J. Dairy Sci. 107:1472–1484
https://doi.org/10.3168/jds.2023-23506

Effects of sunflower oil infusions of *Asparagopsis taxiformis* on in vitro ruminal methane production and biohydrogenation of polyunsaturated fatty acids

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ABSTRACT

Asparagopsis taxiformis inhibits ruminal methane (CH_4) production due to its bromoform ($CHBr_3$) content. The immersion of *A. taxiformis* in edible vegetable oils allows the extraction and stabilization of the highly volatile $CHBr_3$ in the oil phase. The objectives of this study were to explore the effects of adding sunflower oils with increasing concentrations of $CHBr_3$ on in vitro ruminal methanogenesis and biohydrogenation. Five batches of 48-h in vitro incubations were performed in 14 fermentation bottles, using rumen inocula collected shortly after the slaughter of young crossbred bulls and 1 g of dry matter (DM) from a total diet of mixed feed without added oil (control) or with 60 μL of sunflower oil per gram of DM as the substrate. The treatments were the $CHBr_3$ content in the oil added: 0 μg (B0), 25 μg (B25), 50 μg (B50), 75 μg (B75), 100 μg (B100), and 150 μg (B150) of $CHBr_3$ per gram of substrate DM. Organic matter (OM) degradability, total gas, CH_4, volatile fatty acids (VFA), long-chain fatty acids, and dimethyl acetals (DMA) were analyzed at the end of each incubation. Data were analyzed with a model considering the treatments as the fixed effect and the run as a random block and using orthogonal contrasts. Degradability of OM was higher in the control group and was unaffected by $CHBr_3$ concentration. Total gas production per gram of degraded OM was unaffected by treatments and averaged 205 ± 29.8 mL/g. Methane (mL) production decreased linearly with increasing $CHBr_3$ concentrations, with 33%, 47%, and 87% reductions for B75, B100, and B150, respectively. Total VFA concentration was unaffected by oil inclusion but was reduced by 20% in $CHBr_3$-containing treatments, although without any dose-response pattern. The molar percentage of acetate decreased linearly, whereas propionate and butyrate increased linearly with the increasing $CHBr_3$ dosage. Including oil in the diet decreased the branched-chain fatty acids and DMA content. Increasing $CHBr_3$ concentrations did not affect branched-chain fatty acids, but linearly increased most of the identified DMA. Adding oil to the control diet increased the 18:2n-6, whereas increasing the concentration of $CHBr_3$ had no effect on 18:2n-6 but decreased linearly the 18:0 and increased the *trans*-18:1 isomers. The results obtained provide evidence that oil immersions of *A. taxiformis* can successfully inhibit ruminal production of CH_4 in vitro at doses of 100 and 150 μg/g DM, and simultaneously modulate biohydrogenation.

Key words: bromoform, biohydrogenation, methane, rumen

INTRODUCTION

Livestock are an important source of greenhouse gas (**GHG**) emissions, contributing to approximately 7.1 Gt of CO_2eq per year (Gerber et al., 2013). The GHG resulting from the production of ruminants contributes 5.7 Gt CO_2eq per year, with 47% of the sector's emissions being methane (CH_4; Opio et al., 2013). The CH_4 is an end product of ruminal fermentation representing between 2% and 12% of gross energy intake and, when released into the atmosphere, is a potent GHG (Johnson and Johnson, 1995).

Different strategies have been studied to reduce CH_4 production (Martin et al., 2010; Knapp et al., 2014; Beauchemin et al., 2020). One of these strategies relies on supplementing certain macroalgae species contain-

Received March 19, 2023.
Accepted September 26, 2023.
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ing secondary metabolites, which are particularly successful in reducing ruminal CH_4 production (Vucko et al., 2017; Sofyan et al., 2022). One of the most promising species to reduce CH_4 production at low levels of biomass inclusion is the red *Asparagopsis taxiformis* (Glasson et al., 2022).

Asparagopsis taxiformis reduces CH_4 production (mL/g substrate OM) due to the presence of secondary metabolites, mainly halogenated compounds, of which bromoform ($CHBr_3$) has the most relevant contribution (Machado et al., 2016b). These metabolites are naturally produced by red algae as a defense mechanism (Kladi et al., 2004; Paul et al., 2006). Halomethane compounds react with the reduced vitamin B_{12}, decreasing the efficiency of the cobamide-dependent methyl transferase (Wood et al., 1968), which is a crucial step in methanogenic pathways in ruminal archaea. Although halomethanes are highly effective in inhibiting CH_4 production, their use in ruminant nutrition has been limited due to their toxicity to animals and humans, which raises some concerns (Glasson et al., 2022). Low dosages of naturally occurring $CHBr_3$ supplied by *A. taxiformis* have shown to be effective in reducing digestive CH_4 emissions in dairy cows, although often coupled with decreased DMI and milk production (Stefenoni et al., 2021) and reduced ruminal functionality (Andreen et al., 2023; Terry et al., 2023).

