Animal 18 (2024) 101249

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Effect of the dietary supplementation with sunflower oil-enriched bromoform from *Asparagopsis taxiformis* on lambs' growth, health, and ruminal methane production

F. Sena [a,b], A.P. Portugal [c], M.T. Dentinho [b,c,d], J. Costa [c], A. Francisco [b,c,d], S. Moradi [a,b], K. Paulos [c], D.M. Soares [a,b,d,e,f], D. Henriques [c], A. Oliveira [g], H. Ramos [g], R. Bexiga [a,b,d], J.J. Correia [a,b,d], G. Alexandre-Pires [a,b,d], T. Domingos [e,f], S.P. Alves [a,b,d], R.J.B. Bessa [a,b,d,*], J. Santos-Silva [b,c,d]

[a] *Faculdade de Medicina Veterinária, Universidade de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal*
[b] *CIISA – Centro de Investigação Interdisciplinar em Sanidade Animal, Av. Da Universidade Técnica, 1300-477 Lisboa, Portugal*
[c] *Polo de Investigação de Santarém, Instituto Nacional de Investigação Agrária e Veterinária (INIAV-Santarém), 2005-048 Vale de Santarém, Portugal*
[d] *Laboratório Associado para Ciência Animal e Veterinária (AL4AnimalS), Av. Da Universidade Técnica, 1300-477 Lisboa, Portugal*
[e] *Terraprima-Serviços Ambientais, Centro de Negócios do Porto Alto, Fração S, Avenida das Nações Unidas, n°97, 2135-199 Samora Correia, Portugal*
[f] *MARATEC – Marine, Environment and Technology Centre, LARSyS, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1, 1049-001 Lisboa, Portugal*
[g] *seaExpert, Ltd., Travessa do Farrobim 15, 9900-361 Horta, Faial, Azores, Portugal*

ARTICLE INFO

Article history:
Received 24 February 2024
Revised 2 July 2024
Accepted 5 July 2024
Available online 10 July 2024

Keywords:
Meat
Methanogenesis
Rumen mucosa
Seaweed
Toxicity

ABSTRACT

The red seaweed *Asparagopsis taxiformis* has a potent antimethanogenic effect, which has been proven both *in vitro* and *in vivo*. Vegetable oil immersions of this seaweed (hereafter **Bromoil**) help stabilise the bromoform (**$CHBr_3$**) responsible for its antimethanogenic effect. We evaluate the effects of increasing the levels of $CHBr_3$ in lamb diets on growth performance, methane (**CH_4**) production, animal health and meat quality. Twenty-four Merino Branco ram lambs were fed a ground complete compound feed, supplemented with 50 mL/kg DM of sunflower oil with different $CHBr_3$ content. The treatments were defined by the $CHBr_3$ doses in the oil: 0 mg (control – **B0**), 15 mg (**B15**), 30 mg (**B30**) and 45 mg (**B45**) of $CHBr_3$ per kg of feed DM. The feed was prepared daily by mixing Bromoil with the compound feed. At the end of the experiment, the lambs were sacrificed, the ruminal content was collected for *in vitro* fermentation to evaluate CH_4 production and organic matter (**OM**) degradability, and the rumen mucosa was sampled for histological examination. Meat samples were collected for chemical composition and $CHBr_3$ analysis. The half-life of $CHBr_3$ in the air-exposed feed was 3.98 h making it very difficult to establish the practiced level of $CHBr_3$ supplementation. Lambs-fed treatments B30 and B45 decreased DM intake by up to 28%. Average daily gain was also reduced due to $CHBr_3$ supplementation, with B45 showing results 40% lower than B0. DM feed conversion ratio was similar for all treatments. The degradability of OM, the volume of total gas and of gas without CH_4 were unaffected by the experimental treatments, evaluated by the *in vitro* method. However, the volume of CH_4 decreased by up to 75% for treatments above 30 mg/kg DM, while the yield of CH_4/g OM degraded was reduced by up to 78% with treatments above 30 mg/kg DM. Meat chemical composition was not affected by Bromoil supplementation and no traces of $CHBr_3$ were found in meat samples. During this experiment, the animals presented normal health and behaviour. However, postslaughter examination of the rumen showed distinct lesions on the ventral region of the rumen mucosa of animals supplemented with Bromoil. These lesions were more severe in the animals receiving treatments B30 and B45. This research determined that although concentrations of $CHBr_3$ in the diet above 30 mg/kg DM helped to reduce CH_4 emissions, it negatively affected the performance and rumen wall.

Implications

The macroalga *Asparagopsis taxiformis* is a promising nutritional supplement to reduce methane emissions by ruminants. Supple-

* Corresponding author.
E-mail address: rjbbessa@fmv.ulisboa.pt (R.J.B. Bessa).

https://doi.org/10.1016/j.animal.2024.101249
1751-7311/

menting lambs' diets with a vegetable oil macerate of this macroalga efficiently reduced *in vitro* methane emissions by up to 75%, without any bromoform residues in the meat. However, our results also showed that more than 15 mg of bromoform/kg of DM in the diet can reduce lambs' intake and growth rate, and that the integrity of the rumen mucosa can be seriously affected. More research is needed to find ways of limiting these constraints so that this nutritional strategy can be safely applied in production.

Introduction

The livestock sector constitutes an important source of greenhouse gas emissions, with an estimated annual contribution of 7.1 Gt CO_2eq (Gerber et al., 2013). Methane (**CH_4**) is one of the greenhouse gases, with a global warming potential of about 25 times higher than carbon dioxide (**CO_2**) over a period of 100 years (Reay et al., 2010). Methane is also a major end-product of ruminal fermentation of carbohydrates, representing an energy loss to the host animal of between 2 and 12% of gross energy intake (Johnson and Johnson, 1995). Reducing CH_4 emissions from livestock is thus currently a main challenge for scientists, public decision-makers, animal industry and farmers. The primary factors affecting the level of CH_4 emissions from ruminants are the amount of feed consumed and the composition of the diet (Johnson and Johnson, 1995). The increase of energy density of the diets, the improvement of forage quality and the use of dietary supplements such as lipids, plant secondary metabolites, chemical inhibitors and alternative electron sinks are dietary strategies that can be implemented to reduce CH_4 emissions from ruminants (Beauchemin et al., 2020).

Recently, the red macroalga *Asparagopsis taxiformis* (hereafter, **AT**) has been recognised as possessing a potent rumen antimethanogenic effect *in vitro* (Machado et al., 2016a; Machado et al., 2018) and *in vivo* (Li et al., 2018; Roque et al., 2021). Its antimethanogenic properties are attributed to naturally occurring halogenated compounds, mainly bromoform (**$CHBr_3$**), produced by the seaweed as a defence mechanism (Kladi et al., 2004; Machado et al., 2016b). Bromoform inhibits methanogenesis through an irreversible reaction with reduced vitamin B_{12} coenzyme-M methyltransferase, inhibiting cobamide-dependent methanogenesis, similar to other halogenated analogues (Machado et al., 2016b). Freeze-drying the biomass is the most effective process used to preserve and stabilise the highly volatile $CHBr_3$ present in the AT biomass (Vucko et al., 2017). However, this process has high economic and environmental costs. The production of $CHBr_3$-enriched macerated oil (**Bromoil**) by immersing AT biomass in edible vegetable oils has been proposed as an alternative to freeze-drying (Magnusson et al., 2020). Bromoil has been shown to efficiently extract and stabilise $CHBr_3$ for at least up to 24 weeks, when stored hermetically sealed and away from light at temperatures up to 40 °C (Tan et al., 2022).

The effectiveness of Bromoil in inhibiting ruminal methanogenesis has been evaluated *in vitro* by Kinley et al. (2022) and Sena et al. (2024). Both studies concluded that Bromoil was effective in reducing ruminal CH_4 emissions, resulting in a 92.4% decrease with a dietary level of $CHBr_3$ of 117 mg/kg DM (Kinley et al., 2022), and a decrease of 87.0% with 150 mg/kg DM (Sena et al., 2024), after 48 h of fermentation.

As far as we know, the use of $CHBr_3$-enriched vegetable oil in an *in vivo* experiment was reported only once (Alvarez-Hess et al., 2023). In that study, dairy cows were supplemented with a Bromoil from *A. armata* to supply 16.8 mg of $CHBr_3$/kg of DM intake. Methane emissions, assessed by modified SF_6 (sulphur hexafluoride) tracer technique, were reduced by 44 and 39%, with no impact on milk production. These results further support the idea that Bromoil can be an effective alternative to using freeze-dried biomass to supplement ruminant diets. To our knowledge, this is the first time that the impact of Bromoil on rumen health and performance of lambs fed a finishing diet are reported.

The main objectives of this research were to evaluate the effect of increasing levels of Bromoil in lamb diets on growth performance, CH_4 production, carcass and meat quality.

Material and methods

Experimental design and animals

Animal care and management procedures in this experiment followed the European Directive 2010/63/UE, which concerns the protection of animals used in scientific experiments. The procedures were approved by the ORBEA of the National Institute for Agrarian and Veterinary Research, process number 4/2023. For the present experiment, 24 Merino Branco ram lambs were used. The lambs were born on October 2022 in the Instituto Nacional de Investigação Agrária e Veterinária, I.P. (INIAV – Fonte Boa) flock and raised by their mothers until weaning, which occurred on 2 January 2023. After weaning, the lambs were confined and randomly distributed to individual pens with an area of 1.25 m^2, and with permanent access to clean water. Lambs were blocked in two groups, according to their initial live weight (i.e. high initial live weight and low initial live weight), and randomly assigned to one of four treatments (six lambs per treatment). The average weaning weight was 17.1 ± 3.13 kg. The feeding experiment lasted for 5 weeks after 1 week of adaptation to the experimental conditions and basal diet, i.e., without Bromoil supplementation.

