All heifers had *ad libitum* access to clean, fresh water. Grab samples were collected from each mixer load of the main diet and bulked weekly for nutrient analysis by wet chemistry (Table 1).

Table 1. Composition of feedlot total mixed rations feed to Angus heifers during the 81-d feeding period.

	Diet (feeding days)[1]			
Item	Starter (0-7)	Intermediate I (8-15)	Intermediate II (16-22)	Finisher (23-81)
Ingredient, % as Fed				
Tempered Barley	37.4	50.7	66.9	81.0
Cereal hay	8.1	6.0	3.6	-
Oaten Chaff	16.2	12.0	7.3	5.5
Whole cottonseed	13.6	10.6	9.7	5.5
Mill run	12.4	9.9	3.4	-
Canola Oil Blend	0.4	0.9	1.3	1.7
Liquid Supplement	6.3	6.3	6.3	6.3
Analysed nutrient composition (DM-basis)[2]				
Dry Matter (%)	80.2	81.3	81.5	80.1
Organic Matter, % DM	93.0	94.0	94.0	94.8
Ash, % DM	7.0	6.0	6.0	5.3
Crude Protein, % DM	12.9	13.1	11.8	11.4
Fat, % DM	4.6	4.6	4.7	5.2
TDN, % DM	71.4	77.6	80.4	81.1
NDF, % DM	42.0	33.8	28.5	28.0
Starch, % DM	22.3	37.0	44.3	46.0
ME, Mcal/kg DM	2.71	2.98	3.11	3.14
NE_m, Mcal/kg DM	1.68	1.81	1.95	2.07
NE_g, Mcal/kg DM	1.07	1.19	1.31	1.42
Ca, % DM	1.18	0.88	0.97	1.05
P, % DM	0.43	0.45	0.41	0.31
Monensin (as formulated), ppm	25	25	25	25

[1] Diets were formulated using Concept 5 software. [2] TDN = total digestible nutrients; NDF = neutral detergent fiber; ME = metabolizable energy; NE_m = Net energy for maintenance; NE_g = Net energy for gain.

Table 2. Formulated bromoform ($CHBr_3$) inclusion (mg/kg DM) in experimental diets

	Treatment			
Experiment days	Control	Low Asp-Oil	Medium Asp-Oil	High Asp-Oil
Adaptation period				
1-7 (Starter)	0	4.2	8.5	12.7
8-15 (Intermediate I)	0	8.5	17.0	25.5
16-22 (Intermediate II)	0	12.7	25.5	38.2
Finisher period				
23-81	0	16.9	34.0	51.0

3.2 Measurements of methane production

CH_4 production was measured by confining each heifer in an individual open circuit respiration chamber (Hegarty *et al.* 2012) for 23 hours on days 6 (starter diet), 14 (first intermediate diet), and 21 (second intermediate diet) in the adaptation period, and on days 34, 48, 62 and 76 in the finisher period (Figure 2). Production of CH_4, O_2, and CO_2 was measured every 10 minutes, and CH_4 production data was corrected for recovery (mean 97.5 ± 2.05%) of a known quantity of pure CH_4.

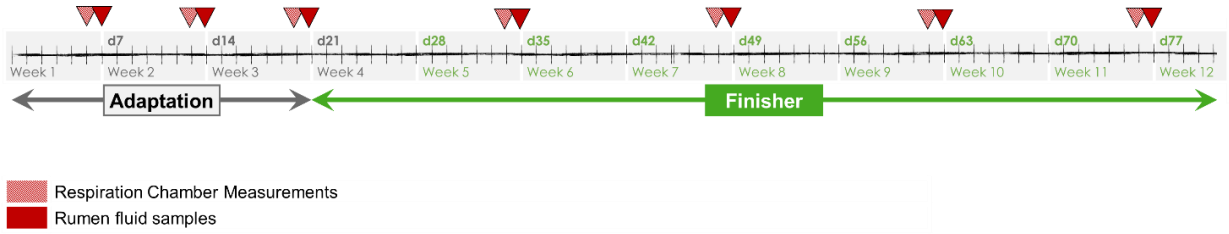


Figure 2. Timeline of experiment indicating 7 sampling periods for methane and animal measures.

3.3 Other animal samples and measures

Pre-feeding body weight was measured on day 0, and immediately before the cattle entered the respiration chambers on days 6, 14, 22, 35, 48, 62 and 76 using a single calibrated animal weighing box (Ruddweigh 600mm Weigh Beam 2000kg weighing capacity, Ruddweigh, Guyra, NSW, Australia) After the cattle left the respiration chambers, they were returned to their individual pens, fed, and 3 hours after feeding were weighed for a post-feeding measure of liveweight on days 7, 15, 23, 36, 49 and 77. At the same time, samples of rumen fluid, faeces and blood were collected. Rumen fluid was sampled by oral intubation, tested for pH (EcoScan Portable pH/ORP meter with TPS pH Sensor) and reduction potential (Mettler Toldeo SevenEasy S20 pH meter with TPS Intermediate Junction Redox Sensor) immediately after sampling, and then subsampled for measurement of volatile fatty acid (VFA) profiles, rumen ammonia, and rumen protozoa enumeration.

A venous whole blood sample was analysed total leucocytes, neutrophils and lymphocytes (Abbott-Cell-Dyn Counter 3700, Abbott Diagnostic Division, Vienna, Austria). A serum sample of venous blood was analysed for haptoglobin, triiodothyronine (T3), thyroxine (T4). A single sample of serum, pooled from all finisher samples for each animal was analysed for iodine and bromide. A faecal sample was collected from the rectum, and analysed for faecal cortisol metabolites (all sampling days); and faecal starch, $CHBr_3$, bromide and iodide content (days 35 – 77 only). Rumen temperature was monitored continuously using smaXtec rumen boluses (Bolus TX-1442A, smaXtec animal care GmbH, Austria).

3.4 Slaughter and carcase measurements

All cattle were transported to slaughter on the same day (day 81 (block 1), day 80 (block 2)). The abattoir was 450 km from the experimental facility. The heifers were lairaged overnight and slaughtered in treatment groups. Immediately post slaughter and dressing, hot standard carcass weight (HSCW, kg) was determined according to AUS-MEAT carcass standards (AUS-MEAT Limited 2005). Post-chilling, carcasses were evaluated by two independent accredited Meat Standards Australia (MSA) graders (Meat Standards Australia 2007) for hump height (mm); fat colour; meat colour; MSA marbling score; rib fat depth (mm); ossification score; ultimate pH (pHu); eye muscle area (EMA, cm^2). Hump heights were measured using a 5 mm graduated metal ruler(Meat Standards Australia 2007). Fat colours were scored against the AUS-MEAT fat colour reference standards against the intermuscular fat positioned laterally to the rib eye muscle using a 0 (white) to 9 (deep yellow) scale (AUS-MEAT Limited 2005). Meat colour was scored using the AUS-MEAT colour reference standards on the bloomed rib eye muscle (longissimus thoracis et lumborum) using a 1 (light pink) to 7 (deep purple) scale (AUS-MEAT Limited 2005). MSA

marbling score was evaluated in chilled carcases against MSA reference standards at the quartering site and was estimated by scoring the amount and distribution of intramuscular fat deposited between individual fibres, on a scale ranging from 100 to 1100 (Romans and Ziegler 1985; AUS-MEAT Limited 2005; Meat Standards Australia 2007). Rib fat depth was measured using a graduated metal ruler, at the quartering site positioned between the 12th and 13th rib (AUS-MEAT Limited 2005). Ossification score was determined a scale between 100 and 590 in accordance with the guidelines described by the United States Department of Agriculture (Romans and Ziegler 1985). Ultimate pH and loin temperature were measured in the rib eye muscle (longissimus thoracis et lumborum) at the time of carcase grading. Carcass temperature and pH were measured using an MSA approved temperature and pH probes (TPS MC-80 or TPS WP-80M pH Meter, TPS Pty Ltd., Springwood, Brisbane, Qld, 4127, Australia). Eye muscle area was determined for each carcase by measuring the longissimus thoracis et lumborum at the quartering site using a standardised grid (AUS-MEAT Limited 2005). Carcase grading data was then used to calculate an MSA index value as described by McGilchrist *et al.* (2019) to estimate the predicted eating quality of each carcase.

The rumen was collected immediately post-slaughter, cleaned and scored for gross pathology for papillae colour and shape and damage to the ventral sac of the rumen by a trained veterinary pathologist (Jonsson *et al.* (2020), Table 3). A sample of normal and lesioned (where evidenced) rumen wall was taken for histological analysis (Jonsson *et al.* 2020).

Table 3. Subjective, categorical scoring system for gross pathology in the ventral sac of the rumen of cattle at post mortem examination (Jonsson et al. 2020).

Feature	Score	Description
Papillae Colour		
0	A	Black/Brown
1	B	Grey/Brown
2	C	Grey/Brown small area with pink tips
3	D	Grey/Brown small area with pink tips
4	E	Pink
5	F	Yellow
Papillae Shape		
0	A	Long + Thin
1	B	Long + Oval
2	C	Short + Thin
3	D	Short + Oval
4	E	Short + Brittle
Ventral Sac		
0	A	No evidence of any damage
1	B	Small areas bare of papillae
2	C	Large areas bare of papillae
3	D	Small areas of excoriation/scaring
4	E	Red/Bloody areas
5	F	Parakeratosis

Shelf-life was evaluated by microbial analysis, visual evaluation of raw product under UV, cooked product evaluation and packaging shelf life evaluation every 10 days for 90 days (shelf-life target), using separate samples of striploin from each carcase on each test day. Organolepetic evaluation of uncooked striploin samples tested for colour, smell, texture, and purge, while the cooked samples were evaluated for appearance, aroma, flavour, mouthfeel,

tenderness, juiciness, overall acceptability and packaging acceptability by a single assessor on each day. Enumeration of enteromesophilic bacterial colonies growing under aerobic conditions (standard plate counts, SPC) was conducted on each test day according to AOAC methods 990.12 for SPC and 998.08 for *E. coli*.

3.5 Consumer sensory analysis of eating quality

At boning, portions (from the anterior end) of the striploin (*M. longissimus dorsi lumborum)* were cut and vacuum packed for sensory testing, and stored at 1 to 3 ° C until fabrication of test steaks. Left and right carcass sides were rotated for sensory selection to eliminate a side effect. An additional 25 mm block was also taken from the striploin of the alternate side to allow for “Links” to be made. 25 mm slices were fabricated to MSA specified grill samples of approximately 38 x 65 mm. Five sample steaks were prepared from each sample striploin, vacuum sealed, chilled, and stored at -20 ° C until sensory testing. Shear force samples were also fabricated from the posterior end of the sensory striploin (P3) to ensure that results were taken in between sensory samples.

In accordance with MSA protocols (Anonymous 2008; Watson *et al.* 2008), samples were then allocated to “Picks”, a set of 42 samples to be evaluated by 60 untrained consumers with 10 consumers evaluating each of the 42 samples and all consumers being served 7 samples. The first sample served was a presumed mid-eating quality “Link” followed by 6 test samples. The test samples were served in accordance with a 6 x 6 Latin Square design that ensured that each sample was served in 5 different presentational order positions and within 5 subsets of 12 consumers. Further, each of the 6 were assigned from different “products”, in this case striploins grouped on the basis of expected quality from low to high with each product served an equal number of times in each order position and before and after each other product. Consumers in each pick ate a control sample, 3 treated samples (Low, Medium and High Asp-Oil), a perceived low-quality striploin from Brahman cattle and a perceived high-quality striploin from Angus cattle. One position from every animal was eaten in each pick and every animal was eaten in all 3 picks with position rotated across the picks, to ensure a balanced pick design. To ensure integrity, sample codes were “blind” to the sensory organisation with sensory data only connected to animal, grading and genomic data after receipt of the consumer results.

The MSA consumer taste panel used untrained consumers to score meat samples for tenderness, juiciness, like flavour and overall acceptability. While the consumers were untrained, they were screened to include only people who preferred steak cooked to medium doneness, ate beef at least once a fortnight and were aged between 18 and 70 years old.

Frozen steaks were thawed and then cooked in accordance to the MSA Grill cooking sheet to achieve a medium degree of doneness using a double-sided clam shell SilexTM S-Tronic 161K grill. After cooking, steaks were rested, halved and served to untrained consumers where samples were scored using a 100 mm scale for tenderness anchored by the words not tender/very tender, juiciness not juicy/very juicy and dislike extremely/like extremely for both like flavour and overall acceptability. The MSA consumer panel also rated the samples for eating quality based on the following scoring system: unsatisfactory (2 star), good everyday (3 star), better than everyday (4 star) with the premium eating quality (5 star). The four sensory scores were weighted to provide a single meat quality score (MQ4) based on a linear discriminant function to provide the best allocation of samples to the four quality

grades. The weightings were 0.3, 0.1, 0.3 and 0.3 for tenderness, juiciness, like flavour and overall acceptability, respectively. Each sample had 10 consumer scores for each sensory trait. The highest and lowest two scores were 'clipped' to remove outliers and the middle six averaged to produce the clipped mean scores for the sensory traits that were used in the database for sensory analysis with all 10 retained for consumer analysis.