The high volatility of $CHBr_3$ makes freeze-drying the algal biomass a key procedure in supplementing *A. taxiformis* as a CH_4 inhibition strategy (Vucko et al., 2017). Despite this, even the freeze-dried biomass loses $CHBr_3$ during storage, as shown by Stefenoni et al. (2021). Immersions of algal biomass in oil can be a viable processing alternative to the more expensive and logistically challenging processing techniques such as freeze-drying, allowing for stabilizing $CHBr_3$ concentrations for at least 12 wk (Magnusson et al., 2020).

Oils containing $CHBr_3$ (hereafter referred to as bromoils) are expected to inhibit ruminal methanogenesis similarly to algal biomass, as recently reported in vitro for an all-forage diet (Kinley et al., 2022). The kinetics of $CHBr_3$ release into the rumen medium can differ from algal biomass, and there is the possibility of synergy between $CHBr_3$ and the oil's PUFA on methanogenesis inhibition. Thus, we hypothesized that bromoil will effectively reduce CH_4 production even with the lowest dosages of $CHBr_3$. In contrast, the effect of $CHBr_3$ on ruminal biohydrogenation (**BH**) of PUFA has not been studied. Biohydrogenation is the extensive incorporation of H_2 in the carbon chains of fatty acids (**FA**), producing SFA (Harfoot and Hazlewood, 1997). The inhibition of methanogenesis by $CHBr_3$ was demonstrated to increase H_2 availability (Kinley et al., 2020). Thus, we hypothesize that $CHBr_3$ will promote the ruminal BH of UFA in the rumen by increasing H_2 availability. The present research aimed to determine the effects of increasing the dosage of $CHBr_3$ supplied as bromoil on CH_4 inhibition and PUFA hydrogenation in the rumen using an in vitro batch system.

MATERIALS AND METHODS

Because no living animal subjects were used, this analysis did not require approval by an Institutional Animal Care and Use Committee.

Algal Biomass Processing

Asparagopsis taxiformis was harvested in the gametophyte lifecycle stage by seaExpert (Feteira, Horta, Portugal) in May 2021 at Angústias, Faial Island, Azores, Portugal (38°31′45″N, 28°37′09″W). The algae were collected by diving between 3 to 6 m. The biomass was stored in a dark, cooled container and promptly transported to land, arriving no more than 1 h after collection. After rinsing, the fresh biomass was sectioned, manually homogenized, and immersed in 250-mL Schott bottles containing 100 mL of refined sunflower oil, in an 8:10 wt/vol ratio. The biomass used in each bottle (80 g) was the maximum physically possible, given the capacity of the bottles used. The bottles were then transported to Faculdade de Medicina Veterinária (University of Lisbon), where the $CHBr_3$ concentration in the oil was evaluated before its use. During storage, the bottles with the oil and *A. taxiformis* were preserved in the dark and at room temperature.

Bromoform Quantification in the Oil

The concentration of $CHBr_3$ in the oil immersion of *A. taxiformis* was determined with a procedure adapted from the one reported by Magnusson et al. (2020). Briefly, 1.5 mL of bromoil was mixed with methanol and internal standard (naphthalene, 10 μg/mL) and analyzed by GC-MS using a Shimadzu GC-MS QP2010-plus instrument (Shimadzu Corp., Kyoto, Japan) equipped with a fused silica capillary column (Supelcowax10, 30 m × 0.25 mm i.d. × 0.20 μm film thickness, Supelco Inc., Bellefonte, PA). Quantification of $CHBr_3$ was performed with an ionization energy of 70 eV using the selected ion monitoring mode targeting the m/z 173 and m/z 128 quantifier ions for the $CHBr_3$ and naphthalene, respectively. The $CHBr_3$ concentration was calculated from its peak area ratio over the internal standard peak area and converted to concentration by reference to standard curves.

In Vitro Ruminal Fermentation

Five series of 48-h batch incubation were conducted in an Ankom RF Gas Production System (Ankom Technology) to assess the effect of the increasing concentration of $CHBr_3$ supplied as bromoil on ruminal fermentation parameters, total gas production and CH_4 concentration in the headspace gas.

The ruminal content used for the incubations was collected, in 5 consecutive weeks, at a commercial slaughterhouse (Santacarnes, Santarém, Portugal) from 3 young crossbred young bulls (15 mo of age) from the same farm, at the day of the incubation. Approximately 500 mL of ruminal content was individually collected soon after the digestive viscera became available at the abattoir (15 min after slaughter), stored in previously heated insulated plastic containers, and immediately transported to the Instituto Nacional de Investigação Agrária e Veterinária (INIAV; Vale de Santarém, Portugal) laboratory (about 10 km distance) in a larger container filled with tepid water. At the INIAV laboratory, the ruminal content from the 3 animals were strained through 4 layers of cheesecloth, pooled, maintained at 38°C, and flushed with CO_2 until being used in the filling of the fermentation bottles. The pH was measured before starting each incubation, averaging 7.03 ± 0.17 (mean ± SEM) for the 5 runs.