The lambs were fed a basal diet *ad libitum*, with ingredients and chemical composition presented in Table 1, which were analysed from feed samples. The treatments resulted from the incorporation of four levels of $CHBr_3$ in the diets: 0 mg/kg DM (**B0** – control), 15 mg/kg DM (**B15**), 30 mg/kg DM (**B30**) and 45 mg/kg DM (**B45**). To obtain the necessary concentrations of $CHBr_3$ in the diet, Bromoil was diluted daily in commercial sunflower oil to guarantee the same level of 50 mL/kg DM of oil supplementation in all diets. The diets were prepared daily by adding the oil to the basal diet with an experiment mixer, and supplementation with Bromoil

Table 1
Ingredients and analysed chemical composition of the basal diet fed to lambs.

Ingredients	Composition (g/kg)
Corn	70
Barley	75
Wheat	75
Dehydrated citrus pulp	75
Dehydrated sugar beet pulp	75
Soybean hulls	70
Soybean meal	100
Sunflower meal	31.5
Calcium carbonate	14
Sodium bicarbonate	8
Salt	4.5
Premix	2
Dehydrated alfalfa	400
Chemical composition	
DM[1]	895
CP[2]	144
Ether extract[2]	28
NDF[2]	371
ADF[2]	223
Starch[2]	222
Sugar[2]	101

[1] as feed.
[2] DM basis.

in the assigned animals began considering the orts and offering 110% of the previous day intake. The lambs were weighed at seven-day intervals before feed distribution. On the slaughter day, the lambs were weighed before leaving the sheepfold.

Preparation of bromoform-enriched sunflower oil from Asparagopsis taxiformis

The stock Bromoil was prepared by maceration of gametophytes of AT in sunflower oil. The gametophytes were harvested by seaExpert, Ltd. (Feteira, Horta, Portugal) on May 2022 at Angústias, Faial Island, Azores, Portugal (38°31′45″ N, 28°37′09″ W). The algae were collected by diving at a depth of 3–6 m, drained and immersed in sunflower oil at 50/50 kg/L ratio and kept in eight 150 L opaque containers. After being transported to land, the containers were stored for 28 days, at which time, the AT macerated oil (i.e. Bromoil) was removed by gravimetry and stored in 5 L opaque containers that were transported to the INIAV – Fonte Boa facilities. From each 150 L container, a composite sample of oil was collected to quantify the $CHBr_3$. The average concentration of $CHBr_3$ in stock Bromoil was 2.97 mg/mL.

Slaughter procedure

Slaughter occurred on three different days (9, 13 and 15 February 2023) at the INIAV – Fonte Boa experimental abattoir, 500 m away from the sheepfold. The slaughter procedures followed regulation number 1099/2009 from the Council of the European Union (EU, 2009). After stunning, with a penetrative captive bolt device, the lambs were killed by exsanguination, the jugular veins and carotid arteries were sectioned and blood samples were collected using a 6 mL AQUIGEL K3/EDTA tube for hemogram and 9 mL serum separator (Deltalab, Barcelona, Spain) tubes for blood biochemical parameters.

After evisceration, the ruminal contents were immediately collected and gently homogenised. A portion of ruminal content was strained through four layers of cheesecloth, and, in the liquid phase, the pH was measured using a pH meter (Metrohm 744). About 1 L of the homogenised ruminal content was transferred to screw cap plastic containers and immediately transported to the laboratory for *in vitro* CH_4 evaluation, according to a procedure adapted from Chaves et al. (2011). The rumen was washed under tap water and observed for lesions, and samples were taken for histological analysis.

The carcasses were weighed to obtain hot carcass weight and transferred to a cooled camera at -4 °C. Twenty-four hours after slaughter, the carcasses were re-weighed for cold carcass weight and graded according to the EU grading procedures for ovine carcasses (European Commission, 2008). The carcasses were split in two halves and in the left side samples of the *Longissimus lumborum* muscle were collected, corresponding to the portion between the first and fifth lumbar vertebrae. The samples were minced with a food processor (Moulinex-123 A320R1, Group SEB Portugal Lda, Lisbon, Portugal) (3 × 5 s), vacuum packed and stored at -20 °C until the chemical composition and pH determination.

Stability of the bromoform in feed

To assess changes in the concentration of $CHBr_3$ in the diet during a 24 h exposure to air, which corresponds to the period between the distribution of meals, a diet similar to that used in the experiment was prepared in triplicate. For this test, the concentration of $CHBr_3$ incorporated in the feed was 58.6 mg/kg DM. After Bromoil incorporation, initial feed samples were taken for immediate $CHBr_3$ determination and the remaining portions were sampled after being placed in trays in contact with air for 3, 6, 10, 15 and 24 h, and immediately processed.

In vitro ruminal fermentation

Three series of 24 h batch incubations, one on each slaughter day, were conducted in an ANKOM RF Gas production System (Ankom Technology, New York, USA) to assess the effect of increasing concentrations of $CHBr_3$ supplied as Bromoil on ruminal fermentation parameters, total gas production and CH_4 concentration in the fermentation flask headspace. The ruminal digesta of each lamb collected at the abattoir was processed individually. After being strained though four layers of cheesecloth, the liquid phase was maintained at 38 °C and flushed with CO_2 until being used in the filling of the fermentation flasks. The pH of the ruminal fluid of each lamb was measured. One gram of ground feed substrate was weighed and transferred to each fermentation flask. The substrate was prepared on the day of the slaughter in the same way and at the same concentration as those used during the experiment with the lambs, matching the diets to those consumed by the lambs donating the ruminal fluid. 60 mL of buffer solution, prepared according to Menke et al. (1979), was added to each fermentation flask and kept in warm water (39 °C) for 20–30 min. After this time, 2 mL of reducing solution preheated to 39 °C was added to each fermentation flasks. Thirty mL of the filtered ruminal liquid was then added to the mixture. Before closing, the headspace of the fermentation flasks was flushed with CO_2. The modules were tightly secured, and the fermentation flasks were placed in a water bath (39 °C) with agitation (50 rpm) for 24 h.

After 24 h, the incubation was stopped by transferring all fermentation flasks to a cold-water bath and gas samples were collected using a 20 mL polypropylene syringe (B. Braun, Omnifix) to 9 mL vacuum tubes (Vacutest Kime). The pH of the fermentation flask content was measured using a pH meter (Metrohm 744). The total contents of each fermentation flask were transferred to glass tubes and centrifuged for 10 min at 1 000 × g. The residue was transferred to a preweighed crucible filter (grade 0, porosity 160–250 μm, VitraPOR, ROBU, Hattert, Germany) mounted in a Kitasato flask and washed using distilled water and vacuum. The solid residue was used for quantification of the DM and organic matter (**OM**) that remained at the end of the fermentation trial.

Total gas volume (mL) was calculated from cumulative pressure according to the ANKOM RF gas production system operator's manual.

Analytical procedures

Proximal and chemical composition of the basal diet

Basal diet samples collected during the experiment were analysed for DM (ISO6496, 1999), CP (ISO5983, 1997) and ether extract (ISO6492, 1999). Starch and total sugars were determined according to Clegg (1956). For NDF and ADF determination, the method used by Van Soest et al. (1991) was followed, in which NDF was quantified with sodium sulphite, without α-amylase and expressed with the ash residue.

Bromoform in the oil, diets and meat

The concentration of $CHBr_3$ in Bromoil was determined by gas chromatography (**GC**) MS using a procedure adapted from Magnusson et al. (2020) and reported by Sena et al. (2024). The concentration of $CHBr_3$ in the feed was determined after mixing 1 g of feed with 100 mL of methanol and 20 μL of internal standard (naphthalene at 1 mg/mL) and left at 4 °C for 72 h. The supernatant was then centrifuged, and an aliquot was injected into the GC–MS. To determine $CHBr_3$ in meat, 5 g of raw meat was mixed with 9.5 mL of methanol and 500 μL of internal standard (naphthalene

at 10 μg/mL) and left at 4 °C for 72 h. The supernatant was then centrifuged, and an aliquot was filtered through a 0.2 μm syringe filter and injected into the GC–MS. A Shimadzu GC–MS QP2010-Plus instrument (Shimadzu Corp., Kyoto, Japan) equipped with a fused silica capillary column (Supelcowax10, 30 m × 0.25 mm × 0.20 μm film thickness, Supelco Inc., Bellefonte, PA, USA) was used for all determinations. The quantification of $CHBr_3$ was performed in selected ion monitoring mode, and the electron impact mass spectra were recorded with an ionisation energy of 70 eV. In selected ion monitoring mode, *m*/*z* 173 and *m*/*z* 128 were the quantifier ions for $CHBr_3$ and naphthalene, respectively. The ion source temperature was maintained at 230 °C, the interface temperature at 280 °C and the GC injector at 180 °C. 1 μL of sample was injected in splitless mode. Helium was used as the carrier gas at a flow rate of 1 mL/min, with the oven temperature stabilised at 40 °C for 1 min and increased at a rate of 16 °C/min until it reached 250 °C, where it remained for 1 min. Bromoform concentration was calculated from its peak area ratio over the internal standard peak area and converted to concentration by reference to standard curves. For the analysis of $CHBr_3$ in oil, feed or meat, different calibration curves were created, with at least nine concentrations ranging from 1 to 40 μL/mL, 0.01 to 1 μg/mL or 0.001 to 0.05 μg/mL, respectively. Calibration standards were injected before and after the samples.

