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Dose-response of Asparagopsis extract and diet quality effects enteric methane emissions for sheep fed high-forage diets

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ABSTRACT

Asparagopsis spp. (Asparagopsis) receives considerable attention, due to the antimethanogenic properties it exhibits when included in diets of ruminant livestock. However, only one previous study has investigated the use of Asparagopsis in sheep *in vivo,* offered as part of a modified total mixed ration, reporting up to 80 % enteric methane (CH_4) abatement at an inclusion of 3 % (organic-matter basis). The objective of this study was to investigate the interaction of bromoform ($CHBr_3$) dose and basal diet quality on CH_4 abatement in sheep, when fed a high forage basal diet with supplementary pellets containing $CHBr_3$ extracted from *Asparagopsis armata* in oil (Asp-oil). Sixty merino wethers were used in a split plot design of five supplementary pellet treatments (100 g/d) of increasing $CHBr_3$ concentrations (A0, 0 mg $CHBr_3$/kg DM; A1, 36 mg $CHBr_3$/kg DM; A2, 72 mg $CHBr_3$/kg DM; A3, 108 mg $CHBr_3$/kg DM; and A4, 144 mg $CHBr_3$/kg DM) and fed either a high-quality (neutral detergent fibre (NDF) 40 %) or low-quality (NDF 55 %) forage basal diet. Wethers were housed in individual pens for 117 days to allow for measurements of feed intake and production parameters. Five measurements of CH_4 production (MP, g CH_4/d), 14 days apart, were conducted with open circuit respiration chambers. Wool growth (g/d), intake of basal diet and supplementary pellets (kg DMI/d), and average daily gain (kg/d) were production parameters. There was no significant interaction between diet × Asp-oil level on CH_4 yield (MY, g CH_4/kg DMI), but independently each had a significant effect. In comparison to their respective control (A0), A4 resulted in a 12 % and 19 % MY abatement for high-quality and low-quality diets, respectively. Average daily gain was significantly affected by diet ($P < 0.001$) but not Asp-oil inclusion. Wool growth rate was affected by diet ($P = 0.05$) only. This study has demonstrated that feeding sheep Asp-oil can reduced MP, regardless of diet quality. However, basal diet continues to influence MY. Future grazing trials are necessary to validate the results of this animal house experiment.

1. Introduction

It is predicted that by 2030, the Australian sheep flock will contribute approximately 23 % to Australia's agricultural enteric methane (CH_4) emissions (Department of Climate Change, Energy, the Environment and Water, 2023). However, there has been minimal research conducted in small ruminants with regard to CH_4 abatement. The biggest challenge for the Australian sheep sector is that the majority (approximately 98 %) of production is pasture based (Badgery et al., 2023) thereby creating hurdles for accurate CH_4 abatement prediction and delivery of potential abatement strategies. Methane production (MP, g CH_4/d) is influenced by feed intake (Fouts et al., 2022) such that increases in feed intake result in more MP (Shibata et al., 1993). However, MP cannot be explained by intake alone and quality of feed needs to be taken into account (Moe and Tyrell, 1979). Sheep consume roughage diets to a constant level of rumen fill (Thornton and Minson, 1972). Low-quality forage diets (< 60 % digestibility), characterised by higher lignin content, can result in longer rumen retention time and lower daily intake (Niderkorn and Baumont, 2009). Contrastingly, high-quality forage diets (> 65 % digestibility) have a shorter rumen retention time, thereby resulting in animals eating more (Campling and Balch, 1961; Conrad et al., 1964; Montgomery and Baumgardt, 1965).

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Increased rumen retention time is associated with increased CH_4 yield (MY, g CH_4/kg DMI, Blaxter et al., 1961; Van Soest, 1975) due to greater fermentation time of structural carbohydrates (Pinares-Patiño et al., 2011). As a result both feed intake and rumen retention time, influenced by feed quality, need to be considered for predicting CH_4 emissions. Seasonal changes in pasture quality result in differing nutritient profiles. Therefore, there is no "one size fits all" approach in understanding possible MP and MY from sheep in pasture systems. A knowledge gap continues to exist for understanding how feed additives can be implemented into pasture-based systems (Beauchemin et al., 2020). Supplementary feeding to manage nutritional gaps in pasture systems is common for sheep under Australian conditions (Rowe, 2003). However, to our knowledge there has been no previous work investigating the use of feed additives into supplementary practices as a direct CH_4 abatement strategy for sheep.

Asparagopsis spp. (Asparagopsis) has received much attention due to promising abatement potential (Beauchemin et al., 2020). The specialised gland cells in Asparagopsis accumulate and release the halogenated compound bromoform ($CHBr_3$, Paul et al., 2006; Abbott et al., 2020). Bromoform interacts with reduced vitamin B_{12} to inhibit the cobamide-dependent methyl group in the final step of methanogenesis (Wood et al., 1968) thereby reducing MP. Previous work using kiln-dried Asparagopsis has reported enteric CH_4 abatement in sheep of up to 80 % (Li et al., 2016). There have been concerns regarding the commercial applicability of freeze-dried or kiln-dried Asparagopsis due to limited shelf-life stability (Magnusson et al., 2020). As a result, a vegetable oil-based product (Asp-oil) has been developed to improve shelf-life stability (Tan et al., 2023) and has comparable results to the freeze-dried medium (Kinley et al., 2022). Asp-oil has demonstrated a 99 % CH_4 abatement in beef cattle when fed in a total-mixed ration (TMR, Cowley et al., 2023, 2024) and 44 % in dairy cows fed via a grain supplement twice daily (Alvarez-Hess et al., 2023, 2024).

At present there have been no *in vivo* studies conducted in sheep using Asp-oil. Bromoform is short-lived in the rumen (Romero et al., 2023; Indugu et al., 2024) and requires continuous intake to be effective. The integration of Asp-oil into intensive beef and pasture-based dairy systems is relatively straight forward, where the additive can be administered in a TMR or in a grain supplement during milking, respectively. A knowledge gap exists in understanding the effectiveness of Asp-oil in a supplementary feeding regime under more extensive conditions, such as those in sheep production. Basal pasture diet quality may also affect the efficacy of the inhibitor. Consequently, transposing outcomes from high grain content studies and/or intensive feeding systems (Alvarez-Hess et al., 2023, 2024; Cowley et al., 2023, 2024) to predict extensive pasture-based CH_4 abatement may not be appropriate.

Several knowledge gaps exist for integrating Asparagopsis as a feed additive strategy in sheep pasture-based systems. It is unknown whether a supplementary pellet can be considered as an effective Asp-oil delivery mechanism. There is a knowledge gap in understanding the influence of pasture quality and Asp-oil inclusion rate on potential CH_4 abatement, and its potential interactions with productivity. The primary objective of this study was to investigate the effect of different levels of Asp-oil, in supplementary pellets, on CH_4 abatement in Merino superfine wethers fed forage basal diets of contrasting fibre and crude protein (CP) content.