On the day of each incubation run, 1 g DM of a ground (1-mm sieve mill) feed substrate was weighed into each fermentation bottle. The feed substrate was a TMR diet, designed for a young bull's fattening experiment (the "MBp" diet described by Santos-Silva et al., 2022), that comprised of 600 g/kg DM of a biodiverse forage haylage and 400 g/kg DM of concentrate ingredients, including corn (52 g/kg DM), wheat (48 g/kg DM), barley (48 g/kg DM), dehydrated citrus pulp (51 g/kg DM), dehydrated beet pulp (48 g/kg DM), soybean hulls (48 g/kg DM), soybean meal (32 g/kg DM), and sunflower meal (24 g/kg DM). The TMR contained on DM basis 166 g/kg of CP, 101 g/kg of starch, 52 g/kg of total sugars, 351 g/kg of NDF, and 22 g/kg of ether extract. The feed substrate was dried at 65°C and milled to pass a 1-mm sieve in a hammer mill (Christy and Norris Lda, serial number 19682, Ipswich, UK).

The treatments tested were control (**CON**, only basal feed substrate, 1 g DM) and 6 treatments of basal feed substrate (1 g DM) plus 60 μL of sunflower oil without (**B0**) or with $CHBr_3$ (**B25**, **B50**, **B75**, **B100,** and **B150**). The volume of sunflower oil was pipetted onto the basal substrate before adding the buffer solution and the ruminal content. Bromoils with increasing $CHBr_3$ concentrations were prepared by diluting the original bromoil with sunflower oil to achieve $CHBr_3$ concentrations of 0.42, 0.83, 1.25, 1.67, and 2.50 mg/mL, corresponding to concentrations of 25, 50, 75, 100, and 150 μg of $CHBr_3$ per gram of substrate DM, supplied in 60 μL of bromoil, respectively.

The buffer solution was prepared according to Menke et al. (1979), and 60 mL was added to each fermentation bottle. The 14 fermentation bottles (2 per treatment) were kept in warm water (39°C) for 20 to 30 min. After this time, 2 mL of reducing solution preheated to 39°C was added to each fermentation bottle. Thirty milliliters of the pooled ruminal liquid was then added to each fermentation bottle, resulting in 92 mL of final incubation volume. Before closing, the headspace of the fermentation bottles was flushed with CO_2. The head modules were tightly secured, and the fermentation bottles were placed in a water bath (39°C) with agitation (50 rpm).

After 48 h, the incubation was stopped by transferring all fermentation bottles to a cold-water bath. Gas samples were collected with a 20-mL polypropylene syringe (B. Braun, Omnifix) in 9-mL vacuum tubes (Vacutest Kima).

These procedures were repeated for 5 runs, except treatment B150, which was repeated in 4 runs, with 2 replicates of each sample. In all runs, the treatments and Ankom RF modules were randomly allocated to the incubation bottles and were randomly distributed in the water bath.

OM degradability

The OM of the feed substrate was determined according to ISO standard 5984 (ISO, 2022). For the determination of OM in the fermentation bottles, their contents were centrifuged for 10 min at 1,000 × *g* at 10°C. The supernatant was saved for VFA analysis, whereas 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. Approximately 500 mg of the washed solid residues of each fermentation bottle were saved for long-chain fatty acid (**LCFA**) analysis. The remaining residue portion was weighed and used for DM and OM determination. The degradability of the feed substrate OM was computed by reconstituting the final OM weight in the residue by considering the subsample collected for LCFA analysis.

Gas and CH_4 Measurements

Total gas volume (mL) was calculated according to the Ankom RF Gas Production System operator's manual. Briefly, the cumulative pressure registered by this system is converted to moles of gas produced using

the ideal gas law and then converted to the volume of gas produced (mL) by applying the Avogadro's law.

The gas present in the headspace at the end of the incubation was sampled and analyzed using a GC equipped with a flame ionization detector to measure the CH_4 proportion. Two hundred microliters of gas was injected in splitless mode into a HP 6890A GC (Hewlett-Packard, Avondale, PA) equipped with a TG-Bond Q capillary column (Thermo Scientific; 30 m × 0.32 mm i.d. × 10 μm film thickness), using helium as the carrier gas.

Methane production (mL) was calculated using the total gas production and the proportion of CH_4 determined by GC at the end of the incubation, assuming that the gas samples were representative of total gas produced. Gas without CH_4 (**Gas-wCH_4**) was calculated by subtracting the volume of CH_4 from the volume of total gas.

FA Analysis

The VFA were prepared according to Francisco et al. (2020) but using iso-caproic as the internal standard (6:0 at 50 mmol/L). Then, VFA were analyzed by GC with flame ionization detection (**GC-FID**) using a Shimadzu GC-2010 Plus (Shimadzu Corp., Kyoto, Japan) equipped with a Nukol capillary column (30 m × 0.25 mm ii.d. × 0.25 μm film thickness, Supelco Inc., Bellefonte, PA).