Organic matter of solid residue and methane proportion in the gas

At the end of each incubation period, the residue of fermentation flasks was weighed and used for DM and OM determination according to ISO5984 (2022) to compute the disappearance of OM. A sample of the gas present at the headspace of each fermentation flask was collected and analysed using a GC equipped with a flame ionisation detector to measure the CH_4 proportion. A volume of 200 μL of gas was injected in an HP 6890A GC (Hewlett-Packard, Avondale, PA, USA). The GC was equipped with a TG-Bond Q capillary column (Thermo Scientific USA 30 m × 0.32 mm internal diameter × 10 μm film thickness). The analysis was conducted with splitless injection and maintaining the oven at 150 °C for 7.5 min. The injector was kept at 200 °C and the detector at 255 °C. Helium was the carrier gas with a flux of 1.4 mL/min. Total CH_4 production (mL) was calculated using the total gas production and the proportion of CH_4 determined by GC at the end of the incubation. Gas without CH_4 was calculated by subtracting the volume of CH_4 from the total gas volume.

Blood chemical analysis

Standard haematological analysis was performed using a Procyte Dx (Idexx) equipment. Biochemical analysis was performed for creatine, urea, albumin, total protein, aspartate aminotransferase and γ-glutamyl transferase concentrations, using a Respons920 (Diasys) equipment, according to International Federation of Clinical Chemistry recommended standard methods. Reference ranges for both haematology and biochemistry were based on Smith et al. (2015).

Meat DM, protein, total lipids and pH

The *Longissimus lumborum* samples used for chemical analysis were allowed to thaw during 24 h under refrigeration at -4 °C. 5 g of minced meat suspended in 50 mL of potassium chloride (0.1 M) were used to measure pH, using a Metrohm 733 pH meter (Metrohm AG, Switzerland) equipped with a combined glass electrode (ISO2917, 1999). DM and CP of *Longissimus lumborum* were determined according to ISO1442 (1997) and ISO5983 (1997), respectively.

Rumen mucosa histopathology and ultrastructure analysis

Sample pieces (5 × 5 cm) of the lambs' ventral sac rumen wall were carefully collected at the slaughterhouse, in order to ensure the integrity of all layers of the rumen wall and kept for fixation in a 10% buffered formalin solution for 2 days. For histological procedures, the previously described sample pieces were embedded into paraffin to create the formalin-fixed paraffin-embedded blocks. The rumen wall formalin-fixed paraffin-embedded blocks were cut with a microtome (Leica RM2125, Germany) in three micrometre-thick slices. The sections were placed onto microscope glass slides and then stained with haematoxylin-eosin and examined under a light microscope (Olympus BX-51, Japan). High-quality images were captured using an Olympus camera (U-CMAD-2, Japan) to identify the presence of alterations in cell structure, inflammation process or any other lesions.

For the electronic microscopy scanning procedures of intact tissues, the previously described sample pieces were rinsed twice in 0.1 M cacodylate buffer (pH 7.4), then dehydrated through a graded ethanol series, and finally dried using the critical point method. The sample pieces were then sputter-coated with gold and mounted on stubs. Specimens were observed with a JEOL5200-LV microscope (JSM Electron Microscopes, Tokyo, Japan) and under a SEM-UR-LBDB Hitachi microscope (SU8010 High-Technologies Corporation, Ibaraki, Japan), and digital images were acquired.

Statistical analysis

The experiment was conducted in a randomised block design, with the animal as the experimental unit. Data were analysed using the Proc Mixed of SAS (SAS Institute Inc., Cary, NC, USA). The heterogeneity of variances of the variables was tested, and when significant ($P < 0.01$), it was accommodated in the models using the group option of the repeated statement of the Proc Mixed. The decay of the $CHBr_3$ concentration in feeds during 24 h of exposure to air was studied by adjusting a first-order exponential decay with a plateau function on Graphic Prism software. Individual average daily weight gain was determined by regression, relating live weight to the day of the test. Average daily weight gain, adjusted for initial live weight, was studied using a model in which diet and block were the fixed effects. DM and nutrient intakes were studied using a model which considered repeated measures over time in each animal and the diet and initial live weight block as fixed effects. The model used an autoregressive covariance structure, selected according to the Akaike information criteria. Feed conversion ratios, live weight at slaughter, and carcass and meat traits were studied using a model where the diet and initial live weight block were the fixed effects. The results of the *in vitro* trial were studied using a model that considered the two fermentation flasks from the same animal as subsamples, the diet as a fixed effect and slaughter day as a random block. The covariance structure used to model the subsampling was the compound symmetry, selected according to the Akaike information criteria. In all the models and variables, when the treatment effect was significant ($P < 0.05$), the least squares means were compared to determine which treatments were effectively different. In the tables, the values for the least squares means and SEs are presented.

Results

Feed intake, growth and carcass traits

The results observed for intake, average daily gain and feed conversion ratio are presented in Table 2. DM intake decreased with

Table 2
Effect of the stepwise dietary inclusion of bromoform ($CHBr_3$) through a sunflower oil macerate of *Asparagopsis taxiformis* on intake, growth performance and carcass traits of the lambs.

	Treatment[1]					
Trait	B0	B15	B30	B45	SEM	*P*-value
Intake (g/day)						
DM	1 103^{c}	1 018^{bc}	873^{ab}	791^{a}	68.7	0.013
CP	154^{c}	147^{bc}	123^{ab}	111^{a}	9.91	0.014
Ether extract	31.4^{c}	29.4^{bc}	25.4^{b}	13.2^{a}	2.06	<0.001
NDF	419^{b}	380^{a}	336^{a}	308^{a}	25.6	0.022
Sugar	115^{c}	102^{bc}	84^{ab}	77^{a}	6.9	0.002
Starch	262^{c}	215^{b}	177^{ab}	167^{a}	14.6	<0.001
$CHBr_3$ (mg/day)	-	16.2^{a}	27.7^{b}	36.3^{c}	2.65	<0.001
Average daily gain (g/day)	254^{b} ± 38.4	207^{ab} ± 31.1	216^{b} ± 7.3	152^{a} ± 20.6		0.042
Feed conversion ratio[2]	5.7 ± 1.11	5.1 ± 0.46	4.6 ± 0.46	6.6 ± 1.61		0.596
Slaughter live weight (kg)	25.1	24.3	24.2	22.0	0.88	0.090
Hot carcass weight (kg)	12.0^{b}	11.1^{b}	11.4^{b}	9.7^{a}	0.40	0.001
Dressing (%)	48.1^{c}	45.0^{ab}	46.8^{bc}	43.8^{a}	0.84	0.008

Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] Diet including 0 mg (B0), 15 mg (B15), 30 mg (B30), and 45 mg (B45) of $CHBr_3$ per kg of diet DM.
[2] DM basis.

dietary $CHBr_3$ concentration ($P = 0.013$), being the highest in B0 and the lowest in treatment B45, with treatments B15 and B30 presenting intermediate values. Compared to B0, B30 decreased DM intake by 21% and B45 decreased DM intake by 28%. CP, ether extract and sugar intakes did not differ between B0 and B15 but were significantly lower in treatments B30 and B45 ($P < 0.05$). Only ether extract intake was significantly lower in treatment B45 compared to B30. Neutral detergent fibre intake was the highest in B0 ($P = 0.022$), while the other treatments presented similar values among them.

Average daily gain decreased with dietary $CHBr_3$ concentration ($P = 0.042$), following a pattern similar to the one described for feed intake, except that the average daily gain in treatment B30 differed from that of B45. In treatment B45, the average daily gain was 40% lower than B0. The DM feed conversion rate did not differ among treatments ($P = 0.596$).

Carcass traits of lambs are also presented in Table 2. Slaughter weight was similar across all treatments ($P = 0.090$). Hot carcass weight was lower (-19%) in treatment B45 than in the other treatments that presented similar values among them, averaging 11.5 ± 0.40 kg ($P = 0.001$). Dressing percentage was lower in treatment B45 than in B0, and B15 and B30 showed intermediate values ($P = 0.008$).

Stability of the concentration of bromoform in the diet offered

The results obtained for the adjustment of one-phase decay model to the concentration of $CHBr_3$ in the diet exposed to air for 24 h are shown in Fig. 1. The fractional rate of $CHBr_3$ decay was 17% per h (95% confidence intervals ranging from 12–23% per h) and the half-life was 3.98 h (95% confidence intervals ranging from 2.9 to 5.7 h). The plateau was reached at circa 6.7% of the initial concentration and at 24 h circa 92% of $CHBr_3$ had disappeared.

In vitro fermentation

The main results from the *in vitro* incubations are reported in Table 3. Degradability of OM during 24 h was unaffected by the supplementation with Bromoil ($P = 0.320$), averaging 632 ± 125.4 g/kg. The volume (mL) of total gas produced and gas production without CH_4 did not differ among treatments, although the value observed in treatment B45 tended to be lower than those of the other treatments ($P < 0.10$). These results normalised to OM degraded were also not significantly different among treatments

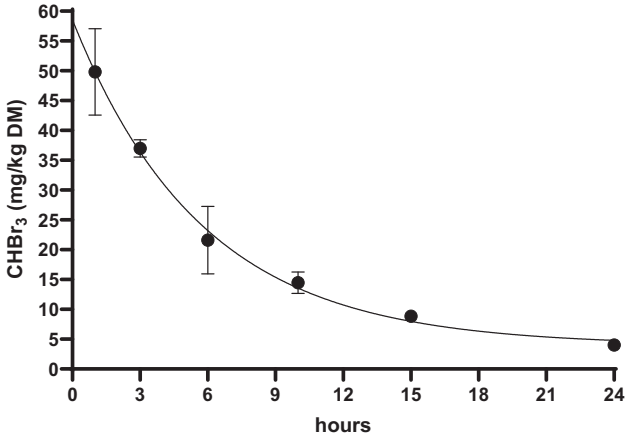

Fig. 1. Changes in bromoform ($CHBr_3$) concentration in the feed given to lambs during 24 h exposure to air and the one phase exponential model (Y = (Y0 – Plateau) *exp(-k*hours) + Plateau, fitted (R^2 = 0.959, RSD=3.64). Equation parameters estimated: Y0 = 58.6; Plateau = 3.94; k = 0.174 and half-life of 3.99 h.