2. Materials and methods

Experimental procedures were approved by the University of New England (UNE) Animal Ethics Committee (Authority no: ARA22–022).

2.1. Experimental design and animals

The study used a split plot design with two contrasting basal diets (main plot), each with five dietary Asp-oil inclusion levels (sub-plot). The experimental unit was an individual sheep, with six sheep allocated to each Asp-oil treatment. A sample size of six sheep was selected based on an expected treatment effect of at least a 15 % reduction in CH_4 emissions (Li et al., 2016).

The trial period was 117 days (d), which included an acclimation period to the animal house for 24 d, a dietary adaptation of 23 d and 70 d of enteric CH_4 and feed intake measurements. Sixty superfine merino weaner wethers (initial liveweight (LW) 22.8 ± 0.4 kg (mean ± sem)) were selected from a commercial flock at UNE, Armidale New South Wales (NSW), Australia, and transported to the UNE Centre of Animal Research and Training (30°29'6.237" S, 151° 38' 16.72" E). On arrival at the animal house, wethers were randomly assigned to individual pens and remained in that pen for the duration of the trial. At the start of their adaptation period (d −24 to d 0), wethers were drenched for internal parasites (Avomec Dual® (abamectin 0.1 % w/v and closantal 5 % w/v) Boehringer Ingelheim, Germany; Duocare® LV Plus SE (fenbendazole 2.5 % w/v, levamisole hydrochloride 4 % w/v and selenium 0.05 % w/v) Virbac, France; and Tridectin® (levamisole 4 % w/v, moxidectin 0.1 % w/v and albendazole 2.5 % w/v) Virbac, France) according to label recommendations. During the adaptation period, wethers were fed a 40:60 mix of lucerne (*Medicago sativa*) and wheaten chaff (*Triticum spp*) *ad libitum*, allowing for a 5 % refusal, and offered approximately 50 g of a proprietary pellet (All-Purpose, Mort & Co, Guyra NSW). After the adaptation period, wethers were stratified by LW and the average flock LW calculated. Wethers were then allocated to one of 10 groups which were balanced between lighter and heavier wethers to ensure each group had a mean LW of 22.8 ± 0.3 kg (± sem). Each group was then randomly allocated to a basal diet × Asp-oil inclusion level treatment.

2.2. Diets and feeding

Basal diets consisted of two different chaff combinations, designed to represent a high or low-quality pasture equivalent with contrasting NDF and CP content (Table 1). The high-quality (HQ) diet consisted of an 80:20 mix of lucerne chaff (*Medicago sativa*) and wheaten chaff (*Triticum spp.),* and the low-quality (LQ) diet consisted of 100 % wheaten chaff (*Triticum spp*.).

The basal diets were supplemented with pellets containing five different levels of Asp-oil (A0 (negative control, canola oil free of $CHBr_3$), A1, A2, A3 and A4) (Table 2). Asp-oil (SeaFeed™, SeaForest Pty Ltd, Triabunna, Tasmania, Australia, 3555 mg $CHBr_3$/kg WMB) was balanced with canola oil free from $CHBr_3$ to achieve 5 % (by mass) total oil inclusion in all pellets (Table 2). Batches of pellets (8.5 kg) were mixed with Asp-oil applied by a pump (Redline 25 L Rechargeable Trolley Sprayer, Silvan Pty Ltd, Victoria, Australia) mounted on a platform scale (PB Portable Bench Scale, CAS Pty Ltd, Queensland Australia). The pellet-oil combination was mixed for 15 minutes (min) in a 3.5 CU Cement Mixer (Easy Mix Pty Ltd, Queensland, Australia) to ensure homogeneity. Batches of Asp-oil level pellets were mixed in

Table 1
Composition of lucerne and wheaten chaff and supplementary pellet fed to the experimental animals over the 117 d.

	Lucerne Chaff	Wheaten Chaff	All Purpose pellet[a]
Dry matter (DM, %)	83.2	86.3	89.3
Crude Protein (% of DM)	23.7	8.8	17.1
Acid Detergent Fibre (% of DM)	29.4	30.6	14.4
Neutral Detergent Fibre (% of DM)	35.4	55.2	21.3
Digestibility (DMD, % of DM)	72.1	60.5	82.7
ME[b] (Calculated, MJ/kg DM, % of DM)	10.8	8.8	12.4
Fat (% of DM)	4.0	2.7	< 1.0
Ash (% of DM)	3.2	6.5	5.9

[a] All Purpose, Mort & Co, Guyra NSW, prior to Asp-oil addition;
[b] $E = (0.203 \times DOMD\%) - 3.001$

Table 2
Inclusion of canola oil and oil containing *Asparagopsis* bioactives (Asp-oil, by mass) for formulated and observed bromoform levels in pellets.

Asp-oil level	Total oil (g)	Asp-oil (g)	Canola oil (g)	Formulated bromoform (mg $CHBr_3$/kg DM pellets)	Mean (± SD) recovered bromoform (mg $CHBr_3$/kg DM pellets)
A0	425	0	425	0	2 ± 0.7
A1	425	85	340	36	23 ± 6.2
A2	425	170	255	72	45 ± 10.5
A3	425	255	170	108	67 ± 13.3
A4	425	340	85	144	88 ± 13.0

ascending order of Asp-oil inclusion. At the end of each mixing session, the mixer and sprayer were decontaminated using detergent and hot water. Experimental pellets were stored in air-tight containers in a cool room (approximately 4 °C) prior to feeding.

The inclusion of pellets in the diet was limited to 100 g/d to ensure feeding was considered representative of a supplementary regime and not a TMR system. Pellets were offered to the wethers in the feeding troughs once daily (8:00). When pellets were consumed by the individual wethers, the respective Asp-oil level chaff mix was offered on *ad libitum* basis, allowing for 5 % refusals. Orts of pellet and chaff were collected after 23 h and weighed. Pellet and chaff samples were taken at the start, mid and end of the experiment for proximate analysis.

2.3. Animal measurements: liveweight and wool characteristics

Individual wether LW was measured prior to feeding at 14 d intervals using a platform scale (KM1 Electronic Weighing System, Gallagher Pty Ltd, Victoria Australia).

Wool growth was measured over a 117 d period using dyebands (1 % Durafur Black Flakes, Imperial Chemical Industries Sydney) placed vertically on the left mid-side position of the sheep (McCloghry, 1997). Dyebands were applied on d 41 and d 85 of the experiment. On d 113 the area of wool containing the dyebands was removed at skin level using Oster clippers No. 40. Samples were placed into a temperature (19 °C) and humidity (44 %) controlled room until wool growth was calculated. On d 117 all animals were shorn and wool growth (g/d) was calculated by multiplying the proportion within the dyeband by total fleece weight (including belly wool prior to skirting, ± 0.01 kg), and then dividing by 44 representing the number of days between dyebands. Wool quality samples were analysed by Australian Wool Testing Authority, Victoria, Australia for fibre diameter (μm), fibre diameter coefficient of variation (FDCV, %; by Laserscan) and staple length (mm; Australian Wool Testing Authority, 2024).