3.6 Statistical and Data Analysis

3.6.1 Data processing

All data from two heifers were removed from the analysed dataset due to chronic health problems: Heifer #19 (Control) had a behavioural pattern of rapid meal consumption, which likely caused a chronic, sub-acute acidosis which showed signs of becoming acute (i.e. diarrhoea) from day 52, and on day 67 the decision was made to remove her from the experiment for her welfare. Heifer 76 (High Asp-Oil) maintained low intakes (frequently under 2 kg/day) from the Intermediate I diet onwards and subsequently experienced weight loss, indicating maladaptation to the grain diet, however, she showed no other behavioural signs of acidosis or illness during the experimental period. Heifer 76's rumen pH levels measured between 5.0 – 5.5 during 4 of the 7 measurements, which are levels commonly used to diagnose subacute and acute acidosis (Nagaraja and Lechtenberg, 2007). Rumen ammonia levels from Heifer 76 were also indicative of rumen acidosis (Hernández et al. 2014), with levels significantly higher than the rest of the cohort (156 – 721 mg/L vs 0 – 131 mg/L). At slaughter, the gross morphology of her rumen wall indicated a chronic, sub-acute acidosis. Full data from these animals is provided in Appendix 1.

Dry matter intake was calculated using daily records for fresh weight of feed offered and refusals, weekly dry matter content (sampled at mixing) for each diet for feed offered, and daily samples of refusals for each heifer, bulked weekly. Individual animal measurements of intake were removed from the DMI dataset on days where faecal contamination of the feed trough overnight caused refusals to exceed 1 kg fresh weight.

Liveweight gain was calculated in two ways: firstly, by the slope of the linear regression of pre-feeding liveweight measurement (days 0 – 77) (see statistical analysis, below), and secondly, arithmetically, by dividing pre-feeding liveweight change in a period by the time between measures of liveweight. Gain:Feed was calculated as follows:

$$GF_{ij} = LW_{ij} - LW_{i(j-1)} / \sum DMI_{(j-1\ldots j)}$$

Where
GF_{ij} = Gain:Feed for the i^{th} animal in the j^{th} period
LW_{ij} = liveweight for the i^{th} animal in the j^{th} period
$DMI_{(j-1\ldots j)}$ = dry matter intake for the j^{th} period

Total VFA was calculated by sum of all identified peaks from VFA chromatography.

3.6.2 Statistical analysis

Repeated measures of CH_4, rumen function, blood haematology, and performance were analysed with a mixed model linear regression, with block and animal as random effects, as follows:

$$Y_{ijkl} = \mu + A_j + P_j + AP_{ij} + b_k + e_{ijkl}$$

Where

μ = the overall mean,
A_i = the effect of the i^{th} ASP dose (Control, Low, Medium, High) – orthogonal contrasts
P_j = the effect of the j^{th} sampling day (j = 1, …, 81)
AP_{ij} = the interaction between the i^{th} ASP dose and the j^{th} Day
b_k = the effect of the k^{th} block, and
e_{ijkl} = the random error associated with the l^{th} repetition of the i^{th} ASP dose in the j^{th} sampling period in block k ~ $N(0,\sigma^2_e)$.

Several models of variance-covariance were tested for each response, and the model of best fit chosen by the lowest Akaike Information Criterion value.

Post slaughter measures of carcase performance and residues, and pooled serum residue samples were analysed with a mixed model linear regression with block as a random effect:

$$Y_{ijkl} = \mu + A_j + b_k + e_{ijkl}$$

Longitudinal changes in circadian patterns of rumen temperature were analysed with the package *cosinoRmixedeffects* (Hou *et al.* 2021) to estimate the non-linear parameters midline statistic of rhythm (MESOR, a function that depends on the rhythm-adjusted mean), amplitude (half the extent of variation within a day) and acrophase (the time of overall high values recurring in each day, relative to the overall mean).

All other parametric data were analysed using the *lme4* (Bates *et al.*, 2015) package of R (R Core Team, 2016). Statistical significance was declared at $P < 0.05$, in which case a pairwise comparison was performed using Bonferroni's adjustment.

Counts of protozoa were highly left-skewed, and zero-inflated, and so these were analysed with a Kruskal-Wallis test with Dunn's test for multiple comparisons of groups.

4 Results

4.1 Methane emissions

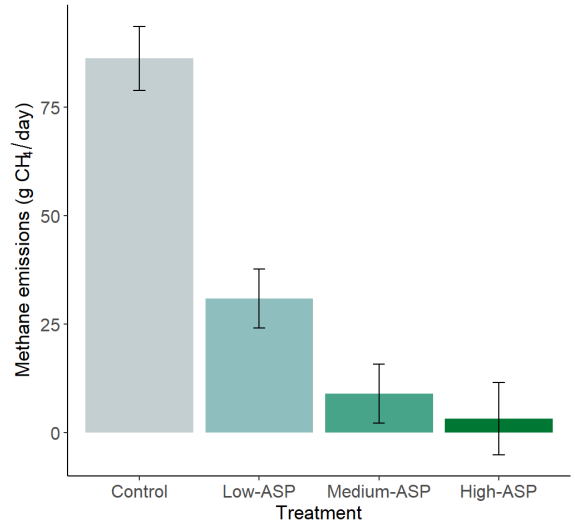
Over the whole feeding period, all Asp-Oils reduced CH_4 production (g CH_4/day) compared to the Control level, by 64.1, 89.7 and 96.3% respectively for the Low, Medium and High Asp-Oils (*P* <0.001, Figure 3, Table 4). A significant treatment by day interaction (*P* = 0.001) was reported for methane production. CH_4 emissions (g CH_4/day) in the Control group of heifers increased over the adaptation period, and into the finisher period (Figure 4), as dry matter intake (DMI) increased. There was no effect of Asp-Oil on day 6 (starter diet), but for all other measurement days, total daily CH_4 emissions of all Asp-Oil groups were significantly lower than for the Control (Figure 4). Over the whole feeding period, CH_4 yield (g CH_4/kg DMI) was reduced by Asp-Oils compared to the Control group (*P* <0.001, Figure 3, Table 4), by 54.5, 85.2, and 95.0 %, by the Low, Medium, High Asp-Oils, respectively. As it was for total emissions, differences in Asp-Oils for CH_4 yield were observed from day 14 onwards (Figure 5).

The High and Medium Asp-Oils groups did not differ in their CH_4 production or yield during the finisher period (Figure 4 & 5; Table 5 & 6), and both achieved maximal inhibition, so that by day 14, CH_4 production and yield did not differ from 0. The Low Asp-Oil demonstrated an increase in CH_4 emission and yield after day 34 of the experiment, such that by day 78 it did not differ from the Control treatment (Figure 4, *P* = 0.371, for total CH4; and Figure 5, *P* = 0.330, for CH_4 yield).

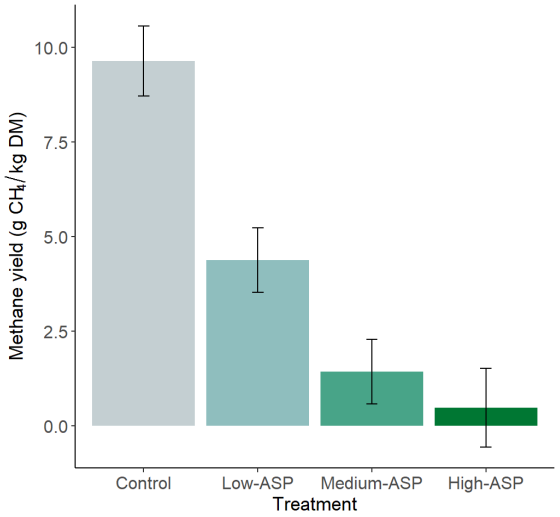
Table 4. Methane (CH_4) emissions, intake (DMI) and liveweight (LW) gain of Angus heifers (least squares mean ± s.e.) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the seven measurements over the whole feeding period (day 0 to 77).

	Asp-Oil Treatment[1]				*P*-value[2]		
Item[4]	Control	Low	Medium	High	T	D	T×D
Total CH_4, g/d	86.29 [b] ± 7.4	30.94 [a] ± 6.8	8.89 [a] ± 6.8	3.16 [a] ± 8.3	0.006	0.3216	0.001
Reduction in total CH_4 from Control	Ref	64.1	89.7	96.3	NA	NA	NA
CH_4 yield, g/kg DMI	9.63 [b] ± 0.9	4.38 [a] ± 0.6	1.43 [a] ± 0.8	0.48 [a] ± 1.0	0.001	0.049	0.235
Reduction in CH_4 yield from Control	Ref	54.5	85.2	95.0	NA	NA	NA
DMI, kg/d	8.90 ± 1.0	8.13 ± 1.0	8.01 ± 1.0	8.87 ± 1.0	0.563	0.000	0.304
DMI, g/kg LW	22.1 ± 1.4	21.0 ± 1.4	20.3 ± 1.4	22.4 ± 1.5	0.526	0.000	0.000
Initial LW[3], kg	346 ± 6.0	346 ± 5.74	344 ± 5.74	336 ± 6.1	0.261	NA	NA
Final LW[4] (d 80/81), kg	462 ± 18.2	461 ± 18.3	441 ± 17.9	443 ± 17.9	0.119	NA	NA
LW gain (kg/day)[3,5]	1.56 ± 0.14	1.36 ± 0.18	1.32 ± 0.18	1.6 ± 0.19	0.412	NA	NA
Average daily gain (kg/day)[3,6]	1.36 ± 0.2	1.24 ± 0.2	1.26 ± 0.2	1.39 ± 0.2	0.945	0.054	0.841
Gain:Feed (kg/kg)[3,7]	0.16 ± 0.0	0.15 ± 0.0	0.154 ± 0.0	0.17 ± 0.0	0.094	0.568	0.874

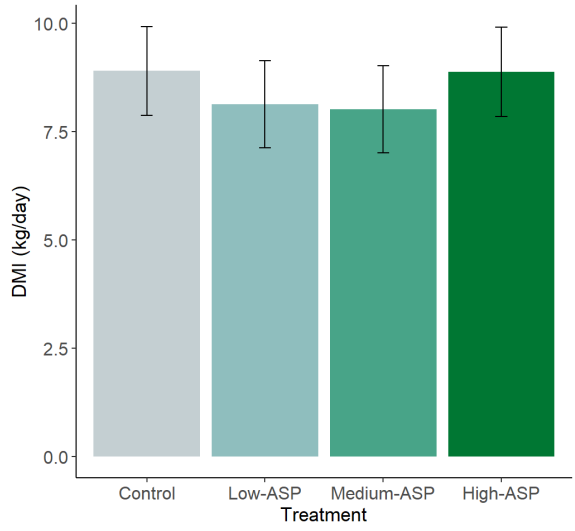
[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4. [2]T: Treatment; D: Day; T×D: Treatment × Day; [3] Liveweight measured pre-feeding; [4]Liveweight measured post-feeding; [5]Liveweight gain (kg/day) determined by regression of liveweight on time (days 0 – 77); [6]Arithmetic mean of average daily gain for 7 measurement periods (days 0 – 77); [5]Mean of period liveweight change/feed intake for 7 measurement periods (days 0 – 77); [a, b]Means within a row showing different superscript letters depict significant main effects of Asp-Oil treatment (alpha = 0.05, Bonferroni-adjusted); CH_4 measured on days 6 (starter diet), 14 (intermediate I diet), and 21 (intermediate II diet) of the adaptation period and finisher diet on days 34, 49, 63 and 77.