($P > 0.05$). The volume (mL) of CH_4 produced ($P < 0.001$) and the yield of CH_4 per g of OM degraded ($P = 0.001$) were not significantly different between B0 and B15 but decreased in treatments B30 and B45. Only the proportion of CH_4 in total gas produced was significantly different between B30 and B45 ($P < 0.001$). When compared to B0, there was a 75% decrease in the volume of CH_4 in B30 and a 56% decrease in B45. When expressed in mL/g OM degraded, there was a 78% and a 60% decrease in CH_4 in treatments B30 and B45, respectively. The inclusion of Bromoil did not affect the pH at the end of the incubation ($P = 0.732$), which averaged 6.10 ± 0.247.

Meat quality traits and blood biochemical parameters

Meat quality traits are presented in Table 4. DM, protein, total lipids and pH were not affected by treatments ($P > 0.05$) and averaged 23.3 ± 1.02%, 19.5 ± 1.00%, 2.66 ± 0.752% and 5.63 ± 0.084, respectively. The biochemical parameters of the blood collected at slaughter are presented in Table 5. The treatments had no effect ($P > 0.05$) on the parameters analysed. For albumin, aspartate aminotransferase/glutamic oxaloacetic transaminase, creatine, gama-glutamyl-transferase, total protein and urea, the average values were 3.47 ± 0.062 g/dL, 79.0 ± 6.05 U/L, 0.60 ± 0.025 mg/dL,

Table 3
Effect of the stepwise dietary inclusion of bromoform ($CHBr_3$) through a sunflower oil macerate of *Asparagopsis taxiformis* on *in vitro* ruminal fermentation traits evaluated individually with lambs ruminal inocula.

	Treatment[1]					
Trait	B0	B15	B30	B45	SEM	*P*-value
OM degradability (g/kg)	607	574	648	696	45.6	0.320
Gas production (mL)						
Total gas	216	231	229	167	24.3	0.084
Gas without CH_4	181	195	221	155	22.4	0.072
CH_4	34.0^{b}	34.1^{b}	8.5^{a}	15.1^{a}	3.92	<0.001
CH_4 (%)[2]	15.7^{c}	14.2^{c}	4.5^{a}	8.7^{b}	1.53	<0.001
Gas yield (mL/g OM degraded)						
Total gas	441	514	474	312	73.3	0.284
Gas without CH_4	371	435	459	284	63.4	0.303
CH_4	71.1^{b}	77.8^{b}	15.7^{a}	28.1^{a}	10.97	0.001
Final pH[3]	6.04	6.07	6.11	6.18	0.110	0.732

Abbreviations: OM=Organic matter.
Values within a row with different superscripts differ significantly at $P<0.05$.
[1] Diet including 0 mg (B0), 15 mg (B15), 30 mg (B30), and 45 mg (B45) of $CHBr_3$ per kg of diet DM.
[2] Methane expressed as a percentage of total gas produced.
[3] pH at the end of the fermentation run.

Table 4
Effect of the stepwise dietary inclusion of bromoform ($CHBr_3$) through a sunflower oil macerate of *Asparagopsis taxiformis* on lambs' meat pH, DM, protein and total lipids.

	Treatment[1]					
Trait	B0	B15	B30	B45	SEM	*P*-value
pH	5.60	5.64	5.64	5.62	0.037	0.856
DM (g/100 g meat)	24.0	23.4	23.1	22.7	0.35	0.090
Protein (g/100 g meat)	20.0	19.5	19.3	19.3	0.40	0.537
Total lipids (g/100 g meat)	3.01	2.49	2.74	2.28	0.336	0.459

[1] Diet including 0 mg (B0), 15 mg (B15), 30 mg (B30), and 45 mg (B45) of $CHBr_3$ per kg of diet DM.

Table 5
Effect of the stepwise dietary inclusion of bromoform ($CHBr_3$) through a sunflower oil macerate of *Asparagopsis taxiformis* on lambs' blood biochemical parameters and hemogram.

	Treatment[1]					
Trait	B0	B15	B30	B45	SEM	*P*-value
Biochemical parameters						
Total protein (g/dL)	6.92	6.40	6.18	6.19	0.296	0.330
Albumin (g/dL)	3.54	3.56	3.46	3.32	0.062	0.066
Creatine (mg/dL)	0.57	0.61	0.64	0.58	0.025	0.212
Urea N (mg/dL)	32.3	35.5	30.0	31.4	2.30	0.516
AST/GOT (U/L)	77.9	83.1	79.3	75.6	6.05	0.885
GGT (U/L)	66.9	62.7	57.3	62.6	4.79	0.616
Hemogram						
Erythrocytes ($\times10^6$/μL)	15.5	14.5	14.8	15.5	0.60	0.584
Haematocrit (%)	35.1	37.3	33.7	31.8	2.07	0.451
Haemoglobin (g/dL)	13.2	16.8	12.1	11.6	2.94	0.600
MCV (fL)	22.6^{ab}	25.6^{b}	22.6^{ab}	20.3^{a}	1.08	0.046
MCH (pg/cell)	8.55	11.9	8.12	7.30	2.434	0.537
MCHC (g/dL)	38 ± 11.9	49 ± 10.7	38 ± 1.5	40 ± 1.8		0.822
Leucocytes ($\times10^3$/μL)	6.44	4.28	5.15	4.44	1.029	0.514
Segmented neutrophils[2] (%)	44.0	43.4	45.9	34.2	5.01	0.500
Lymphocytes (%)	54.0	55.5	52.3	62.3	4.73	0.594
Monocytes (%)	2.00	1.12	1.79	3.53	1.246	0.651
Platelets ($\times10^3$/μL)	526	511	1 201	474	327	0.421

Abbreviations: AST/GOT=Aspartate aminotransferase/glutamic oxaloacetic transaminase; GGT=Gama-glutamyl-transferase; MCV=Mean corpuscular volume; MCH=Mean corpuscular haemoglobin; MCHC=Mean corpuscular haemoglobin concentration.
Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] Diet including 0 mg (B0), 15 mg (B15), 30 mg (B30), and 45 mg (B45) of $CHBr_3$ per kg of diet DM.
[2] Segmented neutrophils as percentage of leucocytes.

62.4 ± 4.79 U/L, 6.42 ± 0.296 g/dL and 32.3 ± 2.30 mg/dL, respectively.

The hemogram is also presented in Table 5. Only mean corpuscular volume showed differences among treatments ($P = 0.046$). Treatment B45 had a lower value than B15, and the other treatments had intermediate values. The other parameters analysed were not affected by the treatments ($P > 0.05$), averaging 5.08 ± 1.029 ($\times10^3$/μL), 15.1 ± 0.60 ($\times10^6$/μL), 677 ± 327.0 ($\times10^3$/μL), 13.4 ± 2.94 g/dL, 8.97 ± 2.434 pg, 41.1 ± 6.46 g/dL, 56. 0 ± 4.73%, 2.11 ± 1.246%, for leucocytes, erythrocytes, platelets,

haemoglobin, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, lymphocytes and monocytes, respectively.

Macroscopic evaluation and histopathology of the rumen mucosa

The rumen wall from lambs allocated to treatments B0 and B15 did not exhibit evident macroscopic modifications. In contrast, all the lambs receiving treatments B30 and B45 presented macroscopic alterations with the proliferative aspect that evoked the aspect of spongy mushrooms. Modified rumen wall was also characterised in some areas by the scarcity of the ruminal papilla (Fig. 2, plates A–D).

The histopathologic analysis revealed that in the control group (B0), only one sample exhibited hyperkeratosis in the rumen mucosa (Fig. 2, plate E), while the rest showed no significant abnormalities. However, 50% of the animals from treatment B15 experienced inflammation of the rumen mucosa, diagnosed as acute purulent erosive ruminitis (Fig. 2, plate F). This situation caused rumen papilla atrophy and mucosal surface flattening due to proliferative lesions in the chorion (Fig. 2, plate G). Moreover, these animals presented proliferative preneoplastic lesions that affected the mucous squamous epithelium, leading to basement membrane invasion and the passage of epithelial cells to the chorion (Fig. 2, plate H). Samples from treatment B30 exhibited a significant alteration in the overall condition of the rumen wall. All lambs given the B30 diets suffered from chronic sclerosing ruminitis and hyperkeratosis in the mucosa at the papilla tips (Fig. 2, plate I), and showed the formation of fibrous tissue (Fig. 2, plate J). All animals from treatment B45 were severely affected, also presenting microabscesses (Fig. 2, plate K), chronic granulomatous ruminitis (Fig. 2, plate L), chronic ruminitis with lymphangiectasia of the mucosal chorion vessels (Fig. 2, plate M) and acute ruminitis with mucosal chorion haemorrhages (Fig. 2, plate N).

Electronic microscopy scanning of the inner surface of the rumen mucosa

Photos from electronic microscopy scanning of the inner surface of the rumen mucosa are presented in Fig. 3. The inner surface of the ventral ruminal sac was characterised by the presence of papillae whose outline presented countless irregular concave prismatic surfaces (Fig. 3, plates A to C). When observing these areas from lambs getting treatments B30 and B45, major morphological changes comprised the area of papilla scarcity or even the loss of mucosal ruminal papillae. The development of proliferative structures that evoke spongy mushroom-like structures (Fig. 3, plates D and E) was also evident. In these areas, a condition of reduced and flat mucosal surface occurred (Fig. 3, plates F–H).