2.4. Ruminal pH, volatile fatty acids and protozoa

Rumen fluid was collected 3 h post feeding on d −8 (baseline) and on d 116 by stomach tubing. Immediately after sampling rumen fluid was tested for pH (Eutech ECPH601PLUS, Thermo Fisher Scientific Inc., Australia) and then subsampled for measurement of volatile fatty acids (VFA), with each sample acidified with 3–4 drops of concentrated (98 %) sulphuric acid. Rumen fluid was also subsampled for rumen protozoa (samples fixed with 4 % formal saline (1:4) and stained with brilliant green and 1500 μL of 30 % glycerol). The VFA samples were stored at −20 °C and protozoa samples at room temperature (approximately 20 °C) until further processing and analysis.

2.5. Enteric methane measurements

Open circuit respiration chambers (n = 8) were used to measure CH_4 emissions. Each respiration chamber (1.3 m × 0.78 m × 1.2 m) was constructed from clear polycarbonate and a galvanised steel frame (Goopy et al., 2014). Chamber space was maintained under negative pressure using two vacuum pumps (HG-750, Zhejiang Daming Mechanical and Electrical Co., Ltd., Zhejiang, China) each drawing on four separate chambers. Flow rate (m^3/h) was determined from the total air volume (m^3) over each 22 h measurement period using a gas meter (AL-800 Diaphragm Meter, Elster American Meter, Nebraska, USA), fitted to the exhaust of each chamber.

Methane emissions were measured over 22 h/wether starting on d 33, 47, 61, 76, 90 and 104. Each CH_4 measurement period took 8 d to complete. All wethers were allocated into a different chamber and day for each measurement period to avoid a chamber or day-effect. Wethers were placed into individual chambers at 9:30 and fed the pellet first. At 10:00 wethers received their *ad libitum* chaff and the chamber was closed. After 22 h, feed refusals from each chamber were collected and recorded to calculate net feed intake. Wethers were returned to their individual pens and remained there until the subsequent CH_4 measurement.

Methane concentration was determined by air sampled continuously at 1.5 mL/min into individual tedlar bags (n = 8), each attached to a peristaltic pump in each chamber. Ambient air samples over each measurement period were also collected into a separate tedlar bag, using a peristaltic pump for analysis of background CH_4 concentration. Individual tedlar bags were analysed each morning for CH_4 concentration, by directly injecting air samples into the infrared sensor of a sheep GreenFeed Unit (GreenFeed Number 63, GreenFeed C-Lock Inc) located in an adjacent enclosed room maintained at approximately 20 °C. Methane output values (millivolt) were converted to ppm (parts per million) CH_4 using a coefficient calculated from the weekly infrared calibration data. Methane emissions were reported as MP and MY. Emptied tedlar bags were flushed with air and nitrogen prior to reinstallation for the following 22 h measurement.

The infrared sensor was calibrated weekly by injecting a known CH_4 gas (zero ppm, 104 ppm and 1000 ppm) into the GreenFeed Unit and recording outputs for each concentration. Chamber recoveries for CH_4 were conducted on d 19, 60 and 113 by injecting a known flowrate of a CH_4 standard gas (25 mL/min, 99 % CH_4, Clear Gas Pty Ltd, Victoria, Australia) and measured using a flame ionisation detection device. Recovery values (95–110 % across the eight chambers) were used to correct CH_4 measurements for each individual chamber and extrapolated to 24 h.

2.6. Laboratory analysis and processing

2.6.1. Feed analysis

After each pellet mixing session, a sub-sample of approximately 100 g was collected and stored in glass vials for $CHBr_3$ testing. As part of a preliminary stability study, after the first mixing session an additional 1 kg of pellets for each Asp-oil level was put into zip-lock bags, with half stored at room temperature (20.4 °C) and the other half in a cool room (approximately 3.8 °C). These pellets were tested monthly for $CHBr_3$ concentrations. Recovered $CHBr_3$ concentrations in all pellet samples were determined by GC-MS (Analytical Services Hobart, Tasmania, Australia) following the methodology adapted by Romanazzi et al. (2021) using a static-headspace with GCMS, rather than a direct injection of methanolic extract onto a polar GC column. Nutritive composition for chaffs and pellets were determined by Near Infrared Spectroscopy (FT/003, Agrifood Technology Victoria, Australia).

2.6.2. Ruminal fluid: volatile fatty acids and protozoa

Volatile fatty acids analysis was adapted from Nolan et al. (2010), which was determined on two replicates of each sample centrifuged for 10 min at 10,000 rpm (rev per min) at room temperature. The centrifuged sample (500 μL) was transferred and mixed with 1000 μL Internal Standard (1.6 % v/v isocaproic acid, 12 % v/v metaphosphoric acid and RO water). Analysis for total VFA (tVFA, mM) and individual VFA concentrations was conducted with a Varian CP-38000 Gas

Chromatograph (Artisan Technology Group, UltiMetal packed column, length 6 ft (1.83 M), 1/8 in. OD, 2 mm ID, Chromosorb 101, mesh size 80100, pre-conditioned).

Protozoa counting was adapted from Villar et al. (2020): one drop of the stained sample was transferred onto a 4 × 4 Fuchs-Rosenthal Chamber grid slide (cell depth 0.2 mm, 16 × 1 mm 2 squares) with a cover slip. Large and small Isotricha, and large and small Entodinia were counted using a × 100 magnification for each sample. Two replicates of each sample were counted. Total Protozoan numbers per millilitre of rumen content was calculated from the average of the two-sub samples.

2.7. Statistical analysis

The data from two animals in the LQ – A3 treatment were excluded from analysis due to declining daily feed intakes. Data were analysed using R Studio (R Core Team, 2016). Distributional assumptions of normality of residuals was checked using Shapiro-Wilks test and kurtosis. Data that was not normally distributed (i.e. wool growth and protozoa) were $\log_{10}$ transformed. Individual LW measurements over time were used to determine average daily gain (ADG) by linear regression. Several models of variance-covariance were tested for each variable and the model of best fit was chosen by the lowest Akaike Information Criterion value. Data were analysed in a mixed effect model using the *lme4* package (Bates et al., 2015) that included diet, Asp-oil level and their interaction as fixed effects and animal as random effect, as follows:

$$Y_{ij} = \mu + D_i + A_j + D_i \times A_j + e_{ij}$$

Where Y_{ij} is the dependent variable, μ is the mean of the variable, D_i is the diet effect, A_j is the Asp-oil level effect, $D_i \times A_j$ is the interaction between diet and Asp-oil level and e_{ij} is the residual error.