Methane emissions (g CH_4/day)
75
50
25
0
Control
Low-ASP
Medium-ASP
High-ASP
Treatment

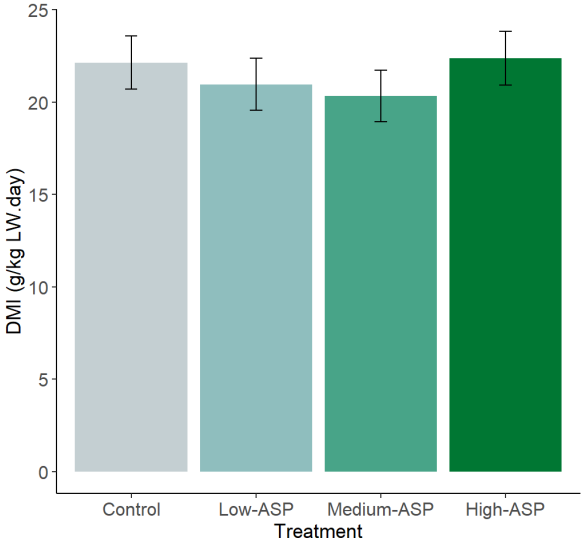
(a)


Methane yield (g CH_4/kg DM)
10.0
7.5
5.0
2.5
0.0
Control
Low-ASP
Medium-ASP
High-ASP
Treatment

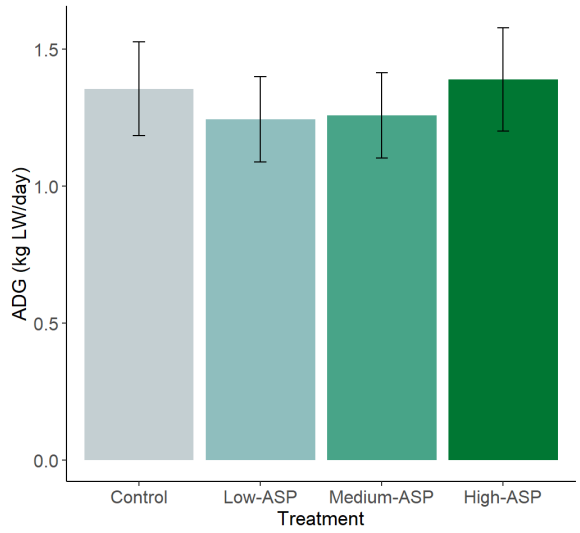
(b)

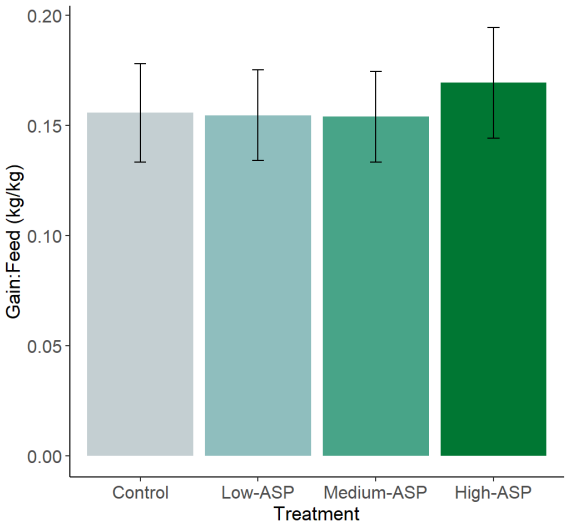

DMI (kg/day)
10.0
7.5
5.0
2.5
0.0
Control
Low-ASP
Medium-ASP
High-ASP
Treatment

(c)


DMI (g/kg LW.day)
25
20
15
10
5
0
Control
Low-ASP
Medium-ASP
High-ASP
Treatment

(d)


ADG (kg LW/day)
1.5
1.0
0.5
0.0
Control
Low-ASP
Medium-ASP
High-ASP
Treatment


Gain:Feed (kg/kg)
0.20
0.15
0.10
0.05
0.00
Control
Low-ASP
Medium-ASP
High-ASP
Treatment

(e) (f)

Figure 3. Methane (CH_4) emissions (a, b), intake (DMI, c, d) average daily gain (ADG, e) and gain:feed (f) of Angus heifers (least squares mean ± s.e.; N = 18) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) over the whole feeding period (day 7 to 77).

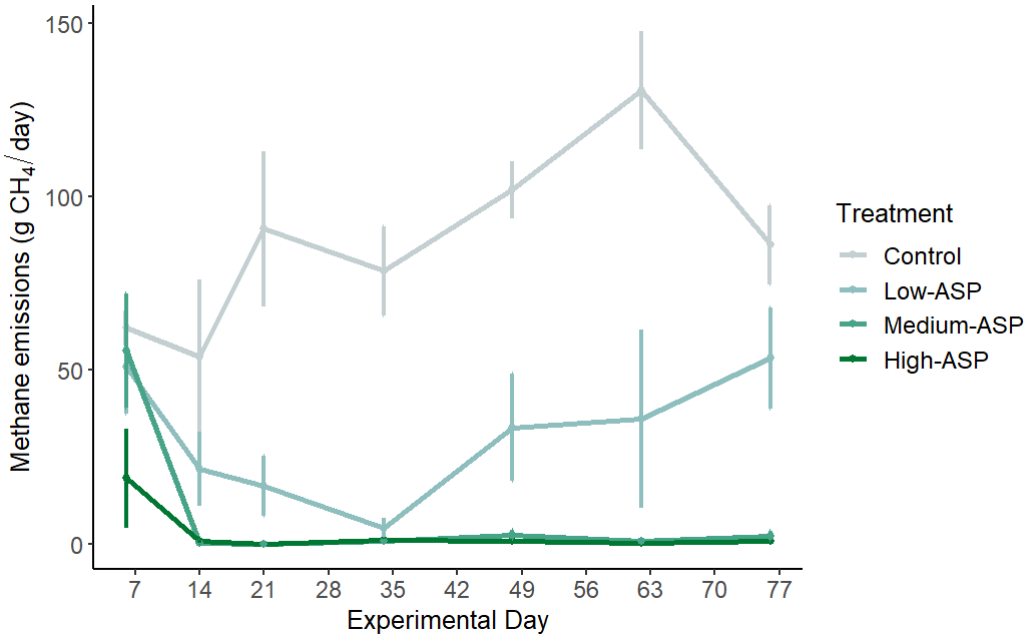


Figure 4. Methane emissions (g CH4 / day) of Angus heifers (observed mean ± s.e.; N = 18) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the seven measurement times during the whole feeding period (day 7 to 77).

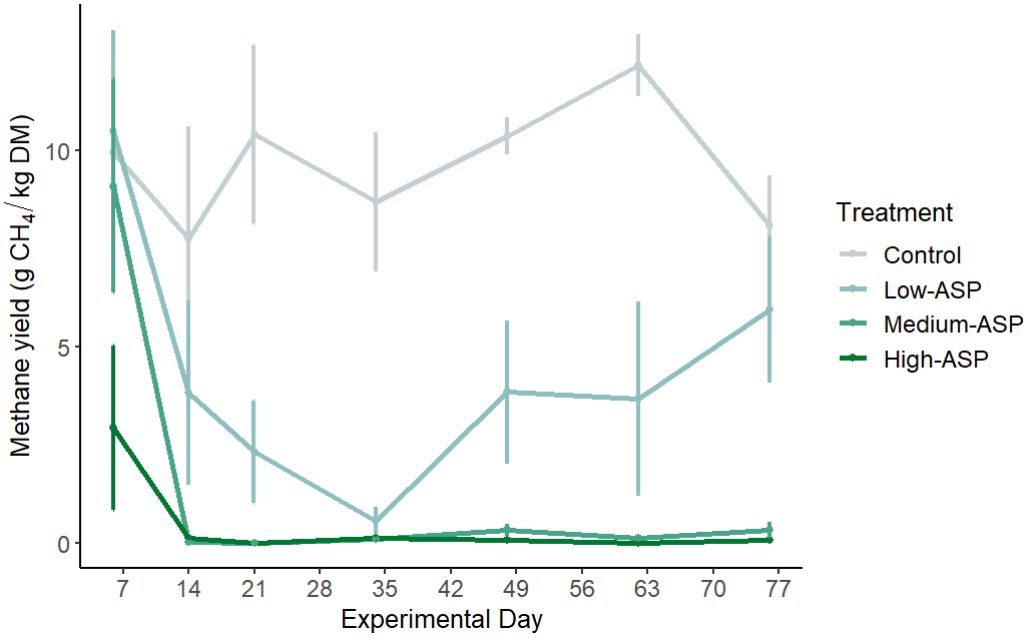


Figure 5. Methane yield (g CH4 / kg DM intake) of Angus heifers (observed mean ± s.e.; N = 18) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the seven measurement times during the whole feeding period (day 7 to 77).

Table 5. Daily methane production (g CH_4/day) of Angus heifers (least squares mean ± s.e.) fed increasing levels of Asparagopsis extract in a canola oil (Asp-Oil) residue on each diet.

	Asp-Oil Treatment[1]						
Diet	Control	Low	Medium	High	*P*[2]		
Adaptation period					T		
Starter	62.20 ± 14.43	50.90 ± 13.24	55.60 ± 13.24	18.90 ± 16.20	0.192		
Intermediate I	53.80 [b] ± 12.06	21.51 [ab] ± 11.07	0.12 [a] ± 11.07	0.62 [ab] ± 13.54	0.020		
Intermediate II	90.75 [b] ± 13.62	15.11 [a] ± 12.84	0.00 [a*] ± 12.84	3.62 [a] ± 14.09	0.000		
					T	D	T×D
Finisher period	99.32[b] ± 8.46	31.91[a] ± 7.76	1.62[a] ± 7.76	0.64[a] ± 9.49	0.002	0.024	0.046

[1]Formulated $CHBr_3$ inclusions in diet DM were (for Starter, Intermediate I, Intermediate II and Finisher, respectively): Control = 0 mg $CHBr_3$/kg DM, n = 4; Low = 4.2, 8.5. 12.7 and 16.9 mg $CHBr_3$/kg DM; n = 5; Medium = 8.5, 17.0, 25.5, and 34.0 mg $CHBr_3$/kg DM, n = 5; High = 12.7, 25.5, 38.2, and 51.0 mg $CHBr_3$/kg DM, n = 4; [2]T: Treatment; D: Day; T×D: Treatment × Day interaction; [a,b]Means within a row showing different superscript letters depict significant main effects of Asp-Oil treatment (alpha = 0.05, Bonferroni-adjusted). CH_4 measured on days 6 (starter diet), 14 (intermediate I diet), and 21 (intermediate II diet) of the adaptation period and finisher diet on days 34, 49, 63 and 77.*For the intermediate II medium treatment the statistical model least squared means were predicted at -1.45, but is presented as 0.00.

Table 6. Methane yield (g CH_4 /kg dietary dry matter intake) of Angus heifers (least squares mean ± s.e.) fed increasing levels of Asparagopsis extract in a canola oil (Asp-Oil) residue on each diet.

	Asp-Oil Treatment[1]						
Diet	Control	Low	Medium	High	*P*[2]		
Adaptation period					T		
Starter	9.95 ± 2.46	10.50 ± 2.25	9.10 ± 2.25	2.92 ± 2.75	0.143		
Intermediate I	7.75 [b] ± 1.94	3.82 [a] ± 1.77	0.02 [a] ± 1.77	0.12 [a] ± 2.17	0.035		
Intermediate II	10.42 [b] ± 1.53	2.16 [a] ± 1.44	0.00 [a*] ± 1.44	0.39 [a] ± 1.58	0.000		
					T	D	T×D
Finisher period	9.80 [b] ± 0.94	3.50 [a] ± 0.86	0.22 [a] ± 0.86	0.07 [a] ± 1.06	0.000	0.050	0.010

[1]Formulated $CHBr_3$ inclusions in diet DM were (for Starter, Intermediate I, Intermediate II and Finisher, respectively): Control = 0 mg $CHBr_3$/kg DM, n = 4; Low = 4.2, 8.5. 12.7 and 16.9 mg $CHBr_3$/kg DM; n = 5; Medium = 8.5, 17.0, 25.5, and 34.0 mg $CHBr_3$/kg DM, n = 5; High = 12.7, 25.5, 38.2, and 51.0 mg $CHBr_3$/kg DM, n = 4; [2]T: Treatment; D: Day; T×D: Treatment × Day interaction; [a,b]Means within a row showing different superscript letters depict significant main effects of Asp-Oil treatment (alpha = 0.05, Bonferroni-adjusted). CH_4 measured on days 6 (starter diet), 14 (intermediate I diet), and 21 (intermediate II diet) of the adaptation period and finisher diet on days 34, 49, 63 and 77. *.*For the intermediate II medium treatment the statistical model least squared means were predicted at -0.16, but is presented as 0.00.

4.2 Feed intake, liveweight gain and feed efficiency

This experiment was not designed to be sufficiently powered to detect differences in intake, growth or feed efficiency. There was no difference between any treatment groups in these measures of productivity (Table 4, Figure 3). To test for true effects of Asp-Oil inclusion on intake, much greater replication is required, preferably in a more commercial-like setting.

4.3 Rumen function

4.3.1 Rumen pH, redox potential, ciliate protozoa enumeration and faecal starch

There was no effect of any treatment on rumen pH or reduction potential (Table 7). Faecal starch digestion was also unaffected by treatment (Table 7). Total protozoa count tended to decrease with increasing Asp-Oil inclusion (*P* = 0.068), so that for the High Asp-Oil treatment, median protozoa count for all genera, and overall, were zero (Rumen fluid samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.

Table 8). The majority of protozoa observed in the Control, Low and Medium Asp-Oil treatments were entodiniomorphs, which also declined with increasing Asp-Oil inclusion (*P* = 0.036). Median counts of holotrichia genera were zero for all treatments.

Table 7. Rumen function measures (least-square means ± s.e.) of Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the seven measurement times during the overall feeding period (day 7 to 77).

Item	Asp-Oil Treatment[1]				*P*-value[2]		
	Control	Low	Medium	High	T	D	T×D
pH	6.39 ± 0.13	6.73 ± 0.12	6.44 ± 0.12	6.29 ± 0.15	0.291	0.079	0.402
Reduction potential	-81.3 ± 34.67	-76.3 ± 34.46	-76.3 ± 34.46	-84.2 ± 34.69	0.856	<0.001	0.885
Faecal starch (g/g)	1.71 ± 0.28	2.31 ± 0.25	2.16 ± 0.27	2.03 ± 0.31	0.896	0.497	0.976

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [2]T: Treatment; D: Day; T×D: Treatment × Day interaction; Rumen fluid samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.

Table 8. Protozoa counts (median [min, max]) of Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the seven measurement times during the overall feeding period (day 7 to 78).