Discussion

The focus of this research was to study the effect of Bromoil supplementation on CH_4 emissions from growing lambs fed a finely ground complete diet. Bromoil is a promising tool to reduce methanogenesis when used as a feed supplement in ruminant production. There are still some questions regarding the impact of Bromoil on animals and their products. These include issues related to productivity, toxicity, animal health effects, meat safety and quality. These questions must be answered before any attempt to spread Bromoil usage in ruminant production. Nevertheless, this research showed for the first time the impact of $CHBr_3$ from AT supplemented through sunflower oil on the rumen status and on animal performance.

Determining the exact amount of $CHBr_3$ fed to experimental animals is difficult due to its high volatility. Nevertheless, it is often assumed that the concentration of $CHBr_3$ in the freeze-dried AT biomass, and hence in the diet, remains stable during the experimental period (Vucko et al., 2017; Roque et al., 2021). However, this might not be true, as demonstrated by Stefenoni et al. (2021). As the stability of $CHBr_3$ in the Bromoil is ensured for up to 24 weeks if stored capped (Tan et al., 2022), we would also expect that Bromoil could ensure the stability of $CHBr_3$ for at least 24 h when mixed with other diet ingredients and exposed to air in the throughs. We checked that hypothesis by analysing the decay of $CHBr_3$ during 24 h. Our data indicated a rapid decrease in the concentration of $CHBr_3$ in the first few hours of exposure to air, with a fractional rate of decay of 17% per h, a half-life of 4 h and more than a 90% loss after 24 h. The continuous variation of $CHBr_3$ in feed makes it very difficult to establish the practical level of $CHBr_3$ supplementation because it depends on individual ingestion behaviour. Hence, ingested $CHBr_3$ was undoubtedly bellow the target values used to prepare the diets. The rapid loss of $CHBr_3$ observed could affect the practical use of Bromoil, requiring frequent supplementation of the diet. Additionally, since $CHBr_3$ is an ozone-depleting gas (Tegtmeier et al., 2015), its release into the atmosphere poses a significant environmental concern regarding the implementation of Bromoil in ruminant production.

Increasing the dosage of $CHBr_3$ in Bromoil decreased DM intake and average daily gain. The use of Bromoil may affect intake simultaneously by the effect of $CHBr_3$ and sunflower oil inclusion in the diets or by synergy of both supplements. Nevertheless, as all the treatments had the same amount of oil, the effects observed must have been due to the presence of $CHBr_3$. The inclusion of AT in the diets of dairy cows (Roque et al., 2019; Stefenoni et al., 2021) and steers (Roque et al., 2021) also depressed DM intake in a dose-dependent manner. Moreover, Li et al. (2018) reported that including 2 or 3% freeze-dried AT using crushed lupins as a carrier in the diet of adult sheep resulted in a relevant refusal of the AT-containing supplement. This effect may be caused by the low palatability of the seaweed, as suggested by Roque et al. (2019). The low palatability of Bromoil can also contribute to the observed depression of DM intake, as it has an intense odour that persisted after the preparation of the diets, in a gradient coinciding with the level of Bromoil inclusion. Reinforcing this fact, we used a high-density, low-volume diet in which all the ingredients, including forage, were grounded. Under these conditions, the odour transmitted by Bromoil was more intense than in low-density bulkier diets, resulting in a reduction of appetite for feed, which was evident in some of the lambs assigned to diets B30 and B45. The dilution of Bromoil in bulkier diets could explain the absence of DM intake depression in dairy cows fed vetch hay *ad libitum* supplemented with a coarse grain mixture and 134 g of Bromoil delivering 480 mg/day of CHBr3. (Alvarez-Hess et al., 2023).

The adaptation of animals to AT, or to $CHBr_3$, might also be determinant in preventing depression in DM intake and productive performance. When this gradual adaptation to $CHBr_3$ was not applied, there were reductions in DM intake and animal performance, as reviewed by Wasson et al. (2022). Adult sheep that maintained DM intake in the experiment reported by Li et al. (2018) had 2 weeks to adapt to the AT-supplemented diets. In the present experiment, lambs were exposed to Bromoil-supplemented diets without an adaptation period, which might have contributed to the reduction in DM intake. The results from the present study could further suggest that the gradual introduction of AT, or Bromoil, in diets could be mandatory to prevent the reduction of intake and weight gain of the animals.

Lamb growth decreased less steadily than feed intake, and the average daily gain was only significantly and strongly depressed compared to B0 in the B45 treatment. The average daily gain values

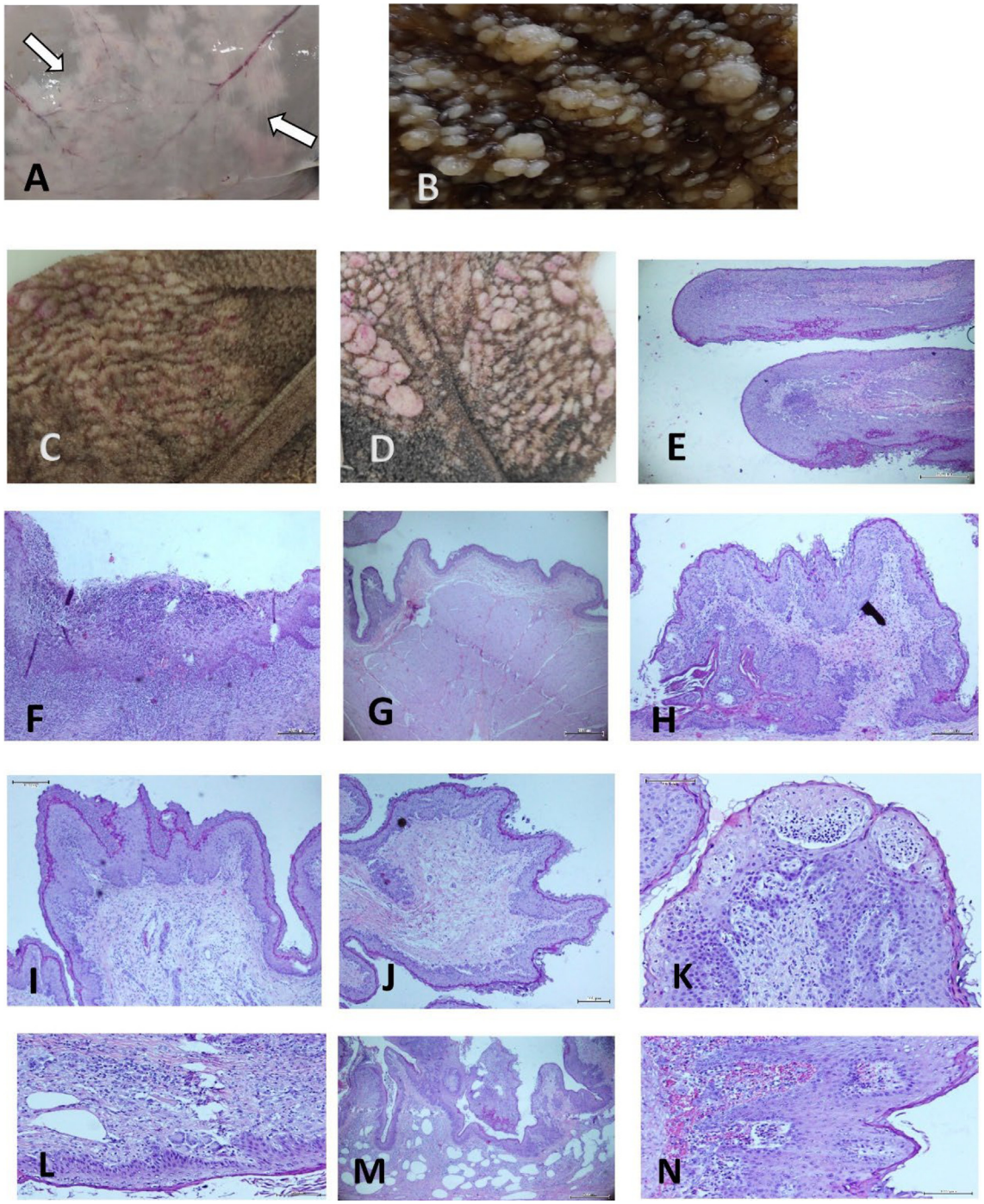

Fig. 2. Macroscopic (Plates A to D) and histopathologic (Plates E to N) images of rumen samples from lambs supplemented bromoform ($CHBr_3$) through a sunflower oil macerate of *Asparagopsis taxiformis*. Plate description: (A) Inner mucosal lesions of the ventral ruminal sac can be observed on the outside mucosa of the rumen. (B) In some areas of the inner ruminal sac, the typical papillae were no longer present, and a spongy aspect can be found. (C) Modified ruminal mucosa was conveyed in some areas by scarcity of papillae. (D) Lesions with a proliferative aspect were present in several areas of the mucosa of the ventral sac of the rumen, which resembled a mushroom cap. (E) The mucosal epithelium presented hyperkeratosis with thickening due to keratinocyte proliferation. Bar = 500 μm. (F) Acute purulent erosive duodenitis, proliferative lesion with superficial loss of epithelial cells and accumulation of purulent exudate on the surface of the mucosa, rich in neutrophils. Bar = 400 μm. (G) Atrophy of the rumen papillae, with smoothing of the mucosal surface. Bar = 500 μm. (H) A proliferative lesion of the mucosal squamous epithelium with invasion of the basement membrane and passage of epithelial cells to the chorion indicating a preneoplastic lesion. Bar = 200 μm. (I) Mucosal epithelium thickening due to hyperkeratosis at the papilla tip, resulting from keratinocyte proliferation. Bar = 200 μm. (J) Chronic sclerosing ruminitis, with the formation of fibrous tissue (fibroblasts and collagen) in the chorion of a papilla. Bar = 200 μm. (K) Acute ruminitis, with the formation of microabscesses in the mucosal epithelium, filled with PN-Neutrophils. Bar = 100 μm. (L) Chronic granulomatous ruminitis led to the formation of granulomas in the mucosal chorion, which was rich in multinucleated giant cells. Bar = 100 μm. (M) Chronic granulomatous ruminitis, with lymphangiectasia of the mucosal chorion vessels. Bar = 500 μm. N) Acute ruminitis with haemorrhage in the mucosal chorion. Bar = 100 μm.