Protozoa was heavily zero-inflated and analysed with Kruskal-Wallis test. Repeated measures of MP and MY were analysed with mixed model linear regression, with linear and orthogonal contrasts performed on Asp-oil level using Holm-Bonferroni adjustment for multiple Asp-oil level groups. Inverse $\log_{10}$ of least square means were reported for wool growth and protozoa.

3. Results

3.1. Liveweight and average daily gain

There was no significant interaction of diet × Asp-oil on final LW (P = 0.98; Table 3). Diet had a significant effect (P < 0.001) on LW such that wethers on the HQ diet had an average final LW of 38.1 ± 7.08 kg (mean ± sem) whereas wethers on LQ diet had an average final LW of 30.5 ± 5.76 kg. Asp-oil had no linear nor quadratic main effect on LW (P = 1.00 for both).

There was no significant interaction of diet × Asp-oil on ADG (P = 0.83; Table 3). Diet had a significant main effect (P < 0.001) on ADG such that wethers on the HQ diet had a higher ADG in comparison to wethers on LQ diet. Asp-oil had no quadratic nor linear effect on ADG (P = 1.00 and P = 0.83, respectively).

3.2. Dry matter intake: total, chaff, pellet, bromoform

There was no significant interaction between diet × Asp-oil level for total dry matter intake (DMI, P = 0.99; Table 3). Diet had a significant effect on total DMI (P < 0.001) such that wethers on the HQ diet ate more (1.14 ± 0.01 kg DM/d, mean ± sem) than wethers on the LQ diet (0.80 ± 0.01 kg DM/d). Asp-oil had neither a linear nor quadratic effect on total DMI (P = 1.00 for both). There was no significant interaction between diet x Asp-oil level for pellet DMI (P = 1.00). Diet had no significant effect on pellet DMI (P = 1.00), and inclusion of Asp-oil had no linear nor quadratic effect (P = 1.00 for both). There was no significant

Table 3
Mean (± sem) dry matter intake (kg/d) bromoform intake (g $CHBr_3$/kg DMI), average daily gain (kg/d), start and final live weight (kg), CH_4 production (g CH_4/d) and CH_4 yield (g CH_4/kg DMI), for Merino wethers fed varying inclusions of oil containing *Asparagopsis* bioactives (Asp-oil) incorporated into a supplementary pellet with either a high-quality or low-quality basal chaff diet.

	High-Quality Chaff					Low-Quality Chaff					*P*-value			
	A0	A1	A2	A3	A4	A0	A1	A2	A3	A4	S.E.M	Diet	Asp-oil	Diet × Asp-oil
													L	*Q*	
Initial liveweight (kg)	22.8	22.8	22.8	22.4	23.2	22.8	23.0	22.7	22.6	22.9	0.90	1.00	1.00	1.00	1.00
Final liveweight (kg)	37.0	38.3	39.2	37.1	39.0	30.1	30.0	30.9	29.6	31.6	1.35	< 0.001	1.00	1.00	0.98
Average daily gain (kg/d)	0.10	0.11	0.10	0.11	0.11	0.07	0.07	0.07	0.07	0.07	0.004	< 0.001	1.00	0.83	0.83
Total intake (kg DM/d)	1.08	1.19	1.14	1.14	1.16	0.81	0.79	0.82	0.80	0.80	0.480	< 0.001	1.00	1.00	0.99
Pellet intake (kg DM/d)	0.089	0.089	0.089	0.089	0.089	0.089	0.089	0.089	0.089	0.089	1.00	1.00	1.00	1.00	1.00
Chaff intake (kg DM/d)	0.99	1.10	1.05	1.05	1.07	0.72	0.74	0.73	0.71	0.71	0.336	< 0.001	1.00	1.00	0.77
Bromoform intake (mg $CHBr_3$/kg DMI)[1]	0.00	2.89	6.01	8.90	11.87	0.00	4.54	8.77	13.65	17.56	0.343	0.001	< 0.001	1.00	< 0.001
Methane Production (g CH_4/d)	16.5	16.8	16.4	16.0	15.5	13.8	12.9	12.5	12.0	10.6	0.69	< 0.0001	0.01	0.60	0.64
Methane Yield (g CH_4/kg DMI)	15.5	14.4	14.6	14.2	13.6	17.3	16.3	14.9	15.5	13.2	0.45	0.01	< 0.0001	0.97	0.12

P-values are Bonferroni-Holm-adjusted. S.E.M: pooled standard error means. Levels of Asp-oil inclusion in pellet: A0 (0 mg $CHBr_3$/kg DM), A1 (36 mg $CHBr_3$/kg DM), A2 (72 mg $CHBr_3$/kg DM), A3 (108 mg $CHBr_3$/kg DM) and A4 (144 mg $CHBr_3$/kg DM). [1]Bromoform intake calculated based on formulated inclusion rates.

interaction between diet x Asp-oil level for chaff DMI (P = 0.77). Diet had a significant effect on chaff DMI (P < 0.001), such that wethers on the HQ diet ate more than wethers on the LQ diet (1.05 ± 0.09 kg DM/d versus 0.72 ± 0.06 kg DM/d, respectively). There was no significant linear nor quadratic effect of Asp-oil on chaff intake (P = 1.00 for both). There was a significant interaction between diet x Asp-oil level associated with $CHBr_3$ intake (P < 0.001), such that wethers on the LQ diet had lower $CHBr_3$ intakes than those on the HQ diet, regardless of Asp-oil treatment.

3.3. Methane production and yield

There was no significant interaction between diet × Asp-oil level for MP (P = 0.64) or MY (P = 0.12; Table 3). Diet had a significant effect on MP (P < 0.0001) such that control (A0) wethers on the LQ diet had a lower MP (13.8 g CH_4/d) in comparison to A0 wethers fed HQ diet (16.5 g CH_4/d). Methane yield was significantly influenced by diet (P = 0.01), such that A0 wethers fed HQ diet had a lower MY (15.5 g CH_4/kg DMI) in comparison to A0 wethers on LQ diet (17.3 g CH_4/kg DMI).

There was a negative linear relationship between increasing Asp-oil treatment on MP abatement (P = 0.01; Fig. 1). In comparison to HQ-A0, a 6 % reduction in MP was observed for wethers fed HQ-A4. In comparison to LQ-A0, a 23 % reduction in MP was observed for wethers fed LQ-A4.

There was a negative linear relationship of Asp-oil on MY (P < 0.0001; Fig. 2). In comparison to HQ-A0, a 12 % MY abatement was observed for wethers fed HQ-A4. In comparison to LQ-A0, a 19 % MY abatement was observed for wethers fed LQ-A4.

3.4. Ruminal pH and fermentation parameters

Ruminal pH for wethers ranged between 6.55 and 6.86 (Table 4). There was no significant interaction between diet × Asp-oil level on ruminal pH (P = 0.96), nor was there any significant effect of diet (P = 0.37) nor Asp-oil level (linear P = 0.64, quadratic P = 0.07).