Item	Asp-Oil Treatment[1]					
	Control	Low	Medium	High	X^2	*P*
Total protozoa (×10^3/mL)	0.531 [0, 23.1]	0.078 [0, 57.8]	0.031 [0, 7.1]	0 [0, 18.6]	7.139	0.068
Large holotrich (×10^3/mL)	0 [0, 0.188]	0 [0, 0.094]	0 [0, 1.41]	0 [0, 13.7]	3.223	0.358
Small holotrich (×10^3/mL)	0 [0, 0.125]	0 [0, 0.094]	0 [0, 0.91]	0 [0, 0.06]	0.974	0.808
Entodiniomorphs (×10^3/mL)	0.500[a] [0, 23.1]	0.078[ab] [0, 57.8]	0.016[b] [0, 7.1]	0[b] [0, 18.6]	8.522	0.036

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [a,b]When *P*-value for Asp-oil level was significant (≤ 0.05) pairwise comparison was performed using the Dunn test, in that case means within a row showing different superscript letter depict significant effects of Asp-Oil treatment; Rumen fluid samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.

4.3.2 Volatile fatty acid molar proportions and rumen ammonium concentration

There was no effect of treatment on total concentration or molar proportions of volatile fatty acids (VFA) or rumen ammonium concentration (Table 9). Molar proportions of total VFA, acetate, propionate, and iso-valerate declined slightly with days on feed, whereas molar proportions of butyrate, iso-butyrate, valerate and caproate increased slightly with days on feed. When analysed by diet (data not presented), there was no effect of treatment on individual or total VFA molar concentrations nor proportions, nor acetate:propionate ratio within the Starter (P > 0.50), Intermediate I (P > 0.05), Intermediate II (P > 0.05), or Finisher diets (P > 0.05), except for the molar proportion of valerate (P = 0.045), which had a significantly higher molar proportion in total VFA of Medium Asp-Oil cattle on the Finisher diet, compared to other groups.

Table 9. Rumen ammonium concentration and volatile fatty acid (VFA) molar proportions of Angus heifers (least squared means ± s.e.) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) over the whole feeding period (day 7 to 78).

	Asp-Oil Treatment[1]				*P*-Value[2]		
	Control	Low	Medium	High	Tr	D	Tr×D
Total VFAs (mmol/L)	75.9 ± 5.85	68.8 ± 5.41	74.3 ± 5.41	68.3 ± 6.24	0.627	0.004	0.435
Acetic acid (% total VFA)	57.2 ± 1.33	55.5 ± 1.19	53.9 ± 1.19	52.5 ± 1.46	0.169	<0.001	0.862
Propionic acid (% total VFA)	21.6 ± 2.15	24.4 ± 2.01	24.8 ± 2.01	23.8 ± 2.26	0.666	0.001	0.646
Butyric acid (% total VFA)	17.0 ± 2.12	16.1 ± 1.95	17.3 ± 1.95	19.8 ± 2.38	0.473	<0.001	0.816
Iso-Butyric acid (% total VFA)	0.6 ± 0.12	0.6 ± 0.11	0.5 ± 0.11	0.4 ± 0.13	0.413	0.011	0.415
Valeric acid (% total VFA)	1.7 ± 0.31	1.7 ± 0.28	1.9 ± 0.28	1.6 ± 0.34	0.754	0.055	0.541
Iso-Valeric acid (% total VFA)	0.7 ± 0.21	1.0 ± 0.19	0.7 ± 0.19	0.5 ± 0.24	0.078	<0.001	0.668
Caproic acid (% total VFA)	1.1 ± 0.22	0.8 ± 0.20	1.1 ± 0.20	0.8 ± 0.24	0.859	<0.001	0.931
Acetate:Propionate	2.9 ± 0.24	2.5 ± 0.22	2.5 ± 0.22	2.4 ± 0.25	0.544	0.238	0.841
Ammonium-N (mg/L)	39.7 ± 8.96	41.4 ± 8.22	51.0 ± 8.22	33.6 ± 10.05	0.200	0.135	0.411

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [2]T: Treatment; D: Day; T×D: Treatment × Day interaction; Rumen fluid samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.

4.4 Animal Health

4.4.1 Rumen wall gross and histological morphology

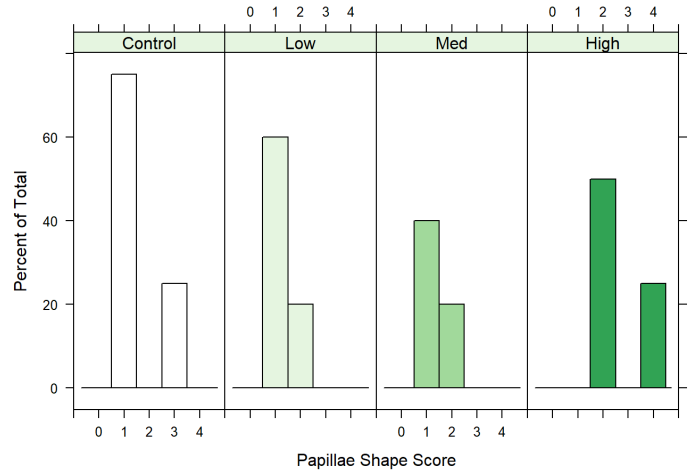
There was no evidence of treatment differences in scores of rumen papillae colour or shape, or damage to the ventral sac of the rumen (Table 10, Figure 6).

Table 10. Scores (mean ± s.e.) of papillae colour and shape, and ventral sac damage in Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) after an 81-day feeding period.

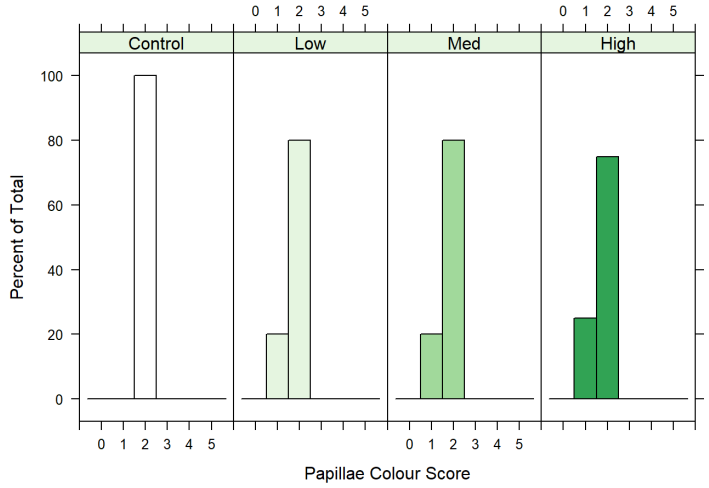
	Asp-Oil Treatment[1]				
Item	Control	Low	Medium	High	*P*-Value
Papillae colour [2]	1.0 ± 0.21	0.8 ± 0.19	0.8 ± 0.19	0.8 ± 0.23	0.730
Papillae shape[3]	0.5 ± 0.48	0.7 ± 0.44	0.4 ± 0.44	1.3 ± 0.52	0.490
Ventral sac damage [2]	0.6 ± 0.80	0.5 ± 0.76	1.5 ± 0.76	0.5 ± 0.81	0.410

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [2]Scored on 1-5 scale; [3]Scored on 1-4 scale; s.e.: Standard error of the mean.

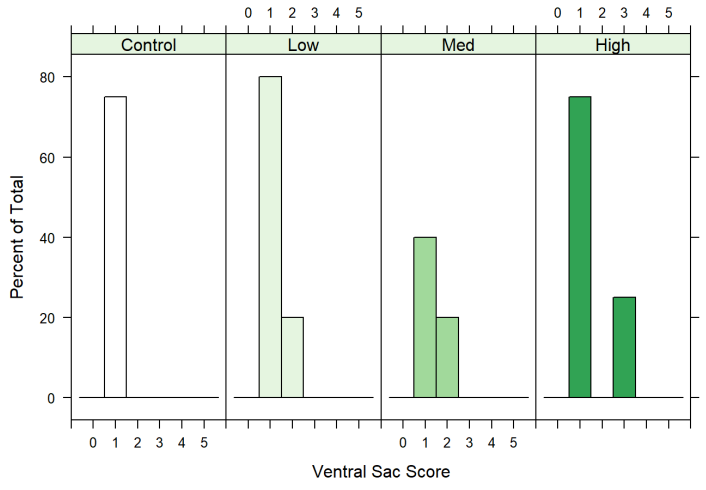
Focal or multifocal small aggregates of lymphocytes, plasma cells and neutrophils in the submucosa, as well as areas with fewer or shorter papillae were consistently present in all groups. In the Control group, 3 out of 4 rumens were considered histologically normal, and one rumen displayed ruminitis scarring. In the Low Asp-Oil group, 3 out of 5 rumens (60%) contained microscopical parakeratosis, without evidence of gross lesions. In the Medium Asp-Oil group, one rumen was grossly and histologically normal (20%); mild to severe parakeratosis was observed in 3 out of 5 rumens (60%), ulceration in 1 out of 5 rumens (20%) and mild scarring in 2 out of 5 rumens (40%). In the High Asp-Oil group, 2 out of 4 rumens showed mild histological lesions of parakeratosis (60%).



(a)



(b)



(c)

Figure 6. Qualitative scores of rumen papillae shape (a) and colour (b), and damage to the ventral rumen sac (c) from Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) for an 80- or 81-day feeding period. Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n

= 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively

4.4.2 Blood haematology

There was no effect of treatment on the results of any of the blood cell differential analyses (Table 11). Haematocrit (%), haemoglobin, leucocyte, monocyte and erythrocyte concentration were significantly ($P < 0.05$) affected by day, such that the mean monocyte concentration increased from 0.633 to 0.877 × 10^6/mL, and haematocrit, haemoglobin and erythrocyte concentration declined from 34.2 to 32.0, 11.8 to 11.3, and from 7.45 to 7.14 ×10^6/mL, respectively, over the duration of the experiment, across all treatment groups, ranges which were not biologically relevant. Platelet and leucocyte concentrations showed significant interactions of day and treatment, but there was no main effect of treatment or day, and so these were not biologically meaningful.

Table 11. Blood cell concentrations of plasma (least squared means ± s.e.) from Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) over the whole feeding period (day 7 to 77).

	Asp-Oil Treatment[1]				P-Value[3]		
Cell type[2]	Control	Low	Medium	High	Tr	D	Tr×D
Leucocytes (×10^6/mL)	10.1 ± 0.75	10.6 ± 0.69	10.4 ± 0.69	10.5 ± 0.81	0.740	0.014	0.162
Neutrophils (×10^6/mL)	3.2 ± 0.31	3.4 ± 0.29	3.9 ± 0.29	4.6 ± 0.32	0.366	0.744	0.070
Lymphocytes (×10^6/mL)	5.8 ± 0.50	6.1 ± 0.46	5.2 ± 0.46	5.0 ± 0.57	0.658	0.050	0.636
Monocytes (×10^6/mL)	0.8 ± 0.10	0.7 ± 0.10	0.8 ± 0.10	0.8 ± 0.11	0.884	0.016	0.589
Eosinophils (×10^6/mL)	0.2 ± 0.10	0.3 ± 0.10	0.4 ± 0.10	0.2 ± 0.12	0.751	0.200	0.354
Basophils (×10^6/mL)	0.2 ± 0.04	0.2 ± 0.04	0.1 ± 0.04	0.1 ± 0.04	0.780	0.748	0.810
Erythrocyte (×10^9/mL)	7.3 ± 0.22	7.2 ± 0.20	7.4 ± 0.20	7.2 ± 0.24	0.651	0.041	0.749
Haemoglobin g/dL	11.7 ± 0.38	11.5 ± 0.35	11.6 ± 0.35	11.6 ± 0.43	0.686	<0.001	0.051
Haptoglobin (mg/L)	342 ± 65.9	432 ± 64.5	421 ± 60.5	439 ± 74.1	0.760	0.007	0.784
Haematocrit (%)	33.3 ± 1.14	32.8 ± 1.04	33.2 ± 1.04	32.9 ± 1.27	0.964	<0.001	0.902
Mean corpuscular volume (fL)	45.6 ± 2.40	46.3 ± 2.20	44.5 ± 2.20	43.9 ± 2.69	0.841	NA	NA
Mean corpuscular haemoglobin (pg)	15.9 ± 0.70	16.2 ± 0.64	15.6 ± 0.64	16.5 ± 0.77	0.271	0.302	0.210
Mean corpuscular haemoglobin concentration (g/dL)	35.0 ± 0.64	35.1 ± 0.59	35 ± 0.59	35.8 ± 0.70	0.224	0.316	0.385
Platelets (×10^6/mL)	594 ± 66.9	591 ± 64.0	703 ± 63.9	759 ± 68.39	0.256	0.433	0.009

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [2]T: Treatment; D: Day; T×D: Treatment × Day interaction; Blood samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.