Long-chain FA and alk-1-enyl (vinyl) ether chains of microbial plasmalogens were analyzed in their methyl ester derivatives (i.e., FA methyl esters and dimethyl acetals [**DMA**], respectively) which were prepared according to Alves et al. (2013). Briefly, freeze-dried samples of the washed solid residues of the fermentation bottles were directly transesterified using sodium methoxide 0.5 *M* in methanol as the alkaline catalyst, followed by adding HCl 1.25 *M* in methanol as the acid catalyst. Nonadecanoic acid (19:0) was used as the internal standard (1 mg/mL) and was added before methylation. The FAME were extracted with hexane and analyzed by CG-FID using a Shimadzu GC-2010 Plus (Shimadzu Corp., Kyoto, Japan) equipped with an SP-2560 capillary column (100 m × 0.25 mm i.d. × 0.20 μm film thickness, Supelco Inc., Bellefonte, PA). The chromatographic conditions were similar to those described by Vítor et al. (2021). Fatty acid methyl esters were identified by comparison of peak retention times with those of commercial standard mixture (FAME mix 37 components from Supelco Inc., Bellefonte, PA) and with published chromatograms (Alves et al., 2013).

Statistical Analysis

Data were analyzed using the Proc MIXED of SAS (SAS Institute Inc., Cary, NC) with a model that included treatment as the fixed effects, run as the random effect. Orthogonal contrasts were used to analyze data, where the effect of adding sunflower oil to the diet was evaluated with a contrast that compares the CON with B0; the global effect of including $CHBr_3$ was assessed with a contrast that compares the B0 with the all the bromoil-containing treatments. Finally, polynomial orthogonal contrast designing for unequal $CHBr_3$ dose spacing was used to test if among the bromoil treatments (i.e., B25, B50, B75, B100, and B150) a linear and quadratic response to $CHBr_3$ dosage was observed. The results presented in Tables 1, 2, 3, 4, and 5 express the least squares means, the standard error of means, and the contrast *P*-values for the variables analyzed. The level of significance considered to declare differences between treatments was $P \leq 0.05$, and the level of significance considered for the tendencies was $0.05 < P < 0.10$.

RESULTS

OMD, Total Gas, and CH_4 Production

Organic matter degradability (**OMD**) and gas production are presented in Table 1. The amount of OM degraded in the fermentation bottles, and hence OMD, were higher in CON than in B0, but there was no difference between B0 and the average of bromoil treatments. Nevertheless, among bromoil treatments the OMD tended to decrease linearly ($P = 0.07$) with increasing $CHBr_3$ dose.

Total gas production and Gas-wCH_4 were not affected by sunflower oil inclusion, nor by $CHBr_3$. Total gas production per gram of degraded OM averaged 205 ± 29.8 mL, and Gas-wCH_4 averaged 182 ± 24.7 mL of gas per gram of degraded OM.

Methane produced (mL) did not differ between CON and B0, but the CH_4 percentage in the gas samples was lower in B0 than in CON. Increasing $CHBr_3$ concentrations led to a linear decrease in both CH_4 production (mL) and percentage in the gas samples. Methane production observed in B75 (11.2 mL), B100 (7.4 mL), and B150 (2.3 mL) represented respectively reductions of 37%, 58%, and 87% compared with CON (17.9 mL). The CH_4 production, expressed as mL per gram of degraded OM, decreased from 32.9 in CON to 21.9, 17.3, and 4.27 in B75, B100, and B150, respectively, corresponding to a reduction of 33%, 47%, and 87%.

Table 1. Effect of stepwise inclusion of $CHBr_3$ supplied through *Asparagopsis taxiformis* oil supplementation on in vitro ruminal total gas and methane production, and OM degradability

	Treatment[1]								Contrast[2]			
Item	CON	B0	B25	B50	B75	B100	B150	SEM	SO	Br	L	Q
OM deg,[3] mg	570	545	552	552	541	535	534	11.1	0.030	0.79	0.07	0.60
OMD,[4] %	68.5	65.5	66.3	66.3	65.0	64.3	64.2	1.33	0.030	0.79	0.07	0.60
Total gas, mL	112	115	119	114	107	109	106	15.4	0.76	0.69	0.22	0.56
Gas-wCH_4,[5] mL	94	101	102	98	96	101	104	12.7	0.47	0.96	0.70	0.50
CH_4, mL	17.9	14.4	17.0	16.0	11.2	7.44	2.26	3.191	0.21	0.08	<0.001	0.97
CH_4, %[6]	14.8	12.1	13.6	13.1	10.1	7.39	2.29	1.387	0.046	0.002	<0.001	0.93
Total gas, mL/g OM deg	201	205	214	208	199	211	199	29.8	0.87	0.93	0.49	0.76
Gas-wCH_4, mL/g OM deg	168	179	184	179	177	194	195	24.7	0.54	0.60	0.36	0.68
CH_4, mL/g OM deg	32.9	25.4	29.2	28.8	21.9	17.3	4.27	6.12	0.15	0.14	<0.001	0.83
Final pH	6.74	6.77	6.79	6.79	6.80	6.81	6.78	0.025	0.45	0.70	0.64	0.41

[1]Treatments: CON = control; B0 = control plus 60 μL of sunflower oil without $CHBr_3$; or with 60 μL of oil supplying 25 μg (B25), 50 μg (B50), 75 μg (B75), 100 μg (B100), or 150 μg (B150) of $CHBr_3$/g substrate DM.