There was no significant interaction between diet × Asp-oil level for tVFA (P = 0.31; Table 4). Diet had a significant effect on tVFA (P < 0.0001) such that wethers on the HQ diet had higher tVFA in comparison to wethers fed the LQ diet. There was no significant interaction between diet × Asp-oil level for any of the individual VFA concentrations such as acetate (P = 0.26), propionate (P = 0.31), butyrate (P = 0.41), iso-butyrate (P = 0.98), valerate (P = 0.70) and iso-valerate (P = 0.47), and acetate-to-propionate ratio (A:P, P = 0.92). Diet also had a significant effect on acetate (P < 0.0001), propionate (P < 0.0001), iso-butyrate (P < 0.001), valerate (P < 0.0001) and A:P ratio (P < 0.0001; Table 4). Increasing inclusion of Asp-oil resulted in a linear reduction in acetate (P = 0.02). Asp-oil level had a significant linear (P < 0.0001) and quadratic effect on A:P ratio (P = 0.01).

There was a no significant interaction between diet × Asp-oil level for total Protozoa (P = 0.20), large Entodinia (P = 0.10) and small Entodinia (P = 0.09), large Isotricha (P = 0.70) and small Isotricha (P = 0.50; Table 5). There was a significant effect of diet on large Entodinia (P = 0.03), small Entodinia (P = 0.01) and large Isotricha (P = 0.003). Protozoa counts were not significantly affected by the inclusion of Asp-oil.

3.5. Wool growth and quality

There was no significant interaction of diet × Asp-oil level on wool growth (P = 0.12), mean fibre diameter (P = 0.16), FDCV (P = 0.53) nor staple length (P = 0.17; Table 6). Diet significantly increased wool growth (P = 0.05), mean fibre diameter (P < 0.0001), and staple length (P < 0.0001) for wethers fed the HQ diet in comparison to the LQ diet. Asp-oil level had no significant linear nor quadratic effect on wool growth (P = 1.00 and P = 0.13, respectively), mean fibre diameter (P = 1.00 for both), FDCV (P = 1.00 for both), curvature (P = 1.00 for both) and staple length (P = 1.00 for both).

3.6. Bromoform recovery from pellets

All Asp-oil pellets lost $CHBr_3$ concentration over the five months, regardless of storage conditions (Fig. 3 and Fig. 4). At the five-month mark, Asp-oil pellets stored at room temperature demonstrated a $CHBr_3$ loss ranging from 95 % to 97 % compared with month one. At the five-month mark for pellets stored in the cool room, $CHBr_3$ concentration losses ranged from 75 % to 80 %.

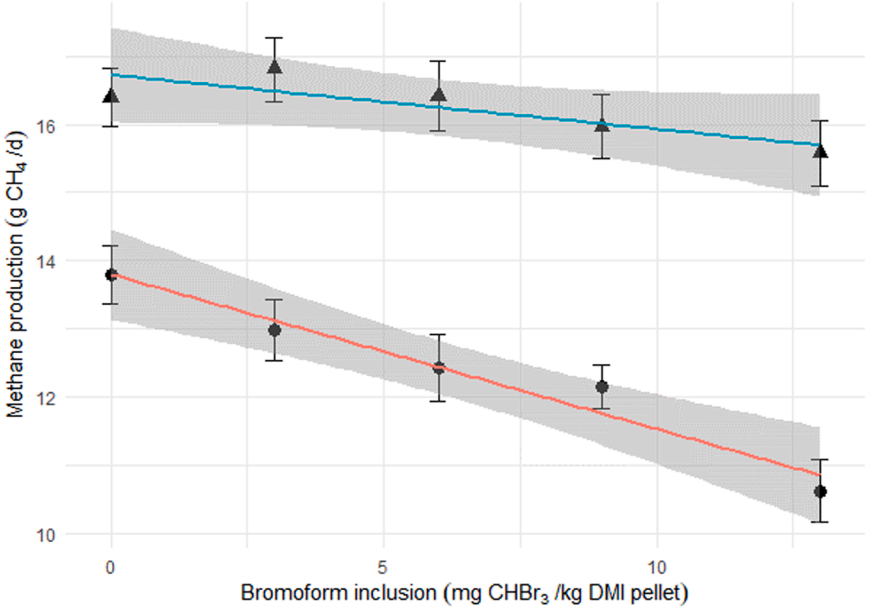

Fig. 1. The relationship between mean methane production (g CH_4/d) and bromoform inclusion (mg $CHBr_3$/kg DMI pellet) for Merino weaned wethers over 117 days for high-quality (triangle, blue line of best fit) and low-quality (circle, orange line of best fit) basal chaff diets. Error bars represent standard error. Shaded grey represents confidence interval of 95 % for line of best fit.

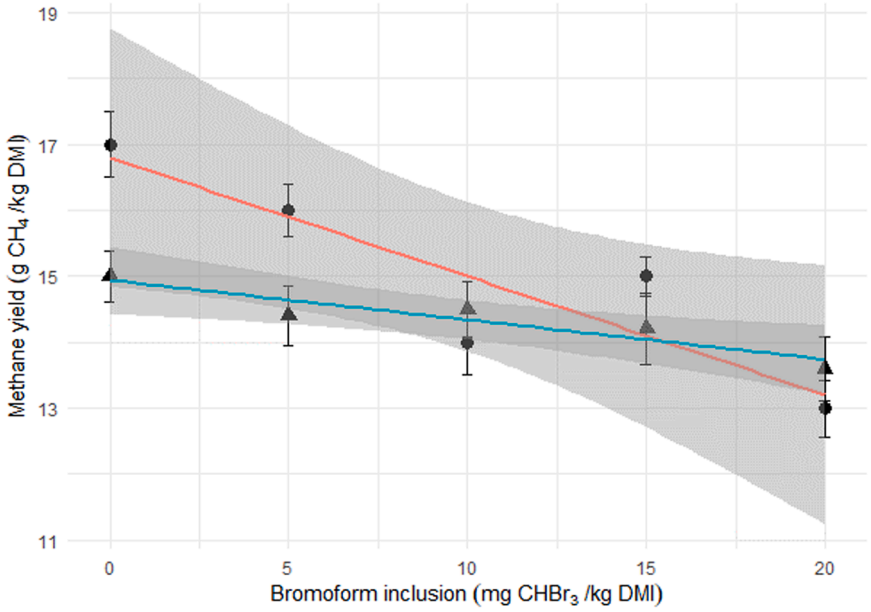

Fig. 2. The relationship between mean methane yield (g CH_4/kg DMI) and bromoform inclusion (mg $CHBr_3$/kg total DMI) for Merino weaned wethers over 117 days for high-quality (triangle, blue line of best fit) and low-quality (circle, orange line of best fit) basal chaff diets. Error bars represent standard error. Shaded grey represents confidence interval of 95 % for line of best fit.

Table 4
Mean (± sem) total volatile fatty acid and the proportions of acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and acetate: propionate for Merino wethers fed varying inclusions of oil containing *Asparagopsis* bioactives (Asp-oil) in a supplementary pellet with either a high-quality or low-quality basal chaff diet.