4.4.3 Body temperature

The MESOR of rumen temperature did not differ between treatments (Figure 7). Amplitude differed significantly between all treatments ($P < 0.001$), such that the amplitude of the High Asp-Oil treatment was greatest, the Medium Asp-Oil was smallest, and the Control and Low Asp-Oil treatments were intermediate. This pattern of variation between treatments in the amplitude of the circadian rhythm does not appear to be related to the inclusion rate of Asp-Oil. Similarly, the acrophase differed significantly between all treatments ($P < 0.001$), such that the acrophase of the Low Asp-Oil group occurred much later, and was much more highly variable, than that of the Control, Medium and High Asp-Oil groups, which were relatively delayed with respect to the overall mean. As for the amplitude parameter, this pattern of variation does not appear to be related to the inclusion rate of Asp-Oil.

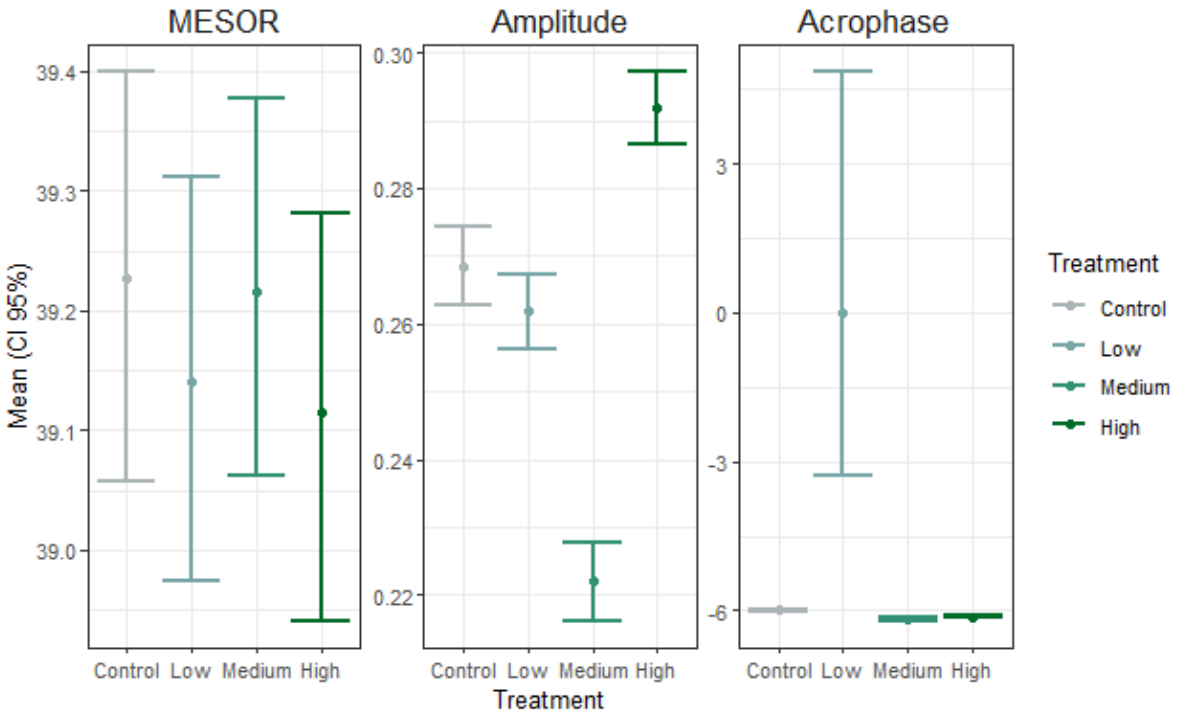


Figure 7. Least square means of circadian rumen temperature rhythm parameters MESOR[1] (°C), Amplitide[2] (°C) and Acrophase[3] (h) with 95% confidence intervals of Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil)[4] overall during an 81-day feeding period. [1]Midline Statistic Of Rhythm, a non-linear function that depends on the rhythm-adjusted mean; [2]half the extent of variation within a day; [3]the time of overall high values recurring in each day, relative to the overall mean; [4]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively.

4.4.4 Hormone and vitamin B_{12} metabolism

Thyroxine and Vitamin B_{12} were highly variable, but there was no indication of a negative effect of Asp-Oil inclusion rate on their concentration in serum, nor on Triiothyronine concentration (Table 12). Faecal cortisol was not affected by Asp-Oil treatment (Table 12).

Table 12. Least square means of serum concentrations of Thyroxine, Triiothyronine and Vitamin B_{12} and faecal cortisol concentration in Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) over the whole feeding period (day 7 to 77).

	Asp-Oil Treatment[1]				P-Value[2]		
	Control	Low	Medium	High	Tr	D	Tr×D
Thyroxine, ng/mL	67.0 ± 8.83	71.0 ± 8.35	74.9 ± 8.34	84.7 ± 9.12	0.235	0.475	0.444
Triiothyronine, ng/mL	2.36 ± 0.464	2.34 ± 0.430	2.63 ± 0.430	3.42 ± 0.481	0.178	0.046	0.083
Faecal cortisol, ng/g	0.90 ± 0.115	0.82 ± 0.098	0.77 ± 0.103	0.75 ± 0.122	0.276	0.084	0.104
Serum Vitamin B_{12}, pg/mL	59.2 ± 9.88	63.8 ± 9.07	54.0 ± 9.07	70.8 ± 11.09	0.466	0.133	0.208

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [2]T: Treatment; D: Day; T×D: Treatment × Day interaction; Faecal and serum samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.

4.5 Mass balance of bromoform, bromide and iodide

4.5.1 Oil bromoform, iodide and bromide

Oil bromoform, iodide and bromide concentrations throughout the experiment are presented in Table 13.

Table 13. Concentrations of bromoform, bromide, iodine and moisture in undiluted canola oil stock solution containing Asparagopsis extract.

Item (As-Fed basis)	Initial	Day 42	Day 78
Bromoform (mg/kg)	3.58	3.35	3.33
Iodine (mg/kg)	163	158	153
Bromide (mg/kg)	11,688	44,018	50,654
Moisture (%)	0	0.4	0.4

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively;

4.5.2 Serum iodine and bromide

While the effect of Asp-Oil inclusion on serum iodine was statistically significant, the lack of a linear or quadratic pattern of change with reference to the Control treatment indicated that there was no biologically relevant effect (Table 14). Serum bromide content increased linearly as Asp-Oil content increased (Table 14). $CHBr_3$ content of serum was not analysed.

Table 14. Responses (least squares mean ± s.e.) of iodine and bromide concentration in serum of Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the finisher period (day 35 – 77, pooled samples).

	Asp-Oil Treatment[1]				
Item	Control	Low	Medium	High	P-Value
Iodine, mg/L	1.17^{ab} ± 1.252	1.42^{b} ± 0.249	1.16^{ab} ± 0.249	0.86^{a} ± 0.253	<0.001
Bromide, mg/L	18.7^{a} ± 1.33	26.2^{b} ± 1.22	37.2^{c} ± 1.22	41.6^{c} ± 1.49	<0.001

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [a,b]Means within a row showing different superscript letters depict significant main effects of Asp-Oil treatment (α= 0.05, Bonferroni-adjusted); Serum samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.

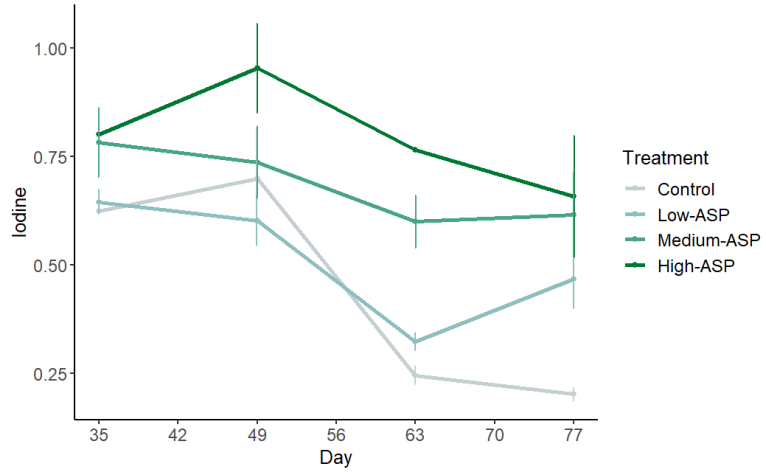
4.5.3 Faecal bromoform, iodine and bromide

Finisher period faecal iodine concentration increased linearly with Asp-Oil inclusion, but there was no effect on finisher period faecal bromide concentration (Figure 8, Table 15). There was a significant negative effect of time on faecal iodine and bromide concentration, declining with days on feed in all treatment groups (Figure 8). $CHBr_3$ could not be detected in any samples of faeces from the finisher period (limit of detection < 2 mg $CHBr_3$/kg; data not shown).

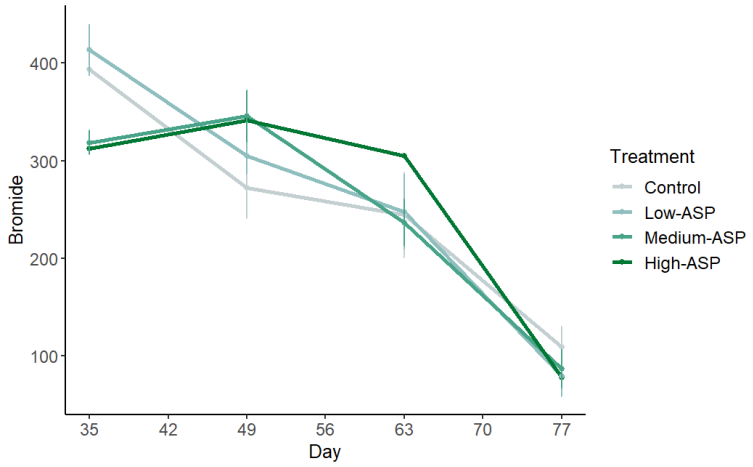
Table 15. Responses (least squares mean ± s.e., mg/kg) of iodine and bromide concentration in faeces of Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the finisher period (day 35 – 77).

	Asp-Oil Treatment[1]				P-Value[2]		
Item	Control	Low	Medium	High	T	D	T×D
Iodine	0.45^{a} ± 0.053	0.51^{ab} ± 0.044	0.68^{bc} ± 0.044	0.80^{c} ± 0.056	0.507	<0.001	0.208
Bromide	247.4 ± 22.12	256.2 ± 19.59	243.3 ± 19.59	250.6 ± 22.46	0.410	<0.001	0.473

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; [2]T: Treatment; D: Day; T×D: Treatment × Day interaction; [a,b]Means within a row showing different superscript letters depict significant main effects of Asp-Oil treatment (α= 0.05, Bonferroni-adjusted); Faecal samples collected on days 7 (starter diet), 15 (intermediate I diet), and 22 (intermediate II diet) of the adaptation period and finisher diet on days 35, 50, 64 and 78.



(a)



(b)

Figure 8: Observed concentrations (± s.e.) of iodine (a) and bromide (b) in faeces of Angus steers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) during the finisher period (days 35 – 77).

4.5.4 Carcase bromoform, iodine and bromide

$CHBr_3$ residues were below detectable limits in all carcases at the liver, kidney, fat and striploin (Table 16).

Table 16. Bromoform ($CHBr_3$) concentration[1] (mg/kg) of kidney, liver, fat, and striploin samples from carcases from Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) for an 80- or 81-day feeding period

		Sample site			
Asp-Oil Treatment[2]	Animal	Kidney	Liver	Fat	Striploin
Control					
	34	ND	ND	ND	ND
	R65	ND	ND	ND	ND
	54	ND	ND	ND	ND
	21	ND	ND	ND	ND
Low					
	O96	ND	ND	ND	ND
	95	ND	ND	ND	ND
	11	ND	ND	ND	ND
	69	ND	ND	ND	ND
	75	ND	ND	ND	ND
Medium					
	73	ND	ND	ND	ND
	81	ND	ND	ND	ND
	99	ND	ND	ND	ND
	6	ND	ND	ND	ND
	150	ND	ND	ND	ND
High					
	145	ND	ND	ND	ND
	67	ND	ND	ND	ND
	R96	ND	ND	ND	ND
	O65	ND	ND	ND	ND

[1]Bromoform tolerable daily intake 17.9 µg/kg BW (WHO 2004). [2]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; ND: Not detectable

Bromide concentrations were below detectable limits in all samples of fat (Table 17). In kidney, liver and striploin, there was a linear response of bromide concentration to Asp-Oil level (Table 17). The sampling site with the greatest concentration of bromide was the kidney in all treatments. The highest recorded concentration of bromide occurred in kidney in the High Asp-Oil group, at 31 mg/kg (Table 17), although in most samples, based on expected consumer intake of these products this was below the recommended sustained upper limit (mg/day) of bromide intake (FAO Panel of Experts on Pesticide Residues in Food 1999).

Table 17. Bromide (Br^-) concentration[1] (mg/kg) of kidney, liver, fat, and striploin samples from carcases from Angus heifers (N = 18) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) for an 80- or 81-day feeding period. Individual carcases are presented with observed value, treatments are summarised with least-squared means.