reported for lambs supplemented with treatment B45 were below the usual values observed for Merino Branco lambs in similar conditions (Santos-Silva et al., 2019; Dentinho et al., 2020). Comparatively, the growth rates observed for lambs supplemented with the other three treatments were within the expected range of average daily gain values (Santos-Silva et al., 2019; Dentinho et al., 2020). There are no reports about the effect of Bromoil on diets for growing ruminants. However, Roque et al. (2021) reported that steers

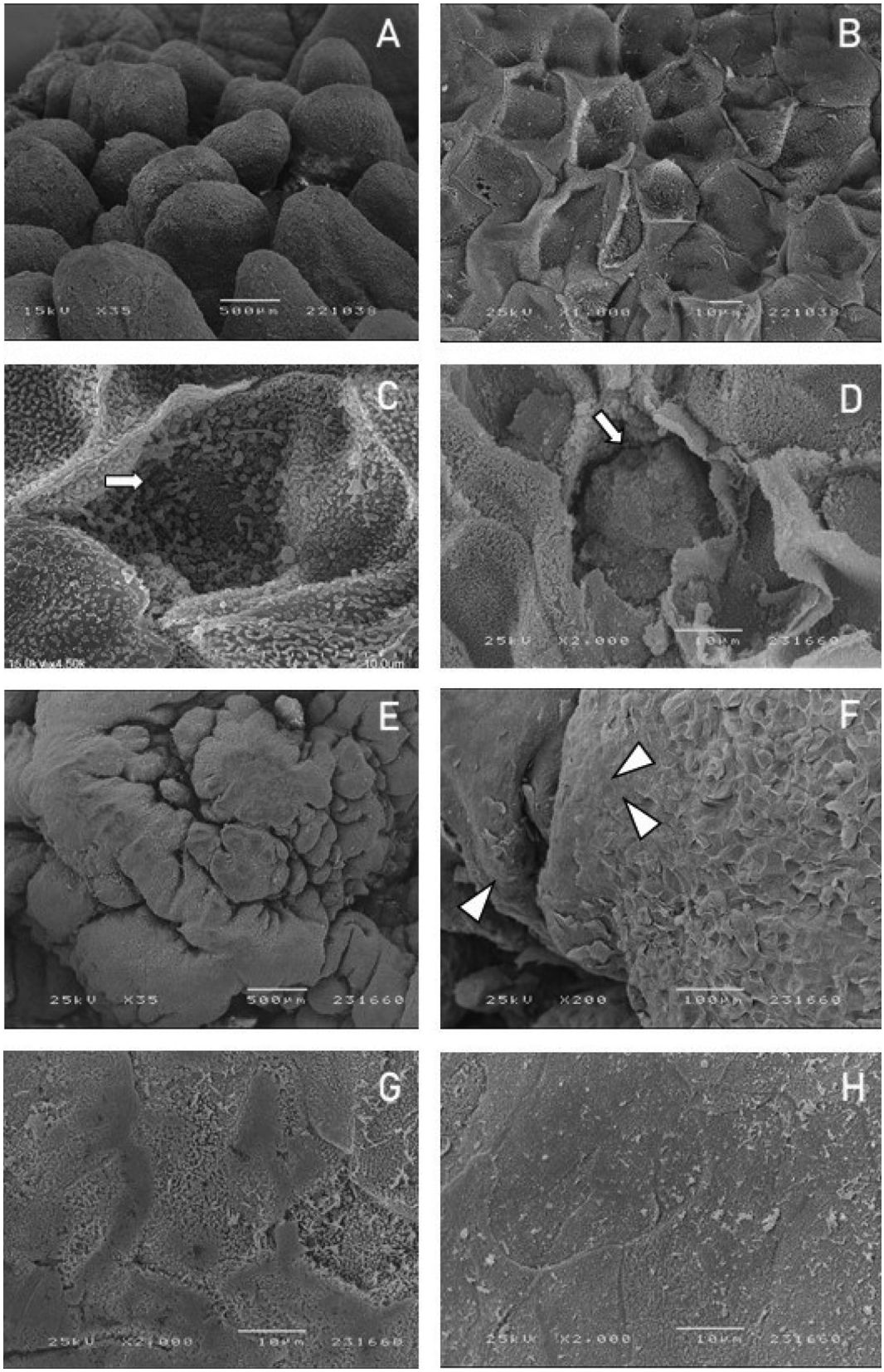

Fig. 3. Scanning electronic microscopy of the inner surface of the rumen mucosa of lambs supplemented bromoform ($CHBr_3$) through a sunflower oil macerate of *Asparagopsis taxiformis*. Plate description: (A) Normal aspect of the ruminal papillae. Bar = 500 μm. (B) Aspect of the irregular concave prismatic surface of the papillae that can house the ruminal microbiome. Bar = 10 μm. (C) Idem, higher amplification - arrow points at the housing of the microbiome. Bar = 10 μm. (D) Ruminal major morphological changes with the loss of mucosal ruminal papillae pattern (arrow) and the development of proliferative mushroom-like structures. Bar = 10 μm. (E) Ruminal mucosa displaying a huge, modified area with loss of papilla pattern and the development of amorphic structures. Bar = 500 μm. (F) In the modified ruminal mucosa occurred the loss of papilla organisation and consequently the typical irregular concave prismatic ultrastructure of the papillae was also lost. Bar = 100 μm. (G) and (H) Display the huge alteration verified in multiple spots of the ruminal mucosa, that no longer present the papillae pattern and display a condition of reduced and flat mucosal surface. Bar = 10 μm.

fed diets supplemented with up to 0.5% of freeze-dried AT (39 mg/kg DM of $CHBr_3$) maintained growth rate despite the linear decrease in feed intake (less than 14% compared to control). This suggests that feed intake might be more responsive to the effects of $CHBr_3$ than growth rate and thus points to an increase in efficiency. We also observed a similar pattern in treatment B30, where the DM intake was diminished by 21% compared to B0, and the average daily gain was non-significantly depressed by only 15%. However, with treatment B45, the depression in average daily gain was drastic (less than 60% that of B0) and even larger in DM intake (less than 72% that of B0), which suggests the occurrence of abnormal health status. The clear reduction of average daily gain observed with treatment B45 was also reflected in the significantly lower hot carcass weight but not on slaughter live weight of the B45 lambs. This indicates a higher offal weight in the B45 lambs, probably due to the hypertrophy of visceral organs like the rumen and could explain the lower dressing percentage observed.

In this experiment, the anti-methanogenic effect of Bromoil was assessed *in vitro*, using the individual ruminal content of lambs as inoculum, collected right after slaughter, thus maintaining the animal as the experimental unit. Each ruminal inoculum was incubated using the diet fed to the donor as substrate, thus being fully adapted to the level of $CHBr_3$ supplementation. The data obtained for CH_4 production and OM fermentation after the 24 h *in vitro* incubation will only be interpreted as a proxy of the *in vivo* conditions. Other experiments in which CH_4 emissions can be measured directly in live lambs should confirm these results.

The effect of $CHBr_3$ supplementation using Bromoil as a carrier in an *in vitro* experiment has already been reported by Kinley et al. (2022) and Sena et al. (2024). In both experiments, the threshold value for reducing CH_4 production was between 50 and 75 mg $CHBr_3$/kg DM. in those two experiments, the ruminal inoculum was collected from animals slaughtered in an industrial plant and fed conventional beef finishing diets. Under those conditions, all the $CHBr_3$ in the system came from Bromoil in the substrate. However, in the present experiment, the donor animals were lambs fed experimental diets supplemented with Bromoil for around 35 days. The microbiota could have adjusted to the sustained presence of $CHBr_3$, with consequent inhibition of the methanogenic pathways in the rumen, so, we could assume that a lower dosage of $CHBr_3$ will be required to substantially reduce CH_4 production.

In the present experiment, the supplementation with $CHBr_3$ did not depress the ruminal fermentative activity, as evaluated *in vitro* by the total gas production, gas without CH_4 and OM degradation. Kinley et al. (2022) reported that the supplementation of forage with Bromoil at $CHBr_3$ concentrations up to 175 mg/kg DM did not affect DM degradability of the production of volatile FA but decreased total gas production by 25% after 72 h of incubation. Sena et al. (2024) reported that supplementing a total mixed diet with Bromoil at $CHBr_3$ concentrations up to 150 mg/kg DM had no effect on total gas production or OM degradability but decreased linearly the volatile FA production. Nevertheless, the results are difficult to compare to those of the present experiment due to the differences in $CHBr_3$ dosage or lack of previous adaptation of the ruminal inocula to the presence of $CHBr_3$.

The applicability of a feed supplement to reduce methanogenesis depends on the market availability of the feed and the impacts on various productive aspects, such as production costs, animal productivity, quality and safety of products for human consumption, and feed management. However, it also depends on the impact on animal health and welfare and food safety. No residues of $CHBr_3$ were detected in meat or liver of the lambs supplemented with Bromoil, indicating that the toxicological risk for consumers is negligible. For these levels of supplementation, blood parameters did not reveal any significant changes to the animals' health. The absorption of $CHBr_3$ along the digestive tract may be null or low, and when it occurs, it is probably excreted in urine, as suggested by Glasson et al. (2022). Moreover, the metabolism of $CHBr_3$ supplied by AT in the rumen seems to be quite fast, as reported by Romero et al. (2023). The authors observed that nearly 90% of $CHBr_3$ was degraded to CH_2Br_2 in the first 3 h of incubation, and that this process was independent of the substrate used as feed. The high volatility of CH_3Br and high reactivity of Br with different elements present in the medium prevented a more complete characterization of $CHBr_3$ degradation pathways in the rumen (Romero et al., 2023).