	High-Quality Chaff					Low-Quality Chaff						P-value			
	A0	A1	A2	A3	A4	A0	A1	A2	A3	A4	S.E.M	Diet	Asp-oil		Diet × Asp-oil
													L	Q	
Ruminal pH	6.72	6.76	6.55	6.66	6.69	6.77	6.61	6.72	6.79	6.86	0.04	0.37	0.64	0.07	0.96
Total VFA (mM)	72.0	93.5	94.6	96.0	71.7	62.09	69.4	66.6	51.8	41.7	9.67	< 0.0001	0.09	0.0008	0.31
Proportion of individual VFAs (mM)															
Acetate	48.5	64.3	64.8	66.2	45.5	37.0	44.3	41.3	31.5	22.02	6.58	< 0.0001	0.02	< 0.0001	0.26
Propionate	16.9	20.0	21.8	22.1	19.4	18.0	19.1	18.7	14.7	14.7	4.52	< 0.0001	1.00	0.20	0.31
Butyrate	4.6	6.8	5.5	5.4	4.6	6.5	4.8	5.0	4.4	3.9	2.59	0.29	0.35	1.00	0.41
Iso-butyrate	0.4	0.5	0.5	0.5	0.4	0.1	0.2	0.3	0.18	0.15	0.06	< 0.001	1.00	0.26	0.98
Valerate	1.1	1.6	1.6	1.3	1.2	0.6	0.8	0.8	0.9	0.70	0.18	< 0.0001	0.10	0.08	0.70
Iso-Valerate	0.5	0.4	0.5	0.6	0.6	0.2	0.3	0.6	0.4	0.34	0.19	0.16	0.94	1.00	0.47
A:P	2.9	3.2	3.0	3.0	2.3	2.3	2.3	2.2	2.2	1.5	0.32	< 0.0001	< 0.0001	0.01	0.92

P-values are Bonferroni-Holm-adjusted. S.E.M: pooled standard error means. Levels of Asp-oil inclusion in pellet: A0 (0 mg $CHBr_3$/kg DM), A1 (36 mg $CHBr_3$/kg DM), A2 (72 mg $CHBr_3$/kg DM), A3 (108 mg $CHBr_3$/kg DM) and A4 (144 mg $CHBr_3$/kg DM).

Table 5
Mean total number of protozoa (count/mL) and counts of large Entodinia, small Entodinia, large Isotricha and small Isotricha for Merino wethers fed varying inclusions of oil containing *Asparagopsis* bioactives (Asp-oil) in a supplementary pellet with either a high-quality or low-quality basal chaff diet.

	High-Quality Chaff					Low-Quality Chaff						P-value		
	A0	A1	A2	A3	A4	A0	A1	A2	A3	A4	S.E.M	Diet	Asp-oil	Diet × Asp-oil
Total Protozoa (count/mL)	26788	32875	16406	50313	31510	3021	21473	16354	17934	34115	5045	0.30	0.05	0.20
Large Entodinia (count/mL)	434	7000	599	1745	521	52	551	339	469	729	52	0.03	0.20	0.10
Small Entodinia (count/mL)	26302	23625	15625	48099	28281	2865	19940	15781	17413	32448	7917	0.01	0.80	0.09
Large Isotricha (per mL rumen fluid)	0	0	26	0	0	0	0	0	0	0	7	0.003	0.40	0.70
Small Isotricha (per mL rumen fluid)	52	2250	156	469	2708	104	982	234	52	938	585	0.70	0.30	0.50

S.E.M: pooled standard error means. Levels of Asp-oil inclusion in pellet: A0 (0 mg $CHBr_3$/kg DM), A1 (36 mg $CHBr_3$/kg DM), A2 (72 mg $CHBr_3$/kg DM), A3 (108 mg $CHBr_3$/kg DM) and A4 (144 mg $CHBr_3$/kg DM).

Table 6
Mean (± sem) wool growth (g/d), mean fibre diameter (MFD, μm), fibre diameter coefficient of variation (FDCV, %), and staple length (SL, mm) for Merino wethers fed increasing inclusions of oil containing *Asparagopsis* bioactives (Asp-oil) in a supplementary pellet with either a high-quality or low-quality basal chaff diet.

	High-Quality Chaff					Low-Quality Chaff						P-value			
	A0	A1	A2	A3	A4	A0	A1	A2	A3	A4	S.E.M	Diet	Asp-oil		Diet × Asp-oil
													L	*Q*	
Wool growth (g/d)	11.34	11.34	11.8	13.68	10.61	8.91	7.71	10.35	9.84	15.46	0.226	0.05	1.00	0.13	0.12
MFD (μm)	15.17	15.63	16.6	15.13	14.98	14.3	13.25	13.7	13.92	14.02	0.795	< 0.0001	1.00	1.00	0.16
FDCV (%)	2.37	2.68	2.25	2.83	2.32	2.4	2.12	2.18	2.5	2.35	0.339	0.18	1.00	1.00	0.53
SL (mm)	74	78	82	73	83	75	68	70	71	73	5.0	< 0.0001	1.00	1.00	0.17

S.E.M: pooled standard error means. Levels of Asp-oil inclusion in pellet: A0 (0 mg $CHBr_3$/kg DM), A1 (36 mg $CHBr_3$/kg DM), A2 (72 mg $CHBr_3$/kg DM), A3 (108 mg $CHBr_3$/kg DM) and A4 (144 mg $CHBr_3$/kg DM).

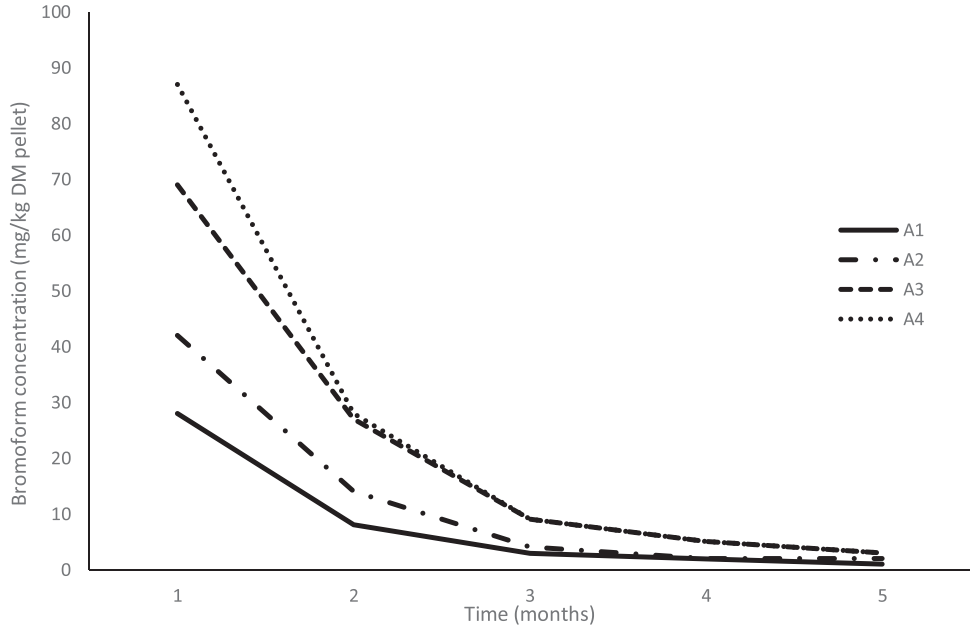

Fig. 3. Bromoform pellet concentration (mg $CHBr_3$/kg DM pellet) for Asp-oil pellets A1 (36 mg $CHBr_3$/kg DM) A2 (72 mg $CHBr_3$/ kg DM) A3 (108 mg $CHBr_3$/kg DM) and A4 (144 mg $CHBr_3$/kg DM) stored at room temperature and tested on a monthly basis.