		Sample site			
Asp-Oil Treatment[2]	Animal	Kidney	Liver	Fat	Striploin
Control					
	34	11	6	ND	ND
	R65	12	6	ND	ND
	54	11	5	ND	ND
	21	14	7	ND	ND
Control mean		12.0[a]	6.0 [a]	0[a]	0[a]
Low					
	O96	17	9	ND	ND
	95	17	8	ND	ND
	11	16	9	ND	ND
	69	19	9	ND	ND
	75	16	9	ND	ND
Low Asp-Oil mean		17.2[ab]	8.9[a]	0[a]	0[a]
Medium					
	73	26	12	ND	5
	81	18	12	ND	6
	99	24	13	ND	6
	6	21	12	ND	ND
	150	18	11	ND	ND
Medium Asp-Oil mean		21.6[bc]	12.1[b]	0[a]	3.6[ab]
High					
	145	29	14	ND	7*
	67	31*	16*	ND	6
	R96	27	13	ND	7*
	O65	28	12	ND	5
High Asp-Oil mean		25.6[c]	12.6[b]	0[a]	4.9[b]
P-value (Treatment)		<0.001	<0.001	-	<0.01

[1]Bromide acceptable daily intake 1 mg/kg BW; with ranges for 1-3-year-old (13 kg) to average adults (70 kg). *Bromide levels may exceed UL (mg/d) with daily consumption of kidney 0.42 kg (<3 years) - 2.3 kg (adult), liver 0.82 kg (<3years) - 4.4 kg (adult), or striploin 1.9 kg (<3years) - 10 kg (adult) (FAO Panel of Experts on Pesticide Residues in Food 1999). [2]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively; ND: Not detectable. [a,b]Means within a row showing different superscript letters depict significant main effects of Asp-Oil treatment within a sampling site (α= 0.05, Bonferroni-adjusted).

Iodide concentrations were below detectable limits in most samples of liver, and all samples of fat and striploin (Table 18). The sampling site of greatest concentration of iodide was the kidney in all treatments, although in most samples, based on expected consumer intake of these products this was below the recommended sustained upper limit (mg/day) of iodine intake (Trumbo *et al.* 2001). There was no significant difference between treatments for iodine accumulation. Two kidney samples in the Low treatment, two in the Medium treatment, and one in the High treatment had iodide concentrations of 0.3 mg/kg – the highest recorded (Table 18).

Table 18. Iodide (I^-) concentration[1] (mg/kg) of kidney, liver, fat, and striploin samples from carcases from Angus heifers (N = 18) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) for an 80- or 81-day feeding period. Individual carcases are presented with observed value, treatments are summarised with least-squared means.

		Sample site			
Asp-Oil Treatment[2]	Carcass ID	Kidney	Liver	Fat	Striploin
Control					
	34	0.2	ND	ND	ND
	R65	0.2	ND	ND	ND
	54	0.2	ND	ND	ND
	21	0.1	ND	ND	ND
Low					
	O96	0.3‡	ND	ND	ND
	95	0.2	ND	ND	ND
	11	0.2	ND	ND	ND
	69	0.2	ND	ND	ND
	75	0.3‡	ND	ND	ND
Medium					
	73	0.3‡	0.1	ND	ND
	81	0.2	0.1	ND	ND
	99	0.2	ND	ND	ND
	6	0.3‡	ND	ND	ND
	150	0.2	ND	ND	ND
High					
	145	0.2	0.1	ND	ND
	67	0.2	ND	ND	ND
	R96	0.2	ND	ND	ND
	O65	0.3‡	0.1	ND	ND

[1]Iodine recommended upper limit (mg/d)(sustained): 1-3 years (0.2); 4-8 years (0.3); 9-13 years (0.6); 14-18 years (0.9); 19+ years (1.1, (Trumbo *et al.* 2001)). ‡Iodide levels may exceed UL (mg/d) with daily consumption of 0.67 kg (<3 years), 1.0 kg (4-8 years), 2.0 kg (9-13 years), 3.0 kg (14-18 years), and 3.7 kg (19+ years).[2]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively. ND: Not detectable. [a,b]Means within a row showing different superscript letters depict significant main effects of Asp-Oil treatment within a sampling site (α= 0.05, Bonferroni-adjusted).

4.6 Carcase Performance

4.6.1 Carcase grading and shear force measurement

There was no effect of treatment on carcase weight, grading or shear force ($P > 0.10$, Table 19). Only 1 carcass failed on pH (>5.7), from the Medium Asp-Oil group.

Table 19. Responses (least squared means ± s.e.) of hot standard carcass weight (HSCW), dressing percentage (Dressing %), P8 fat depth (P8 fat), rib fat depth (Rib fat), eye muscle area (EMA), Meat Standards Australia marbling scores (MSA Marbling), ossification score (Ossification), ultimate pH (pHu), Meat Standards Australia index (MSA index) in Angus heifers (N = 18) fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil) after an 80 – 81 day feeding period.

	Asp-Oil Treatment[1]				
Item	Control	Low	Medium	High	*P*
HSCW, kg	229 ± 7.43	222 ± 7.18	220 ± 7.18	234 ± 7.54	0.157
Dressing percentage, %	49.6 ± 0.60	50.7 ± 0.61	50.2 ± 0.57	49.7 ± 0.57	0.284
P8 fat, mm	10.0 ± 1.15	9.4 ± 1.06	9.8 ± 1.06	9.25 ± 1.30	0.961
Rib fat, mm	6.5 ± 0.96	5.6 ± 0.88	4.2 ± 0.88	5.5 ± 1.08	0.381
EMA, cm^2	63.8 ± 2.23	66.7 ± 2.11	67.5 ± 2.11	71.9 ± 2.30	0.037
MSA Marbling score	307.5 ± 21.36	288.0 ± 19.60	288.0 ± 19.60	310.0 ± 29.97	0.669
AUSMEAT Marbling score	0.5 ± 0.27	0.4 ± 0.25	0.4 ± 0.25	0.8 ± 0.31	0.760
AUSMEAT fat colour score	0.0 ± 0.20	0.3 ± 0.19	0.2 ± 0.19	0.3 ± 0.23	0.826
AUSMEAT meat colour score	3.0 ± 0.48	3.3 ± 0.45	3.5 ± 0.45	3.1 ± 0.49	0.792
Ossification score	145.0 ± 5.95	138.0 ± 5.45	140.0 ± 5.45	140.0 ± 6.67	0.846
pH_u[2]	5.53 ± 0.027	5.57 ± 0.025	5.64 ± 0.025	5.62 ± 0.031	0.038
MSA index	55.6 ± 6.48	50.3 ± 6.00	39.8 ± 6.00	51.8 ± 6.91	0.254
Shear force (kg)	5.53 ± 1.049	4.96 ± 0.967	5.44 ± 0.967	5.09 ± 1.137	0.967

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM; Low = 791 mg $CHBr_3$/kg oil DM; Medium = 1591 mg $CHBr_3$/kg oil DM; High = 2389 mg $CHBr_3$/kg oil DM, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively. [2] For pH_u, the *P*-value of the *F*-test is presented (= 0.038), but a linear contrast with Bonferroni's test was not significant at alpha = 0.05.

4.6.2 Sensory traits

The Control and Medium Asp-Oil groups had the highest scores across most sensory traits. The Medium Asp-Oil samples produced slightly better results in all sensory traits particularly for flavour, in comparison to the Control group. The low and High Asp-Oil groups had lower scores for tenderness and juiciness when compared to the Control. The Medium Asp-Oil treatment had the highest MQ4 score and High Asp-Oil had the lowest MQ4 score. No statistically significant differences in the eating quality traits between the control and any Asp-Oil groups was observed (Table 20). The effect of muscle position was also not significant, although a slight decrease in P4 was observed across the sensory traits, which aligned with data in the current MSA model for the posterior end of the striploin. Coefficients for Low and High Asp-Oil were lower across the sensory variables, with the largest effect occurring in the tenderness trait, -4.47 for Low Asp-Oil and -1.65 for High Asp-Oil in comparison to the Control group. There was also a slight improvement (but not significant) in eating quality in comparison to the control evident in the Medium Asp-Oil treatment group; +0.79 for tenderness, +1.39 for juiciness, +3.87 for flavour, +1.98 for overall liking and +2.13 for meat quality. This was consistent across each of the 3 picks suggesting that with larger numbers the difference may reach significance. There was no additional variance attributable to groups of consumers (picks) after accounting for consumer level variation.

Table 20. Results from a linear mixed effect model with treatment and position as fixed effects and carcase number as a random intercept. For each dependent variable (MQ4, Tenderness, Juiciness, Flavour and Overall liking) there were 18 carcases and 54 samples. Estimated coefficients should be interpreted relative to the baseline level, which for treatment was the Control sample and for position was the anterior position (A1).

	Tenderness		Juiciness		Flavour		Overall liking		MQ4	
Predictors	Estimate	*P*	Estimate	*P*	Estimate	*P*	Estimate	*P*	Estimate	*P*
Asp-Oil Treatment[1]										
(Intercept)	54.19	<0.001	58.9	<0.001	58.16	<0.001	57.35	<0.001	56.8	<0.001
Control	*Reference*		*Reference*		*Reference*		*Reference*		*Reference*	
Low	-4.47	0.638	-1.77	0.773	0.33	0.958	-1.64	0.835	<1.91	0.802
Medium	0.79	0.934	1.39	0.82	3.87	0.533	1.98	0.8	2.13	0.78
High	-1.65	0.869	-1.96	0.762	0.85	0.896	-1.45	0.861	-0.87	0.914
Muscle position										
A1	*Reference*		*Reference*		*Reference*		*Reference*		*Reference*	
A2	-2.36	0.375	-1.01	0.674	-2.89	0.237	-2.41	0.294	-2.4	0.287
P4	-3.09	0.246	-3.37	0.162	-2.38	0.329	-2.88	0.21	-2.84	0.208
Random Effects										
σ^2	62.42		50.76		52.33		46.35		44.64	
τ_{00}	177.96 $_{carcase\_no}$		65.65 $_{carcase\_no}$		66.88 $_{carcase\_no}$		119.57 $_{carcase\_no}$		112.65 $_{carcase\_no}$	
ICC	0.74		0.56		0.56		0.72		0.72	
N	18 $_{carcase\_no}$		18 $_{carcase\_no}$		18 $_{carcase\_no}$		18 $_{carcase\_no}$		18 $_{carcase\_no}$	
Observations	54		54		54		54		54	
Marginal R^2/ Conditional R^2	0.025/0.747		0.034/0.579		0.034/0.576		0.023/0.727		0.025/0.723	

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively.

4.6.3 Strip-loin shelf-life measurement

The shelf-life target of 90 days was achieved by striploins from all carcases in the Control, Low and High Asp-Oil groups, while 3 out of 5 Medium Asp-Oil carcases achieved 80 days of shelf-life acceptability, and 2 out of 5 Medium Asp-Oil carcases achieved 90 days of shelf-life acceptability.

All carcases achieved 90 days of acceptable retail evaluation. In the packaging, more purge was noticed from day 31 onwards while some air bubbles were observed from day 50 onwards. The colour of the striploin was acceptable throughout the shelf life evaluation period. All carcases achieved 90 days of acceptable post-cooking evaluation. The cooked samples displayed a noticeable slight aged smell, aroma and flavour from day 60 onwards, but these were still acceptable throughout the shelf life evaluation period. The cooked striploins of the Control and Low Asp-Oil carcases were slightly tough and chewy in the first 30 days, but this texture improved from day 30 onwards.

There was no effect of Asp-Oil on SPC (Table 21). All control and Low Asp-Oil samples achieved the target SPC for 90 days. Two out of five Medium Asp-Oil samples achieved the target SPC for 90 days, while three High Asp-Oil sample achieved the target SPC (< 1×10^6 cfu/g) for 80 days. Three out of four High Asp-Oil samples achieved the target SPC for 90 days, while one High Asp-Oil sample achieved the target SPC for 80 days. Cell counts of *E. coli* for all samples remained < 10 cfu/g until the end of the monitoring period. All samples tested for coliforms were within the target threshold. All samples recorded < 10 cfu coliforms/g until the end of the monitoring period, except for two carcases from the control group, recording 90 and 330 cfu/g on days 30 and 40, respectively, one carcase from the Low Asp-Oil group, recording 40 cfu/g on day 40, and one carcase from the Medium Asp-Oil group recording 150 cfu/g on day 30.

Table 21. Aerobic plate count of bacterial colonies (SPC, × 10^6 cfu/g, least squares mean ± s.e.) sampled from strip loin of Angus heifers fed increasing levels of Asparagopsis extract in a canola oil carrier (Asp-Oil).

	Asp-Oil Treatment[1]					
Test day	Control	Low	Medium	High	SE	*P*-Value
10	0	0	0	0.24	17.65	1.000
20	0.44	0.05	0	0.26	17.65	0.983
30	0.29	0	0	0.25	17.65	0.989
40	0.37	0	0.01	0.39	17.65	0.985
50	1.10	0.41	0.27	4.04	17.65	0.957
60	1.21	1.49	0.90	1.26	17.65	0.953
70	4.54	15.80	7.65	2.23	17.65	0.824
80	2.06	11.67	2.82	4.04	17.65	0.919
90	8.51	1.48	84.77	10.02	17.65	0.677

[1]Asp-oil levels were Control = 0 mg $CHBr_3$/kg oil DM, n = 4; Low = 791 mg $CHBr_3$/kg oil DM, n = 5; Medium = 1591 mg $CHBr_3$/kg oil DM, n = 5; High = 2389 mg $CHBr_3$/kg oil DM, n = 4, included in the ration at 0.54, 0.86, 1.28 and 2.17 % DM for Starter, Intermediate I, Intermediate II and Finisher diets, respectively. Target SPC: 1×10^6 cfu/g; marginal range: $1 – 10 \times 10^6$ cfu/g; Rejection threshold: > 10×10^6 cfu/g (Australian Country Choice, *Pers. Comm*.).