Throughout the experimental period, the animals consistently maintained normal health, a fact corroborated by the daily visual observations. Although the blood samples collected at slaughter are not representative of the whole experiment, they express the animals' health status after 5 weeks of Bromoil supplementation. Further research on Bromoil supplementation with lambs should take frequent blood samples throughout the experiment. However, upon postslaughter examination of the rumen wall, distinct lesions emerged, concentrated mainly on the ventral region of the rumen. These lesions pointed to an underlying inflammatory process, coinciding with a marked reduction in both the number and size of rumen papillae. It is important to emphasise that these lesions were observed exclusively in the animals assigned to treatments B15, B30 and B45, with the most pronounced effects observed in groups B30 and B45. In these treatments, all animals exhibited a spectrum of lesions, including acute purulent erosive ruminitis, hyperkeratosis, proliferative lesion, atrophy of the rumen papillae, proliferative lesion of the squamous epithelium of the mucosa, chronic sclerosing ruminitis, chronic granulomatous ruminitis, rumen mucosa lymphangiectasia and microabscesses. Furthermore, histological examination of rumen papillae revealed a consistent invasion of inflammatory cells across all animals supplemented with treatments B15, B30 and B45. The fact that these lesions were located on the rumen ventral sac led us to suspect that they might result from the direct contact between the feed and the mucosa. The diet used in this trial was a ground high-density complete diet, the forage (dehydrated alfalfa) was also ground and Bromoil was mixed with the other raw materials. The small particles of the ground feed would be easily hydrated and deposited mainly on the ventral regions of the rumen, which led us to assume that this was the main reason why the changes were located only in this region. Crossing these features with the findings from scanning electron microscopy, it was clear that in these proliferative masses was loss of the papillated area. Consequently, a decrease in the surface of the rumen mucosa occurred, with loss of the papillae revetments. Altogether, a condition of the reduced and flat mucosal surface occurs, presumably with loss of absorptive function. Most of the published studies in which the animals were fed AT do not report on the rumen wall status. The exceptions are one study with sheep (Li et al., 2018) and another with dairy cows (Muizelaar et al., 2021). Li et al. (2018) observed extensive nodular proliferation and discolouration in the rumen mucosa with the blunting of ruminal papillae on wethers fed a complete diet supplemented with AT up to 3% OM intake. Muizelaar et al. (2021) reported that dairy cows fed 333 g of AT DM/day presented a significant area of rumen mucosa with the absence of rumen papillae accompanied by haemorrhages and ulcers. In localised areas, there was a loss of papillae and a thickening of the mucosa. The association between AT and Bromoil intake, and the rumen lesions needs to be better understood, but it suggests that using AT-based supplements in ruminant feeds is not without risk. It is unclear whether the occurrence of mucosa lesions is related to the form in which $CHBr_3$ is carried into the rumen or type of basal diet used. Bulkier diets where $CHBr_3$ can

be diluted in a higher feed volume, could also reduce this risk. There is a need to deepen existing knowledge on this subject.

The potential carcinogenic and ozone-depleting effects of $CHBr_3$ and other halomethane analogues raise some concerns when supplying these substances to animals used for human consumption (Pandey et al., 2021; Glasson et al., 2022). It has been shown that $CHBr_3$ is absorbed by AT- supplemented dairy cows and is excreted in trace amounts in urine and milk (Muizelaar et al., 2021; Alvarez-Hess et al., 2023). The $CHBr_3$ detected in the milk form those cows was below the recommended drinking water for safe consumption (EPA, 2018). Apparently, $CHBr_3$ is not accumulated in the muscle and adipose tissues. In the present experiment, $CHBr_3$ was not detected in any muscle samples, confirming previous results (Li et al., 2018; Kinley et al., 2020; Roque et al., 2021). These results suggest that diets of finishing ruminants can be supplemented with $CHBr_3$ carried by Bromoil without compromising the safety of meat.

Conclusions

The findings of this study indicate that Bromoil with $CHBr_3$ doses of 30–45 mg/kg DM reduced more than 56% of CH_4 emissions evaluated *in vitro* with rumen inocula from individual lambs. No $CHBr_3$ was detected in meat indicating that it can be safe for human consumption. Despite this, animal performance was impaired by the higher doses of $CHBr_3$, with decreased intake and growth. Moreover, the animals fed the highest $CHBr_3$ concentrations presented severe lesions in the ventral region of the rumen wall. The marked loss of $CHBr_3$ from air-exposed feed presents a challenge for the practical farm-level implementation of the Bromoil- supplemented diets and if scaled-up might contribute to the depletion of the stratospheric ozone. Although Bromoil showed a high potential to reduce rumen CH_4 production further research is needed to ensure it can effectively improve farm's environmental sustainability, without negatively affecting animal health and productivity.

Ethics approval

The project was approved by INIAV's ORBEA with the process ORBEA-INIAV/04/2023.

Data and model availability statement

None of the data were deposited in an official repository. The dataset analysed in this study is available from the corresponding author upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

Author ORCIDs

F. Sena: https://orcid.org/0000-0002-6918-5301.
A.P. Portugal: https://orcid.org/0000-0003-0623-437X.
M.T. Dentinho: https://orcid.org/0000-0002-3848-3402.
J. Costa: https://orcid.org/0000-0001-6063-8928.
A. Francisco: https://orcid.org/0000-0002-2559-6379.
S. Moradi: https://orcid.org/0009-0000-9179-2784.
K. Paulos: https://orcid.org/0000-0002-9335-1699.
D.M. Soares: https://orcid.org/0000-0002-9924-769X.
D. Henriques: https://orcid.org/0000-0002-0673-4203.
A. Oliveira: https://orcid.org/0000-0002-5207-535X.
H. Ramos: https://orcid.org/0009-0004-0287-0269.
R. Bexiga: https://orcid.org/0000-0002-2524-9887.
J. Correia: https://orcid.org/0000-0002-1909-4540.
G. Alexandre-Pires: https://orcid.org/0000-0002-9462-4679.
S.P. Alves: https://orcid.org/0000-0002-3171-9566.
T. Domingos: https://orcid.org/0000-0002-6194-0405.
R.J. B. Bessa: https://orcid.org/0000-0003-4109-3488.
J. Santos-Silva: https://orcid.org/0000-0002-9171-0977.

CRediT authorship contribution statement

F. Sena: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **A.P. Portugal:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **M.T. Dentinho:** Writing – review & editing, Methodology, Investigation, Formal analysis. **J. Costa:** Writing – review & editing, Resources, Investigation. **A. Francisco:** Writing – review & editing, Methodology, Investigation, Formal analysis. **S. Moradi:** Writing – review & editing, Methodology, Investigation, Formal analysis. **K. Paulos:** Writing – review & editing, Methodology, Investigation, Formal analysis. **D.M. Soares:** Writing – review & editing, Investigation, Formal analysis. **D. Henriques:** Writing – review & editing, Data curation. **A. Oliveira:** Writing – review & editing, Resources, Methodology, Investigation. **H. Ramos:** Writing – review & editing, Resources, Methodology, Investigation. **R. Bexiga:** Writing – review & editing, Methodology, Investigation, Formal analysis. **J.J. Correia:** Writing – review & editing, Methodology, Investigation, Formal analysis. **G. Alexandre-Pires:** Writing – review & editing, Methodology, Investigation, Formal analysis. **T. Domingos:** Writing – review & editing, Resources. **S.P. Alves:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **R.J.B. Bessa:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **J. Santos-Silva:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of interest

None.

Acknowledgements

None.

Financial support statement

This study was supported by two Ph.D. research grants awarded by Fundação para a Ciência e Tecnologia (FCT) to F. Sena (UI/BD/152817/2022) and to D. Soares (2022.13385.BDANA). Other grants awarded by FCT: PTDC/CAL-ZOO/29654/2017, UIDB/00276/2020 (CIISA) and LA/P/0059/2020 (AL4AnimalS). Funding from projects GEEBovMit (PRR-C05-i03-I-000027 GEEBovMit), Greenbeef (POCI-01-0247-FEDER-047050/LISBOA-01-FEDER-047050) is also acknowledged.

References

Alvarez-Hess, P.S., Jacobs, J.L., Kinley, R.D., Roque, B.M., Neachtain, A.S.O., Chandra, S., Williams, S.R.O., 2023. Twice daily feeding of canola oil steeped with *Asparagopsis armata* reduced methane emissions of lactating dairy cows. Animal Feed Science and Technology 297, 115579. https://doi.org/10.1016/j.anifeedsci.2023.115579.

Beauchemin, K.A., Ungerfeld, E.M., Eckard, R.J., Wang, M., 2020. Review: Fifty years of research on rumen methanogenesis: Lessons learned and future challenges for mitigation. Animal 14, s2–s16. https://doi.org/10.1017/S1751731119003100.

Chaves, A.V., Dugan, M.E.R., Stanford, K., Gibson, L.L., Bystrom, J.M., McAllister, T.A., Van Herk, F., Benchaar, C., 2011. A dose-response of cinnamaldehyde supplementation on intake, ruminal fermentation, blood metabolites, growth performance, and carcass characteristics of growing lambs. Livestock Science 141, 213–220. https://doi.org/10.1016/j.livsci.2011.06.006.

Clegg, K.M., 1956. The application of the anthrone reagent to the estimation of starch in cereals. Journal of the Science of Food and Agriculture 7, 40–44. https://doi.org/10.1002/jsfa.2740070108.