4. Discussion

To our knowledge this is the first study that has quantified the potential CH_4 abatement in sheep *in vivo* using Asp-oil in a supplementary pellet. The present study has demonstrated that inclusion of Asp-oil in supplementary pellets fed daily to sheep can reduce MP. The response of MP and MY to increasing Asp-oil dose was not affected by diet quality. However, diet quality affected absolute MP such that the HQ diet had a larger MP associated with 30 % greater feed intake (DM basis) compared with the LQ diet. Feed intake has been previously reported as a driving factor for CH_4 emisisons (Charmley et al., 2016) with animals eating more also producing more CH_4. The HQ diet had a lower MY in comparison to the LQ diet. Both of these outcomes were expected. Increasing NDF decreases DMI and increases MY, influenced by rumen fill and retention time (Van Soest, 1975; Molano and Clark, 2008; Pinares-Patiño et al., 2011; Huhtanen et al. 2015).

The present study observed a dose-response effect with increasing inclusion of Asp-oil, resulting in a MP abatement of 6 % and 23 % and MY abatement of 12 % and 19 % at the highest Asp-oil inclusion (A4) on HQ and LQ diets, respectively. The observed Asp-oil effect was lower than expected based on previous studies, which have reported a positive dose-response with increasing inclusions of Asparagopsis and CH_4 abatement when administered in a modified TMR, TMR or grain supplementation twice daily (Li et al., 2016; Kinley et al., 2020; Roque et al., 2021; Cowley et al., 2023; Alvarez-Hess et al., 2024). For example, Cowley et al. (2023) reported a 99 % abatement and Alvarez-Hess et al. (2023) achieved a 44 % abatement in MY.

This experiment delivered Asp-oil in supplementary pellets once daily at an upper inclusion of 144 mg $CHBr_3$/kg pellet DM. Bromoform is short-lived in the rumen (Lanigan, 1972; Romero et al., 2023). Previous Asp-oil dose-response trials have been tested in TMR in beef cattle (Cowley et al., 2024), or twice daily grain supplementation in dairy (Alvarez-Hess et al. 2024). However, there is a knowledge gap for the efficacy of CH_4 inhibition outside these forms of delivery (Min et al., 2021). Methane abatement is influenced by the mode of delivery, regardless of whether the same form of Asparagopsis i.e. Asp-oil is used. Cowley et al. (2024) reported a 64 % reduction with 17 mg $CHBr_3$/kg DMI in a TMR, in comparison to Alvarez-Hess et al. (2024) who reported a 21 % reduction with approximately 20 mg $CHBr_3$/kg DMI fed twice daily in a grain supplement. The delivery system is known to impact abatement potential in other feed additives (i.e. Odongo et al., 2007 versus Grainger et al., 2010) and so it can be deemed inappropriate to compare previous Asp-oil TMR studies with the present study. Consequently, the most suitable studies for comparison are dairy trials (Alvarez-Hess et al., 2023, 2024) in which Asp-oil was fed twice daily. The present study limited supplementary pellet feeding to once daily to take into consideration on-farm practicality. However, there is a possibility that the lower feeding frequency was the cause of the lower MY abatement in comparison to Alvarez-Hess et al. (2023, 2024).

Total oil content of the pellets was capped at 5 % DM to avoid

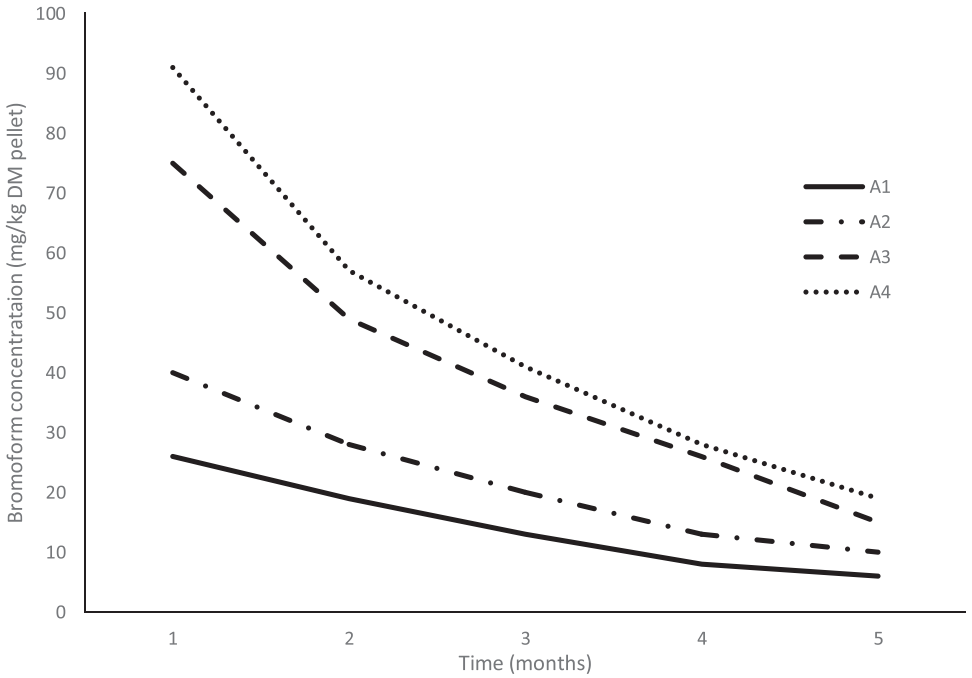

Fig. 4. Bromoform pellet concentration (mg $CHBr_3$/kg DM pellet) for Asp-oil pellets A1 (36 mg $CHBr_3$/kg DM) A2 (72 mg $CHBr_3$/ kg DM) A3 (108 mg $CHBr_3$/kg DM) and A4 (144 mg $CHBr_3$/kg DM) stored in a cool room and tested on a monthly basis.

compromising physical integrity. This resulted in an upper inclusion of 11.9 mg $CHBr_3$/kg DMI and 17.6 mg $CHBr_3$/kg DMI for HQ and LQ diets, respectively. It is possible that with a higher $CHBr_3$ concentration in the Asp-oil, a greater abatement could have been achieved in the present study without compromising on pellet integrity.