5 Discussion

5.1 Methane mitigation potential

In this project, Asp-Oil inclusion in a feedlot diet typical of Australian short-fed production systems for the domestic grain-fed market was highly efficacious at reducing production of CH_4, achieving 98 % mitigation of methane emissions at the Medium Asp-Oil treatment (1591 mg $CHBr_3$/kg oil DM) in the finisher diet (providing 34.0 mg $CHBr_3$/kg DMI). Although statistically the 3 Asp-Oil treatments did not differ, numerically overall, and for the finisher period, maximal CH_4 mitigation was achieved from the High Asp-Oil treatment. The increments of $CHBr_3$ inclusion used in formulation of the treatments were quite large (0.8 mg $CHBr_3$/kg oil DM, equating to 17 mg $CHBr_3$/kg dietary DM in the finisher diet), and so there is scope to refine recommendations for Asp-Oil and $CHBr_3$ inclusion over a narrower range around the Medium and High Asp-Oil treatments.

The results of the present experiment have demonstrated that an extract of *Asparagopsis* in canola oil is equally as efficacious as whole FD-Asp in mitigating methane production in grain-based beef cattle diets, for a similar $CHBr_3$ content. In a previous study, dietary inclusion of 0.38% DM of a FD-Asp product provided 24.2 mg $CHBr_3$/kg DM in a TMR of 70% steam-rolled barley (as-fed) and induced a 98% reduction in CH_4 yield compared to the control (Kinley *et al.* 2020). In another study a dietary inclusion of 0.45% DM of a FD-Asp product provided 35.1 mg $CHBr_3$/kg DM in a finisher diet and induced a 70% reduction in CH_4 yield compared to the control (Roque *et al*. 2021).Those FD-Asp inclusions resulted in similar $CHBr_3$ inclusions to the present Medium Asp-Oil treatment in the finisher diet (34.0 mg $CHBr_3$/kg DM), which achieved a CH_4 yield of 0.22 g CH_4/kg DMI, or reduction of 98% from Control (9.8 g CH_4/kg DMI) in the finisher diet. These results confirm that it is the lipid-soluble bioactive products (mostly $CHBr_3$) in *Asparagopsis* which provide the CH_4 mitigation effect, and suggest that there is likely no additive effect of whole *Asparagopsis* biomass on CH_4 emissions.

Maximal treatment CH_4 mitigation was first achieved by the Medium and High Asp-Oils at the end of the Intermediate I period, where these diets provided 17 and 25.5 mg $CHBr_3$/kg DM, respectively. The Low Asp-Oil treatment achieved a lower level of CH_4 mitigation in the finisher diet at 17 mg $CHBr_3$/kg DM than the Medium Asp-Oil treatment achieved in the Intermediate diet I at the same $CHBr_3$ inclusion. While this could suggest an interaction with fibre content, an alternative explanation could be related to time required for rumen adaptation. Furthermore, the increase in CH_4 emissions and yield in the Low Asp-Oil group from days 48 – 76 may indicate some level of adaptation by the rumen microbiome at this level of $CHBr_3$ inclusion at the end of the feeding period. Li *et al.* (2018) found no reduction in persistence of CH_4 mitigation at 0.5 % OM kiln-dried *Asparagopsis* over 72 days. Further research is required to confirm persistence of methane suppression below or equal to 17 mg/kg $CHBr_3$/kg DM.

From an inventory perspective, *Asparagopsis* has potential to reduce CH_4 emissions by ~ 9.5 g CH_4/kg DM intake in feedlot finisher diets, when considering research to date (Kinley *et al.* 2020; Roque *et al.* 2021) and the present study. Mean baseline (Control group) CH_4 yield across these 3 studies was 10.9 g CH_4/kg DM.

The diets in this experiment were all formulated for equal total fat content (DM-basis), including the Control diet. Certainly oil is known as a CH_4 mitigant in its own right, with each

1 % increase in dietary oil reducing CH_4 yield by 1.02 g CH_4/kg DMI or 4.37 % (Almeida *et al.* 2021). The basal diet in the present experiment was based on tempered barley, 5.2 % fat in the finisher diet, and was broadly representative of a typical Australian feedlot diet. In a previous MLA project (B.FLT.5010), CH_4 yield from Control steers was 5.07 g CH_4/kg DMI, from a similar tempered barley feedlot diet, but with a fat inclusion of 6.9 % in the finisher diet, compared to emissions of 9.96 g CH_4/kg DMI with 5.2 % dietary fat in the present Control finisher diet. The effect of total oil inclusion on CH_4 emissions may therefore be important for nutritionists formulating feedlot diets, considering baseline emissions. An additive effect of Asp-Oil with dietary fat may be possible, as this has been recently demonstrated for another inhibitor of methyl coenzyme M (Gruninger *et al.* 2022). In the Medium Asp-Oil treatment, the diets contained undiluted stock Asp-Oil (3.58 mg $CHBr_3$ /kg oil DM) at 0.77 % DM in the finisher diet, compared to total oil inclusion of 2.17% DM, which provides scope for this product to be incorporated at lower total oil inclusion rates. However, greater understanding of the interactions of Asp-Oil with total oil or dietary fat inclusion is necessary to refine recommendations for formulation of diets using this product.

5.2 Product safety

5.2.1 Animal health

When considering the whole feeding period, this project found no effect of Asp-Oil on blood differential results, which supported previous research using FD-Asp (Li et al. 2018). Previously (Manilal *et al.* 2013), feeding an extract of *Asparagopsis* to Giant Tiger Prawns (*Penaeus monodon*) resulted in an increased haemocyte count, analogous in vertebrates to phagocyte count (which includes leucocytes, neutrophils, lymphocytes, and monocytes, among other cell types) indicating enhanced immune-modulatory efficacy. This result was not replicated in the present study feeding Asp-Oil to cattle, neither did the present study suggest any inflammatory response to treatments, when the whole feeding period was considered. Chronic systemic inflammation or reduced bodily function may result in elevated cortisol excreted in faeces, but there was no effect of Asp-Oil in this study.

Rumen temperature fluctuated substantially during the experiment, as to be expected with cycling heifers, but did not differ between treatments. Variation in the amplitude and acrophase of the circadian rumen temperature rhythm was not related to Asp-Oil treatment. Core body temperature is regulated by the thyroid gland, among other mechanisms, and there was likewise no effect of Asp-Oil treatment on serum concentrations of thyroxine or triiothyronine.

Methylcobalamin is a co-enzyme facilitating the last step of CH_4 synthesis, and is an analogue of Vitamin B_{12}, both synthesised by rumen bacteria and competing for dietary cobalt (Co) supply. Vitamin B_{12} concentration in serum was not significantly affected by Asp-Oil treatment, and typically varies substantially within a herd (Freer 2007).

5.2.2 Rumen wall

At a gross morphological level, there were no differences in scores of rumen damage or abnormality of papillae. The presence 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 and was consistently present in both the Control and the Asp-Oil groups. Focal/multifocal areas with fewer papillae or shorter papillae may be considered a normal finding since these were consistently present in all the groups including Control. There was no parakeratosis observed in Control rumens, but parakeratosis was observed in some heifers from all Asp-Oil groups with a prevalence of 44% in treated animals. However, this change was detectable only at histological examination (microscopic). Ruminal parakeratosis is a common occurrence in animals fed a high-concentrate ration during the finishing period (potentially as high as 40 % within a group). It is also seen in cattle fed rations of heat-treated alfalfa pellets as well as in calves with prolonged ruminal acidosis due to ruminal drinking (milk ingested into the rumen). The lesion is thought to be caused by the lowered pH and the increased concentration of volatile fatty acids (VFAs) in the ruminal fluid. A post-mortem survey of 2116 rumens at slaughter previously revealed the presence of hyperkeratosis/parakeratosis in 58% of the samples (Magrin *et al.* 2021). Lesions with unconfirmed origin were observed in 5 out of 10 sheep on a pelleted lupin-concentrate ration and supplemented with FD-Asp (Li *et al.* 2018). The changes in the rumen mucosa observed in the present study may be consistent with mild ruminal acidosis secondary to carbohydrate ingestion, more than related to the Asp-Oil supplementation. The mild to moderate scarring process observed in some animals is remnant of a previous chronic pathological condition, as they are outcomes of the healing process from ruminitis or ulceration. Given the chronicity of the lesions, it is possible the antigenic and allergic stimulus resolved before the commencement of the study.

5.3 Product transfer

There was no evidence of $CHBr_3$ transfer to meat or faeces in any Asp-Oil treatment in this study. This supports previous research demonstrating that Asparagopsis derived $CHBr_3$ does not transfer to meat and edible offal (Li *et al.* 2018; Kinley *et al.* 2020), faeces (Muizelaar *et al.* 2021) or milk from healthy animals (Roque et al 2019, Stefenoni et al 2021, Muizelaar *et al.* 2021) when offered at effective feed inclusion levels. The greatest evidence of $CHBr_3$ in products from animals fed *Asparagopsis* is in milk, where Control cows generally do not differ in milk $CHBr_3$ content: Stefenoni *et al.* (2021), feeding dairy cows 0.50 % FD-Asp /kg DM containing ~10 mg $CHBr_3$/kg *Asparagopsis* DM, found the resulting milk $CHBr_3$ concentration to be 28.9 µg/L, although this did not differ from the $CHBr_3$ concentration of milk from Control cows, and Roque *et al.* (2019) recorded milk $CHBr_3$ concentration of 0.15 µg/L resulting from feeding both 0.92 and 1.84 % FD-Asp /kg DM, which also did not differ from Control cows. That said, all these values are considerably lower than the 100 µg/L $CHBr_3$ levels recommended as safe for drinking water by WHO (2004). Overall, the research on $CHBr_3$ transfer to date, including this present study, provide strong evidence that feeding *Asparagopsis* and its extracts to ruminants does not pose a risk of $CHBr_3$ toxicity to consumers of those animal products.

Bromine is considered toxic to cattle at concentrations exceeding 200 mg/kg DMI (NASEM 2016). Doses above 5000 mg/kg DM have caused production and health problems in rats and chicks (National Research Council 1980). In the finisher diets of the present study, the Low, Medium and High Asp-Oils contributed 55, 111 and 166 mg bromide/kg DM, respectively – all well below the threshold of maximum tolerable concentration. The increasing dietary bromide concentration observed with increasing Asp-Oil treatment was

reflected in elevated bromide concentrations in serum of all Asp-Oil treatments. Bromine is rapidly excreted through the urine, and there was no evidence of upregulated transfer of bromide to faeces with Asp-Oil treatment in the present study.

Acceptable Human Daily Intake (ADI) of 1 mg bromide/kg BW.day was established by the FAO/WHO Joint Meeting on Pesticide Residues (FAO Panel of Experts on Pesticide Residues in Food 1999) for bromide (Br^-). This was based on a 12-week oral human study using sodium bromide where no neurophysiological or endocrinological effects were observed at the highest tested dose of 9 mg Br^-/kg BW.day (equivalent to 11.6 mg/kg BW.day sodium bromide), using a 10-fold safety factor (52(E)(1)(f)). A technical assessment on a Bromine containing compound, by Food Standards Australia NZ has also concluded a safe limit of consumption at 1 mg/kg BW.d of bromide (Food Standards Australia New Zealand n.d.). In the present study, bromide residues in the carcase increased with increasing Asp-Oil treatment in kidney, liver and striploin. The fat does not appear to be a point of deposition of bromide, based on the results of the present study. The highest carcase bromide concentration recorded (kidney, High Asp-Oil treatment) is unlikely to result in bromide intakes exceeding the recommended upper limit: excessive levels of bromide intake would require daily consumption of 0.42 kg/day (<3 years old) to 2.3 kg/day (19+ years old) of kidney from that carcase. Further research is required to confirm if a pre-slaughter withhold (2-3 d) of Asparagopsis would lower the level of bromide in meat and edible offal.

Finishing cattle have an iodine requirement of 0.50 mg/kg DM, and 50 mg/kg DM has been suggested as a maximum tolerable concentration for beef cattle (NASEM 2016). All diets in the present experiment contained < 10 mg iodine/kg DM. Although iodine concentration in serum varied in the present experiment, there was no relationship with Asp-Oil treatment. Lengthy exposure to high dietary iodine causes goitrogenic and antithyroidal effects (National Research Council 1980). No effect of Asp-Oil treatment was observed on thyroid function in this present study. Increasing Asp-Oil treatment resulted in increased iodine concentration in faeces, such that the faeces of High Asp-Oil heifers was 77% higher than Control heifers, however, most ingested iodine that is not concentrated in the thyroid is excreted via urine (> 90 % of ingested iodine, Institute of Medicine Panel on Micronutrients (2001)).