[2]Contrasts: SO = CON vs. B0; Br = B0 vs. (B25 + B50 + B75 + B100 + B150)/5; L = linear effect of $CHBr_3$ concentration; Q = quadratic effect of $CHBr_3$ concentration.

[3]OM degraded (mg per fermentation bottle).

[4]Degradability of OM.

[5]Volume of total gas without methane.

[6]Methane, expressed as percentage of total gas produced.

Table 2. Effect of stepwise inclusions of $CHBr_3$ supplied through *Asparagopsis taxiformis* oil supplementation on VFA molar percentage (mol %) in fermentation bottle fluid after the 48-h incubation period

	Treatment[1]								Contrast[2]			
Item	CON	B0	B25	B50	B75	B100	B150	SEM	SO	Br	L	Q
Total (mmol/L)	82.0	89.0	64.9	69.8	70.1	77.4	72.2	16.87	0.37	0.004	0.28	0.34
VFA, mol %												
Acetate	63.8	64.8	62.0	61.0	58.0	58.5	55.7	1.54	0.42	<0.001	<0.001	0.55
Propionate	19.0	18.6	19.1	20.3	21.3	22.2	23.6	1.38	0.50	<0.001	<0.001	0.35
Iso-butyrate	1.97	1.94	2.27	2.17	2.13	2.21	2.15	0.15	0.86	0.032	0.59	0.67
Butyrate	9.79	9.54	10.4	10.7	11.3	11.5	12.8	0.97	0.56	<0.001	<0.001	0.45
Iso-valerate	3.04	2.84	3.39	3.13	4.47	2.92	2.85	0.61	0.79	0.396	0.42	0.37
Valerate	1.93	1.80	2.10	2.17	2.22	2.19	2.31	0.16	0.37	<0.001	0.17	0.99
Caproate	0.50	0.46	0.62	0.56	0.54	0.55	0.51	0.060	0.51	0.035	0.12	0.58
Acetate/propionate ratio	3.42	3.56	3.26	3.06	2.82	2.72	2.38	0.272	0.31	<0.001	<0.001	0.65

[1]Treatments: CON = control; B0 = control plus 60 μL of sunflower oil without $CHBr_3$; or with 60 μL of oil supplying 25 μg (B25), 50 μg (B50), 75 μg (B75), 100 μg (B100), or 150 μg (B150) of $CHBr_3$/g substrate DM.

[2]Contrasts: SO = CON vs. B0; Br = B0 vs. (B25 + B50 + B75 + B100 + B150)/5; L = linear effect of $CHBr_3$ concentration; Q = quadratic effect of $CHBr_3$ concentration.

Table 3. Effect of stepwise inclusions of $CHBr_3$ supplied through *Asparagopsis taxiformis* oil supplementation on dimethyl acetal (DMA) composition of fermentation bottle solid phase (expressed as total μg/fermentation bottle)

	Treatment[1]							Contrast[2]			
DMA[3]	CON	B0	B25	B50	B75	B100	B150	SEM	SO	Br	L	Q
Iso-13:0	21.8	14.2	12.9	15.8	17.1	15.4	15.5	1.65	<0.001	0.46	0.42	0.17
13:0	20.9	14.0	12.9	15.5	17.3	15.9	15.0	1.69	0.002	0.41	0.49	0.09
Iso-14:0	17.3	10.6	11.1	12.7	14.8	14.0	15.1	1.83	<0.001	0.018	0.022	0.29
14:0	24.4	16.4	17.0	18.4	21.5	20.0	22.4	1.85	<0.001	0.042	0.020	0.53
Anteiso-15:0	47.3	30.9	31.8	34.7	43.0	38.2	42.6	3.61	<0.001	0.028	0.016	0.30
15:0	14.0	10.2	10.4	10.1	12.3	11.5	12.8	1.36	0.007	0.25	0.06	0.87
16:0	83.4	54.0	49.4	56.7	71.6	60.7	69.3	12.02	<0.001	0.21	0.027	0.27
18:0	8.94	6.29	7.90	7.25	7.83	6.97	9.89	1.10	0.022	0.06	0.09	0.08
18:1	7.87	5.21	4.95	6.66	9.12	6.76	7.41	1.33	0.041	0.05	0.11	0.05
Total	245	162	158	178	214	189	210	19.4	<0.001	0.06	0.019	0.24

[1]Treatments: CON = control; B0 = control plus 60 μL of sunflower oil without $CHBr_3$; or with 60 μL of oil supplying 25 μg (B25), 50 μg (B50), 75 μg (B75), 100 μg (B100), or 150 μg (B150) of $CHBr_3$/g substrate DM.
[2]Contrasts: SO = CON vs. B0; Br = B0 vs. (B25 + B50 + B75 + B100 + B150)/5; L = linear effect of $CHBr_3$ concentration; Q = quadratic effect of $CHBr_3$ concentration.
[3]Dimethyl acetals.