Dentinho, M.T.P., Paulos, K., Francisco, A., Belo, A.T., Jerónimo, E., Almeida, J., Bessa, R.J.B., Santos-Silva, J., 2020. Effect of soybean meal treatment with *Cistus ladanifer* condensed tannins in growth performance, carcass and meat quality of lambs. Livestock Science 236, 104021. https://doi.org/10.1016/j.livsci.2020.104021.

EPA, United States Environmental Protection Agency, 2018. 2018 Edition of the Drinking Water Standards and Health Advisories Tables. EPA 822-F-18-001. Office of Water U.S. Environmental Protection Agency, Washington, DC, USA.

EU, 2009. Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. Official Journal of the European Union L303, 1–30.

European Commission, 2008. Commission regulation (EC) N° 1249/2008 of 10 December 2008 laying down detailed rules on the implementation of the Community scales for the classification of beef, pig and sheep carcases and the reporting of prices thereof. Official Journal of the European Union L337, pp. 3–30.

Gerber, P.J., Hristov, A.N., Henderson, B., Makkar, H., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A.T., Yang, W.Z., Tricarico, J.M., Kebreab, E., Waghorn, G., Dijkstra, J., Oosting, S., 2013. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: A review. Animal 7, 220–234. https://doi.org/10.1017/S1751731113000876.

Glasson, C.R.K., Kinley, R.D., de Nys, R., King, N., Adams, S.L., Packer, M.A., Svenson, J., Eason, C.T., Magnusson, M., 2022. Benefits and risks of including the bromoform containing seaweed *Asparagopsis* in feed for the reduction of methane production from ruminants. Algal Research 64, 102673. https://doi.org/10.1016/j.algal.2022.102673.

ISO1442, 1997. Meat and meat products. Determination of moisture content (reference method). International Organization for Standardization, Geneve, Switzerland.

ISO2917, 1999. Meat and meat products — Measurement of pH — Reference method. International Organization for Standardization, Geneve, Switzerland.

ISO5983, 1997. Animal feeding stuffs - determination of nitrogen content and calculation of crude protein content, Kjeldhal method. International Organization for Standardization, Geneve, Switzerland.

ISO5984, 2022. Animal feeding stuffs - determination of crude ash. International Organization for Standardization, Geneve, Switzerland.

ISO6492, 1999. Animal feeding stuffs - determination of fat content. International Organization for Standardization, Geneve, Switzerland.

ISO6496, 1999. Animal feeding stuffs - determination of moisture and other volatile matter content. International Organization for Standardization, Geneve, Switzerland.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. Journal of Animal Science 73, 2483–2492. https://doi.org/10.2527/1995.7382483x.

Kinley, R.D., Martinez-Fernandez, G., Matthews, M.K., de Nys, R., Magnusson, M., Tomkins, N.W., 2020. Mitigating the carbon footprint and improving productivity of ruminant livestock agriculture using a red seaweed. Journal of Clean Production 259, 120836. https://doi.org/10.1016/j.jclepro.2020.120836.

Kinley, R., Tan, S., Turnbull, J., Askwe, S., Harris, J., Roque, B., 2022. Exploration of methane mitigation efficacy using *Asparagopsis*-derived bioactives stabilized in edible oil compared to freeze-dried *Asparagopsis in vitro*. American Journal of Plant Sciences 13, 1023–1041. https://doi.org/10.4236/ajps.2022.137068.

Kladi, M., Vagias, C., Roussis, V., 2004. Volatile halogenated metabolites from marine red algae. Phytochemistry Reviews 3, 337–366. https://doi.org/10.1007/s11101-004-4155-9.

Li, X., Norman, H.C., Kinley, R.D., Laurence, M., Wilmot, M., Bender, H., de Nys, R., Tomkins, N., 2018. *Asparagopsis taxiformis* decreases enteric methane production from sheep. Animal Production Science 58, 681–688. https://doi.org/10.1071/AN15883.

Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N., 2016a. Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on *in vitro* fermentation and methane production. Journal of Applied Phycology 28, 1443–1452. https://doi.org/10.1007/s10811-015-0639-9.

Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N., 2016b. Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity *in vitro*. Journal of Applied Phycology 28, 3117–3126. https://doi.org/10.1007/s10811-016-0830-7.

Machado, L., Tomkins, N., Magnusson, M., Midgley, D.J., de Nys, R., Rosewarne, C.P., 2018. *In vitro* response of rumen microbiota to the antimethanogenic red macroalga *Asparagopsis taxiformis*. Microbial Ecology 75, 811–818. https://doi.org/10.1007/s00248-017-1086-8.

Magnusson, M., Vucko, M.J., Neoh, T.L., de Nys, R., 2020. Using oil immersion to deliver a naturally-derived, stable bromoform product from the red seaweed *Asparagopsis taxiformis*. Algal Research 51, 102065. https://doi.org/10.1016/j.algal.2020.102065.

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D., Schneider, W., 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. Journal of Agricultural Sciences 93, 217–222. https://doi.org/10.1017/S0021859600086305.

Muizelaar, W., Groot, M., van Duinkerken, G., Peters, R., Dijkstra, J., 2021. Safety and transfer study: Transfer of bromoform present in *Asparagopsis taxiformis* to milk and urine of lactating dairy cows. Foods 10, 584. https://doi.org/10.3390/foods10030584.

Pandey, D., Mansouryar, M., Novoa-Garrido, M., Naess, G., Viswanath, K., Hansen, H., Nielsen, M., Khanal, P., 2021. Nutritional and anti-methanogenic potentials of macroalgae for ruminants. In: Lei, X.G. (Ed.), Seaweed and microalgae as alternative sources of protein. Burleigh Dodds Science Publishing Limited, Cambridge, UK, pp. 1–33. https://doi.org/10.19103/AS.2021.0091.14.

Reay, D., Smith, P., Van Amstel, A., 2010. Methane sources and the global methane budget. In: Reay, D., Smith, P., Van Amstel, A. (Eds.), Methane and Climate Change. Earthscan, London, UK, pp. 1–13.

Romero, P., Belanche, A., Jiménez, E., Hueso, R., Ramos-Morales, E., Salwen, J.K., Kebreab, E., Yáñez-Ruiz, D.R., 2023. Rumen microbial degradation of bromoform from red seaweed (*Asparagopsis taxiformis*) and the impact on rumen fermentation and methanogenic archaea. Journal of Animal Science and Biotechnology 14, 133. https://doi.org/10.1186/s40104-023-00935-z.

Roque, B.M., Salwen, J.K., Kinley, R., Kebreab, E., 2019. Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. Journal of Clean Production 234, 132–138. https://doi.org/10.1016/j.jclepro.2019.06.193.

Roque, B.M., Venegas, M., Kinley, R.D., de Nys, R., Duarte, T.L., Yang, X., Kebreab, E., 2021. Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. PLOS ONE 16, e0247820.

Santos-Silva, J., Francisco, A., Alves, S.P., Portugal, P., Dentinho, T., Almeida, J., Soldado, D., Jerónimo, E., Bessa, R.J.B., 2019. Effect of dietary neutral detergent fibre source on lambs growth, meat quality and biohydrogenation intermediates. Meat Science 147, 28–36. https://doi.org/10.1016/j.meatsci.2018.08.015.

Sena, F., Portugal, P.V., Dentinho, M.T., Paulos, K., Costa, C., Soares, D.M., Oliveira, A., Ramos, H., Alves, S.P., Santos-Silva, J., Bessa, R.J.B., 2024. Effects of sunflower oil infusions of *Asparagopsis taxiformis* on *in vitro* ruminal methane production and biohydrogenation of polyunsaturated fatty acids. Journal of Dairy Science 107, 1472–1484. https://doi.org/10.3168/jds.2023-23506.

Smith, B.P., Van Metre, D.C., Pusterla, N., 2015. Large animal internal medicine. Elsevier Mosby, St. Louis, MO, USA.

Stefenoni, H.A., Räisänen, S.E., Cueva, S.F., Wasson, D.E., Lage, C.F.A., Melgar, A., Fetter, M.E., Smith, P., Hennessy, M., Vecchiarelli, B., Bender, J., Pitta, D., Cantrell, C.L., Yarish, C., Hristov, A.N., 2021. Effects of the macroalga *Asparagopsis taxiformis* and oregano leaves on methane emission, rumen fermentation, and lactational performance of dairy cows. Journal of Dairy Science 104, 4157–4173. https://doi.org/10.3168/jds.2020-19686.

Tan, S., Harris, J., Roque, B.M., Askew, S., Kinley, R.D., 2022. Shelf-life stability of Asparagopsis bromoform in oil and freeze-dried powder. Journal of Applied Phycology 35, 291–299. https://doi.org/10.1007/s10811-022-02876-y.

Tegtmeier, S., Ziska, F., Pisso, I., Quack, B., Velders, G.J.M., Yang, X., Krüger, K., 2015. Oceanic bromoform emissions weighted by their ozone depletion potential. Atmospheric Chemistry and Physics 15, 13647–13663. https://doi.org/10.5194/acp-15-13647-2015.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Vucko, M.J., Magnusson, M., Kinley, R.D., Villart, C., de Nys, R., 2017. The effects of processing on the *in vitro* antimethanogenic capacity and concentration of secondary metabolites of *Asparagopsis taxiformis*. Journal of Applied Phycology 29, 1577–1586. https://doi.org/10.1007/s10811-016-1004-3.

Wasson, D.E., Yarish, C., Hristov, A.N., 2022. Enteric methane mitigation through *Asparagopsis taxiformis* supplementation and potential algal alternatives. Frontiers in Animal Science 3, 999338. https://doi.org/10.3389/fanim.2022.999338.