In the carcase, the liver, fat and striploin are not points of deposition of iodine, but in all carcases, iodine was detected in the kidney although without a relationship to Asp-Oil treatment. In all Asp-Oil treatments, some individual carcases returned maximal iodine concentrations of 0.3 mg/kg in the kidney. This concentration is unlikely to result in excessive iodine intakes by consumers, with the recommended tolerable upper limit of iodine intake only being exceeded with daily consumption of 0.67 kg (<3 years), 1.0 kg (4-8 years), 2.0 kg (9-13 years), 3.0 kg (14-18 years), and 3.7 kg (19+ years) of kidney from those carcases (Trumbo *et al.* 2001). The tolerable upper limit of intake was established by elevated thyroid stimulating hormone concentrations, suggesting a risk of clinical hypothyroidism (Institute of Medicine Panel on Micronutrients 2001), with a *Lowest-Observed-Adverse-Effect Level* of 1,100 µg iodine/day (Institute of Medicine Panel on Micronutrients 2001). Roque *et al.* (2021) reported striploin iodine concentrations of 0.08 and 0.15 mg/kg, in steers fed 0.45 and 0.91 % FD-Asp in dietary DM, respectively, for 147 days. In that experiment, the 0.45 % *Asparagopsis* treatment provided 35mg $CHBr_3$/kg DM, which was similar to the Medium Asp-Oil treatment in the present research. Iodine levels in

Asparagopsis is known to vary widely and as such it is important to be diligent in monitoring the levels delivered to animals and to continue to develop cultivation and processing techniques that provide for minimized iodine as well as bromide content as-fed.

5.4 Product stability

Considering the volatility of $CHBr_3$ and the 78-day duration of the study, the $CHBr_3$ concentration of the Asp-Oil was monitored over time. Asp-Oil was stored at 4 °C and a 7.0 % reduction of $CHBr_3$ concentration was observed over the 78 days. Contrastingly, Magnusson *et al.* (2020) reported that $CHBr_3$ concentration of clarified carrier oil increased over 12 weeks storage at 25 °C. In that research, it was hypothesised that this was a result of continued release of $CHBr_3$ into the oil from particulate *Asparagopsis* biomass. The Asp-Oil used in the present research initially contained substantial sediment, which may have been residual biomass from *Asparagopsis* or from raw canola (as the oil was a cold-pressed extraction). The clear oil supernatant was removed and used for the experiment, while the sediment was discarded, which may potentially explain the different results of Asp-Oil stability in the stock solution. The stability of the Asp-Oil product stored at 4 °C in the present experiment was similar to FD-Asp stored at 4 °C (5.0 % loss of $CHBr_3$), but better than FD-Asp stored at 25 ° C (37.8 % loss, Magnusson *et al.* (2020)). More samples of Asp-Oil, from a range of source batches, should be tested over a range of storage conditions to establish the shelf-life stability of this product.

5.5 Animal performance

In the present experiment (n = 5 replicate animals per treatment), there was no effect of Asp-Oil treatment on DM intake, liveweight gain or feed efficiency, but this experiment was not sufficiently replicated (powered) to detect differences of less than 15% between groups. Faecal starch excretion was unaffected by Asp-Oil, which concurs with previous research demonstrating no effect of FD-Asp inclusion on starch digestibility (Stefenoni *et al.* 2021). Previous assessment of dry matter digestibility *in vitro* found no effect of FD-Asp (Kinley et al. 2016a; Kinley et al. 2016b; Machado et al. 2016; Kinley et al. 2021). Rumen pH was unaffected by treatment, corresponding with previous work in pellet-fed sheep (Li *et al.* 2018)

Total VFA and molar proportions of VFA were unaffected by Asp-Oil. Past literature using FD-Asp contains conflicting results about whether the diversion of H^+ from CH_4 results in an increase in certain VFAs as an alternative hydrogen sink (Ungerfeld 2015). Li *et al.* (2018) and Kinley *et al.* (2020) both found no effect of 1.23 % and 0.37 % (respectively) FD-Asp /kg DM on total VFA production, but reported substantial decreases in acetate:propionate ratio for sheep and cattle fed high roughage diets. Meanwhile, 0.5 % *Asparagopsis*/kg DM reduced total VFA production with no change in acetate:propionate ratio in dairy cows (overall, although there were indications of loss of $CHBr_3$ content and methane reduction potential over the course of that experiment, Stefenoni *et al.* (2021). The lack of consensus in findings regarding diversion of H^+ to beneficial sinks such as propionate, which is an energy pre-cursor in ruminants, indicates a need for more directed research into productive efficiency co-benefits from feeding *Asparagopsis* or its extracts. The present study was not able to confirm or dispute previous reports of productivity gains (Kinley et al. 2020; Roque et at. 2021) through demonstration of more advantageous VFA profiles, and there was no demonstration of improvement in gain:feed ratio. Productivity co-benefits to

antimethanogenic efficacy of Asp-Oil need to be resolved in more highly-powered experiments that permit for full expression of productive potential by cattle.

5.6 Carcase grading and eating quality

There was no effect of Asp-Oil treatment on carcase grading or eating quality, but overall, all animals in this experiment including control had small, lean carcases, low MSA index scores, and low consumer eating quality scores. This is not unexpected, since the use of heifers, intensive research conditions, and frequent sampling events will all contribute to poorer production outcomes than expected in a commercial feedlot setting. There was no effect of treatment on carcase grading measures, apart from a biologically insignificant increase in pH_u in the High Asp-Oil group. The consumer sensory scores were low in comparison to well-grown grain-finished commercial cattle, and had much greater within group variance than is normally observed. This affected the ability to detect between-group differences, where group sizes were small (4 – 5 carcases per group). A production-trial, sufficiently powered to detect 5% difference in productivity and 10-point difference in sensory scores, conducted in a commercially-relevant setting, is essential for confirmation that the use of Asp-Oil in feedlot production systems has no negative impact on meat eating quality.

6 Conclusions/Recommendations

This project was the first to test Asp-Oil in feedlot diets and has confirmed the safety and effectiveness of an *Asparagopsis* extract in a canola oil carrier in Australian short-fed feedlot diets. Through gold-standard respiration chambers, CH_4 emissions were monitored in heifers provided 34 and 51 mg $CHBr_3$/kg DMI and demonstrated 85.2 – 96.3 % reduction in CH_4 production and yield overall, and 97.9 – 99.3 % reduction in the finisher diet, with Medium and High Asp-Oil inclusion levels, respectively.

There was no effect of Asp-Oil treatment on rumen temperature, pH, redox potential, VFA and ammonia production, rumen pathology and histopathology, over the whole feeding period. Although no differences were detected in animal productivity and carcase parameters, the experiment was not sufficiently powered to detect differences in these, and so production responses to Asp-Oil need to be tested in a more highly replicated and commercially-relevant setting.

There was no detectable $CHBr_3$ in any carcase samples, and iodine and bromide residues in kidneys were at levels unlikely to lead to consumers exceeding recommended maximum intakes.

Overall, Asp-Oil was found to be safe and effective at reducing CH_4 emissions and yield by greater than 90%.

7 Key Messages

- Asp-Oil is equally as efficacious as FD-Asp for the same $CHBr_3$ inclusion in feedlot diets.
- Maximal CH_4 yield mitigation (85.2 to 95.0%; overall feeding period) was achieved from the Medium and High Asp-Oil treatments.
- There was no effect of Asp-Oil on animal or carcase performance, but to determine production impacts of Asp-Oil, this product needs to be tested in a highly replicated setting typical of commercial feedlot conditions.
- All Asp-Oil treatment diets were below published upper tolerable limits for cattle for $CHBr_3$, bromine and iodine, and no health effects on the cattle could be ascribed to the Asp-Oil treatments.
- There was no excretion of bromoform to the environment via faeces.
- There was no accumulation of $CHBr_3$ in the carcase, and although iodide and bromide depots in the kidney increased with Asp-Oil treatment, it is unlikely that consumption of these kidneys would lead to a consumer exceeding maximum recommended intakes of iodide or bromide.
- Asp-Oil is a safe, effective, and logistically feasible product for mitigating CH_4 emissions in feedlot diets

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9 Appendix 1. Data of animals removed from analysis

Animal ID	19 (Control)	76 (High Asp-Oil)
Methane (CH_4) emissions		
Total CH_4, g/d	72.83	9.87
CH_4 yield, g/kg DMI	11.74	2.89
Animal performance		
DMI, kg/d	6.14	3.77
DMI, g/kg LW	6.47	3.77
Initial LW, kg	324	356
Final LW (d 80/81), kg	366	342
Average daily gain (kg/day)	0.41	-0.14
Gain:Feed (kg/kg)	0.07	-
Rumen function measures		
pH	6.08	5.69
Reduction potential	-24.50	-103.29
Faecal starch (%)	3.57	3.38
Protozoa counts		
Total protozoa ($\times10^3$/mL)	4.0	2.3
Large holotrich ($\times10^3$/mL)	0.0	2.0
Small holotrich ($\times10^3$/mL)	0.0	0
Entodiniomorphs ($\times10^3$/mL)	4.0	0.3
Rumen volatile fatty acid molar proportions		
Total VFAs (mmol/L)	82	55
Acetic acid (mmol/mmol)	567	285
Propionic acid (mmol/mmol)	175	154
Butyric acid (mmol/mmol))	173	76.3
Iso-Butyric acid (µmol/mmol)	7	4
Valeric acid (µmol/mmol)	15	9
Iso-Valeric acid (µmol/mmol)	9	16
Caproic acid (µmol/mol)	10	3
Acetate:Propionate	3.23	1.84
Rumen ammonium-N (mg/L)	47.40	254.35
Rumen wall morphology		
Papillae colour (1-5 scale)	0.5	0
Papillae shape (1-4 scale)	1	0
Ventral sac damage (1-5 scale)	0	0
Blood cell concentrations of plasma		
Leucocytes ($\times10^6$/mL)	7.91	12.61
Neutrophils ($\times10^6$/mL)	2.22	5.15
Lymphocytes ($\times10^6$/mL)	4.75	6.01
Monocytes ()	0.74	0.85
Eosinophils ($\times10^6$/mL)	0.11	0.49
Basophils ($\times10^6$/mL)	0.09	0.15
Erythrocyte ($\times10^9$/mL)	7.22	8.42
Haemoglobin g/dL	11.53	12.23
Haptoglobin (mg/L)	549.06	311.19
Haematocrit (%)	34.34	35.40
Mean corpuscular volume (fL)	42.04	42.03
Mean corpuscular haemoglobin (pg)	14.08	14.44
Mean corpuscular haemoglobin concentration (g/dL)	33.48	34.24
Platelets ($\times10^6$/mL)	493.53	690.47
Hormone and vitamin B12 metabolism		
Thyroxine, ng/mL	66.91	69.38
Triiothyronine, ng/mL	2.44	1.41
Faecal cortisol, ng/g	1.15	0.90
Serum Vitamin B_{12} , *p*g/mL	33.69	59.93
Serum residues, mg/L		
Iodine	1.29	0.71
Bromide	8.38	29.04
Faecal residues, mg/kg		

Animal ID	19 (Control)	76 (High Asp-Oil)
Iodine	0.42	0.71
Bromide	228.71	269.74
Bromoform ($CHBr_3$) concentration[1] (mg/kg)		
Kidney	ND	ND
Liver	ND	ND
Fat	ND	ND
Striploin	ND	ND
Bromide (Br^-) concentration[1] (mg/kg)		
Kidney	13	14
Liver	6	8
Fat	ND	ND
Striploin	ND	ND
Iodide (I^-) concentration[1] (mg/kg)		
Kidney	0.1	0.1
Liver	ND	ND
Fat	ND	ND
Striploin	ND	ND
Carcase grading		
HSCW, kg	176.4	151.6
Dressing percentage, %	48.1	44.7
P8 fat, mm	6	3
Rib fat, mm	4	2
EMA, cm2	43	74
MSA Marbling score	400	230
AUSMEAT Marbling score	2	0
AUSMEAT fat colour score	1	1
AUSMEAT meat colour score	5	6
Ossification score	140	140
pH_u	5.65	5.76
MSA index	56.23	-
Shear force, kg	4.66	7.42
Striploin SPC, cfu/g		
Day 10	<100	<100
Day 20	150000	<100
Day 30	6700	30000
Day 40	<100	60000
Day 50	22000	13000000
Day 60	2200000	520000
Day 70	15000000	1600000
Day 80	4000000	5400000
Day 90	36000000	7000000
Striploin *E. Coli*, cfu/g		
Day 10	<10	<10
Day 20	<10	<10
Day 30	<10	<10
Day 40	<10	<10
Day 50	<10	<10
Day 60	<10	<10
Day 70	<10	<10
Day 80	<10	<10
Day 90	<10	<10
Striploin Coliforms, cfu/g	<10	<10
Day 10	<10	<10
Day 20	<10	<10
Day 30	<10	<10
Day 40	<10	<10
Day 50	<10	<10
Day 60	<10	<10
Day 70	<10	<10
Day 80	<10	<10
Day 90	<10	<10