VFA Profile

Total VFA concentration (mmol/L) was unaffected by sunflower oil inclusion but was reduced about 20% in $CHBr_3$-containing treatments compared with B0, although without any dose-response pattern (Table 2). Inclusion of sunflower oil to the diet did not affect the molar percentage (mol %) of the individual VFA. However, the bromoils affected the molar proportions of all VFA except that of iso-valeric (iso-5:0). The molar percentage of acetate decreased linearly, whereas propionate and butyrate increased linearly with the increasing $CHBr_3$ dosage. For the iso-butyric, iso-valeric, valeric, and caproic acids, no dose-response pattern was observed.

The acetate/propionate ratio was not affected by sunflower oil inclusion but was lower in bromoil treatments when compared with B0, decreasing linearly with increasing $CHBr_3$ concentrations, from 3.26 (B25) to 2.38 (B150).

Dimethyl Acetals

Nine DMA were detected in the solid phase of fermentation bottles' contents, and their content (μg/fermentation bottle) is presented in Table 3. All the DMA were lower in B0 than in the CON. The iso-14:0, 14:0, and anteiso-15:0 DMA were increased in bromoil treatments. These 3 DMA plus 16:0 and the sum of DMA increased linearly with $CHBr_3$ concentration.

Fatty Acids

The sum of total FA (mg/g OM) in the solid phase of the fermentation bottles was not affected by the inclusion of sunflower oil, averaging of 67.4 ± 4.05 mg/g OM (Table 4). There were also no linear or quadratic effects of the $CHBr_3$ dose.

The concentration (μg/fermentation bottle) of the saturated branched-chain FA (**BC-SFA**) decreased by the inclusion of sunflower oil (Table 4) but was unaffected by $CHBr_3$. Total odd-linear chain SFA (**odd-SFA**) and also decrease with the inclusion of sunflower oil in the substrate and were also unaffected by $CHBr_3$. Individual linear even-numbered SFA (**even-SFA**), listed in Table 4, also decreased with sunflower oil inclusion, except for 22:0 and 24:0. No linear nor quadratic effects of increasing $CHBr_3$ concentrations were observed, except for the 22:0 that increased linearly from B25 to B150.

Total C18 FA (mg/g OM) in the solid phase of the fermentation bottles were not affected by the inclusion of sunflower oil, but tended to increase linearly ($P = 0.06$) with $CHBr_3$ dosage (Table 4). In relation

Table 4. Effect of stepwise inclusions of $CHBr_3$ supplied through *Asparagopsis taxiformis* oil supplementation on fatty acid (FA) composition of fermentation bottle solid phase (expressed as mg/fermentation bottle [F] or as μg/F)

	Treatment[1]								Contrast[2]			
Item	CON	B0	B25	B50	B75	B100	B150	SEM	SO	Br	L	Q
Total FA, mg/F	32.8	31.4	30.2	31.3	34.6	31.6	34.6	2.70	0.65	0.66	0.19	0.77
Total FA, mg/g OM	73.3	64.9	60.2	67.4	70.0	66.9	69.1	4.05	0.07	0.60	0.15	0.23
C18 FA, mg/F	22.9	23.4	22.2	23.1	25.7	23.6	26.4	2.15	0.81	0.66	0.09	0.85
C18 FA, mg/g OM	50.8	48.3	44.2	49.6	52.0	50.0	52.7	3.55	0.48	0.61	0.06	0.31
BC-SFA,[3] μg/F												
Iso-13:0	19.9	15.7	16.3	17.2	18.8	16.6	15.1	2.44	0.032	0.46	0.40	0.16
Iso-14:0	64.9	51.5	51.6	52.7	55.4	49.4	43.7	6.00	0.010	0.80	0.08	0.21
Iso-15:0	176	129	130	134	152	130	128	21.5	<0.001	0.51	0.68	0.23
Iso-16:0	121	85.9	89.4	90.0	97.4	84.9	77.4	11.58	<0.001	0.77	0.11	0.24
Iso-17:0	185	132	140	139	148	129	131	18.7	<0.001	0.65	0.41	0.79
Iso-18:0	32.6	23.5	25.5	24.9	26.5	21.8	24.8	3.68	0.005	0.62	0.62	0.68
Sum iso BC-SFA, μg/F	600	438	453	458	498	432	420	56.2	<0.001	0.67	0.32	0.39
Anteiso-15:0	388	287	295	306	344	303	294	34.7	<0.001	0.31	0.81	0.18
Anteiso-17:0	214	150	160	160	170	148	130	18.8	<0.001	0.74	0.042	0.24
Sum anteiso BC-SFA, μg/F	602	436	454	466	514	451	424	52.0	<0.001	0.43	0.36	0.19
Sum BC-SFA, μg/F	1,201	874	907	924	1,012	884	845	106	<0.003	0.50	0.34	0.27
Odd SFA, μg/F												
13:0	20.8	16.3	17.2	18.2	18.7	18.7	17.0	3.37	0.007	0.19	0.85	0.19
15:0	399	298	309	318	341	310	296	32.9	0.001	0.45	0.53	0.31
17:0	200	150	157	160	168	142	150	18.6	0.003	0.69	0.45	0.89
21:0	19.2	13.3	12.7	15.2	16.4	14.3	14.4	1.15	<0.001	0.29	0.65	0.11
23:0	50.7	35.7	35.4	39.7	42.7	40.0	38.3	3.11	<0.001	0.23	0.64	0.11
Subtotal odd SFA	690	514	531	551	586	525	516	55.7	0.001	0.43	0.36	0.19
Even SFA, μg/F												
12:0	45.5	36.2	35.8	38.1	41.5	38.4	37.0	5.53	0.013	0.48	0.88	0.20
14:0	320	247	252	264	281	253	248	26.0	0.003	0.49	0.66	0.35
16:0	6,853	5,638	5,580	5,711	6,125	5,540	5,798	542	0.024	0.78	0.84	0.72
20:0	247	205	199	209	225	197	212	19.8	0.034	0.79	0.75	0.67
22:0	166	193	161	185	202	193	200	12.6	0.108	0.72	0.047	0.16
24:0	178	155	145	158	170	155	158	10.5	0.114	0.89	0.57	0.28
26:0	80.1	53.7	69.1	63.5	68.2	59.0	62.4	8.56	0.003	0.10	0.42	0.68
28:0	95.0	63.7	65.4	68.1	74.8	68.6	67.3	6.85	<0.001	0.24	0.86	0.23
Subtotal even SFA, mg/F	7.98	6.59	6.51	6.70	7.18	6.51	6.78	0.58	0.025	0.47	0.58	0.40
Other UFA, μg/F												
16:1*c*7/*t*3[4]	22.7	13.0	12.7	14.2	15.3	14.7	14.2	1.28	<0.001	0.34	0.46	0.22
16:1*c*9	10.7	9.11	7.47	10.5	9.11	9.90	11.0	1.57	0.31	0.69	0.09	0.66
Cyclo-17:0[5]	29.5	19.0	19.1	23.7	22.8	19.6	17.9	6.66	0.019	0.62	0.47	0.35
20:1	16.7	19.9	18.2	23.3	23.5	22.1	24.4	2.23	0.28	0.28	0.11	0.40