The present study consisted of basal chaff diets, with HQ and LQ diets consisting of NDF of 40 % and 55 %, respectively. Vetch hay and supplementary grain diets fed by Alvarez-Hess et al. (2023, 2024) had NDF inclusions of 45 % and 36 %, respectively. Forage: concentrate ratio is known to influence CH_4 production (Lileikis et al., 2023). The interaction of differing NDF levels with CH_4 abatement from Asparagopsis has previously been studied in TMR diets (Roque et al., 2021; Kinley et al., 2021) where increased NDF levels resulted in reduced abatement efficacy. It is noteworthy that abatement on a percentage basis were influenced by each study's baseline emissions, which was also apparent in the present study's HQ and LQ diet control (A0) groups. Consequently, relative CH_4 abatement percentages are proportional to each study baseline and are expected to be different amongst studies. As a result, studies should be compared on an abatement yield unit (g CH_4abated/mg $CHBr_3$.kg DMI^{-1}). On such a basis, it is still evident that feeding frequency and NDF influences abatement potential. The current study reported a 0.16 and 0.23 g CH_4 abatement/mg $CHBr_3$.kg DMI^{-1} for Asp-oil fed once daily in diets with NDF values of 40 % and 55 % for HQ and LQ diets, respectively. Despite similar $CHBr_3$ inclusions, Alvarez-Hess et al. (2023, 2024) achieved abatements of 0.78 and 0.45 g CH_4/mg $CHBr_3$.kg DMI^{-1}, respectively. This was likely influenced by feeding frequency and basal diet NDF concentration. Basal diet, delivery system and dose still need to be considered for the CH_4 abatement potential of Asp-oil in future studies. Therefore, caution is warranted when transposing Asp-oil results from different livestock systems.

There was no interaction effect of diet × Asp-oil on tVFA. Total concentration was influenced by diet and Asp-oil level, with a linear reduction in tVFA as Asp-oil inclusion increased. However, the effects of VFA production and absorption as a response of inhibiting methanogenesis cannot be derived from rumen VFA concentrations (Ungerfeld and Pitta, 2024). Previous studies have reported mixed results for tVFA and the inclusion of Asp-oil, some reporting reductions (Li et al. 2016; Alvarez-Hess et al., 2024) and others having no effect (Alvarez-Hess et al. 2023; Cowley et al., 2024). There was no interaction of diet × Asp-oil on the proportion of propionate, acetate or A:P ratio. The inclusion of $CHBr_3$ has been associated with an increase in di-hydrogen production (Martinez-Fernandez et al., 2016; Roque et al., 2019; Cowley et al., 2024). If metabolic hydrogen accumulates, this could favour the production of propionate over acetate (Muñoz-Tamayo et al., 2021; Glasson et al., 2022). A linear reduction in A:P ratio was observed with increasing inclusions of Asp-oil. However, no linear nor quadratic effect was observed for the proportion of propionate itself, suggesting that there is no natural redirection of hydrogen to propionate with MP inhibition observed in this study. Acetate:proprionate ratio varies across studies (Alvarez-Hess et al., 2023, 2024; Cowley et al., 2024), indicating that the concept of hydrogen redirection to more advantageous VFA with the inclusion of Asp-oil cannot be confirmed.

Average daily gain was significantly influenced by diet such that sheep on the HQ diet had larger ADGs than sheep fed the LQ diet. Wool growth was also significantly influenced by diet, such that sheep on the HQ diet had greater wool growth than sheep fed the LQ diet. Average daily gain was not significantly affected by Asp-oil, nor was wool growth. Feed intake is a good indicator of weight gain (Lippke, 1980) and a strong positive correlation between DMI and wool growth has previously been reported for sheep fed contrasting diets (Hynd and Masters, 2002). Previous work has observed that heavier weaners, that consumed more, grew more wool than lighter weaners and resulted in higher staple strength and fibre diameter (Masters et al., 1998). No reductions in total feed intake (kg DM/d) or the proportions of chaff or pellet DMI were affected by Asp-oil concentrations up to 17.6 mg $CHBr_3$/kg DMI. However, previous studies have reported reductions in feed intake with Asp-oil inclusions up to 35 mg $CHBr_3$/kg DMI, albeit with improved feed conversion efficiency (Kinley et al., 2024). This trial was designed and powered to test CH_4 abatement not production parameters. Trials with a higher $CHBr_3$ inclusion levels and greater feeding frequency are required to validate production parameters under a commercial setting.

The Asp-oil medium was created to reduce the volatilisation of $CHBr_3$ and improve stability (Magnusson et al., 2020). It is evident from this study that the recovered $CHBr_3$ concentration in the supplementary pellets was numerically lower than formulated. It is feasible that the

actual $CHBr_3$ concentration at feeding was compromised by the inherent volatility of the compound and inability of the matrix in these trial pellets to minimise loss during storage. A plausible explanation is that the increased surface area: volume ratio of the thinly coated Asp-oil on the pellet may have resulted in greater volatilisation potential than if Asp-oil were to be contained in a beaker and/or mixed within a grain supplement. The detected $CHBr_3$ in the A0 pellets may have been an artefact of the extraction process used in canola oil processing (George et al., 2024). The shelf-life of $CHBr_3$ in Asp-oil has previously been reported and is known to be influenced by light, temperature and exposure to air (Tan et al., 2023). The stability trial conducted as part of this work indicated that $CHBr_3$ concentration in a pellet would decline over five months regardless of storage temperature. This raises challenges for large-scale manufacturing, storage and delivery in grazing systems over time.

5. Conclusion

Although there was no diet × Asp-oil interaction on CH_4 abatement, a linear reduction in MP from sheep was observed when supplemented with Asp-oil pellets of increasing $CHBr_3$ concentration on a daily basis. Asp-oil inclusion up to 17.6 mg $CHBr_3$/kg DMI had no impact on feed intake, ADG or wool growth. Greater CH_4 abatement in sheep may be achievable with a combination of higher $CHBr_3$ concentrations and more frequent feeding. The methodology for including Asp-oil into a pellet needs to be refined to reduce $CHBr_3$ loss in manufacture and for commercial use. Future grazing trials are required to confirm the CH_4 abatement reported from this study.

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Sitienei Daniel Korir: Writing – review & editing, Supervision, Formal analysis. **Cowley Frances C.:** Writing – review & editing, Visualization, Supervision, Methodology, Formal analysis, Conceptualization. **Adam Charlotte:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Doyle Emma:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Preston James:** Writing – review & editing, Supervision. **Kahn Lewis:** Writing – review & editing, Supervision, Data curation. **Tomkins Nigel:** Writing – review & editing, Supervision, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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