[1]Treatments: CON = control; B0 = control plus 60 μL of sunflower oil without $CHBr_3$; or with 60 μL of oil supplying 25 μg (B25), 50 μg (B50), 75 μg (B75), 100 μg (B100), or 150 μg (B150) of $CHBr_3$/g substrate DM.

[2]Contrasts: SO = CON vs. B0; Br = B0 vs. (B25 + B50 + B75 + B100 + B150)/5; L = linear effect of $CHBr_3$ concentration; Q = quadratic effect of $CHBr_3$ concentration.

[3]Branched-chain saturated fatty acids.

[4]Slash (/) indicates that the compounds are coeluted.

[5]Cyclo-17:0, methyl-11-cyclohexylundecanoate.

Table 5. Effect of stepwise inclusion of $CHBr_3$ supplied through *Asparagopsis taxiformis* oil supplementation on C18 fatty acid (FA) profile (expressed as % of total C18 FA)

	Treatment[1]								Contrast[2]			
Item[3]	CON	B0	B25	B50	B75	B100	B150	SEM	SO	Br	L	Q
C18 FA, % of total FA	68.9	74.3	73.3	73.7	74.4	74.5	76.2	1.04	<0.001	0.89	<0.001	0.54
C18 FA, % of total C18 FA												
18:0	85.5	74.2	78.4	77.4	75.2	71.8	69.9	3.33	<0.001	0.85	<0.001	0.75
18:1*t*6/*t*7/*t*8	0.49	0.51	0.55	0.54	0.55	0.57	0.58	0.053	0.55	0.07	0.203	0.73
18:1*t*9	0.29	0.46	0.38	0.38	0.38	0.41	0.37	0.025	<0.001	0.005	0.95	0.38
18:1*t*10	1.47	3.42	1.14	1.25	1.88	1.88	5.05	1.511	0.12	0.21	0.002	0.18
18:1*t*11	2.03	3.40	3.62	3.82	6.16	6.80	6.46	1.234	0.66	0.018	0.002	0.10
18:1*t*12	0.57	0.67	0.67	0.65	0.71	0.74	0.82	0.044	0.021	0.11	<0.001	0.43
18:1*c*9/*t*13/*t*14	2.83	9.18	7.22	8.00	7.39	8.77	8.76	1.141	<0.001	0.13	0.11	0.84
18:1*t*15/*c*11	0.79	0.79	0.79	0.81	0.82	0.79	0.75	0.051	0.84	0.90	0.12	0.18
18:1*c*12	0.24	0.31	0.30	0.29	0.27	0.34	0.31	0.037	0.037	0.81	0.30	0.91
18:1*c*13	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.002	0.18	0.25	0.94	0.72
18:1*t*16/*c*14	0.67	0.56	0.60	0.59	0.59	0.58	0.54	0.065	<0.001	0.10	0.003	0.42
18:1*c*15	0.13	0.09	0.10	0.10	0.09	0.10	0.09	0.017	<0.001	0.64	0.34	0.97
18:2*c*9,*t*12	0.01	0.14	0.09	0.11	0.11	0.12	0.10	0.027	<0.001	0.06	0.72	0.15
18:1*c*16	0.10	0.10	0.10	0.10	0.10	0.10	0.08	0.008	0.97	0.32	0.003	0.05
18:2*t*9,*c*12	0.03	0.15	0.10	0.11	0.12	0.14	0.11	0.027	<0.001	0.07	0.71	0.17
18:2*t*11,*c*15/*t*10,*c*15	0.11	0.06	0.07	0.07	0.07	0.10	0.09	0.017	<0.001	0.13	0.06	0.46
18:2n-6	1.57	3.78	3.80	3.59	3.33	3.99	3.76	0.990	0.009	0.89	0.87	0.83
18:3n-3	1.22	0.62	0.63	0.69	0.70	0.71	0.66	0.115	<0.001	0.49	0.87	0.44
18:2*c*9,*t*11	0.08	0.27	0.31	0.28	0.42	0.64	0.49	0.165	0.14	0.10	0.033	0.19
18:2*t*10,*c*12	0.01	0.50	0.19	0.49	0.31	0.45	0.32	0.211	0.003	0.24	0.67	0.22
Oxo-18:0	0.96	0.84	0.87	0.74	0.83	0.96	0.82	0.140	0.13	0.92	0.72	0.61
Sum *cis*-FA	4.11	10.5	8.53	9.32	8.68	10.1	10.0	1.10	<0.001	0.13	0.12	0.81
Sum *trans*-FA	6.43	8.98	6.96	7.23	10.2	11.0	13.8	1.61	0.10	0.46	<0.001	0.96

[1]Treatments: CON = control; B0 = control plus 60 μL of sunflower oil without $CHBr_3$; or with 60 μL of oil supplying 25 μg (B25), 50 μg (B50), 75 μg (B75), 100 μg (B100), or 150 μg (B150) of $CHBr_3$/g substrate DM.

[2]Contrasts: SO = CON vs. B0; Br = B0 vs. (B25 + B50 + B75 + B100 + B150)/5; L = linear effect of $CHBr_3$ concentration; Q = quadratic effect of $CHBr_3$ concentration.

[3]Slash (/) indicates that the compounds coelute.

to total FAME, the sum of C18 FA (% of total FA) was increased by the sunflower oil inclusion, and also increased linearly with $CHBr_3$ dosage (Table 5).

The results from the C18 FA (expressed as a percentage of total C18 FA) are presented in Table 5. The major constituents of this group are the 18:0, 18:1*t*11, 18:1*t*10, 18:1*c*9, and 18:2n-6. The sunflower oil inclusion in substrate decreased the proportion of 18:0 and 18:3n-3 but increased the proportion of almost all the other C18 FA except for 18:1*t*6/*t*7/*t*8, 18:1*t*10, 18:1*t*11, 18:1*t*15/*c*11, 18:1*c*13, 18:1*c*16, 18:2*c*9,*t*11, and oxo-18:0. When compared with B0 the bromoil-containing treatments presented lower 18:1*t*9 and higher 18:1*t*11. Some of C18 FA were linearly affected by the increase in $CHBr_3$ concentration, such as the 18:0, 18:1*t*16/*c*14, and 18:1*c*16 that decrease linearly with $CHBr_3$ concentration and the 18:1*t*10, 18:1*t*11, 18:1*t*12, and 18:2*c*9,*t*11 that increase linearly with $CHBr_3$ concentration. The 18:0 decreased from 78.4% in B25 to 69.9% in B150, whereas 18:1*t*10 and 18:1*t*11 increased from 1.14% and 3.62% in B25 to 5.05% and 6.46% in B150, respectively.

The total *cis*-C18 FA was increased by the inclusion of sunflower oil in the substrate, and no linear nor quadratic effects of $CHBr_3$ concentration were observed. The total *trans*-FA was not affected by sunflower oil inclusion, although it increased linearly with $CHBr_3$ concentration.

DISCUSSION

The main objective of this experiment was to verify the effectiveness of sunflower oil enriched in $CHBr_3$ from *A. taxiformis* in reducing ruminal CH_4 production. The decrease in CH_4 proportion present at the headspace gas samples collected at the end of incubation and of the estimated CH_4 production decreased linearly with the $CHBr_3$, but only became noticeable in practice for concentrations above 75 μg $CHBr_3$/g substrate DM, with 33%, 47%, and 87% reductions respectively for B75, B100, and B150. Some caution is needed to interpret our data, as the CH_4 determinantions were conducted only in gas samples collected at the end of each fermentation run, thus might be less sensible to the variations that could have occurred in the different stages of the fermentation process. Nevertheless, these results are consistent with those reported by Kinley et al. (2022) that sampled gas at 24, 48, and 72 h and observed a CH_4 reduction at 48 h of 28% and 92% for 78 and 117 μg/g DM.

The effective $CHBr_3$ dosages (>75 μg/g DM) reported by us were, however, higher than those reported by Machado et al. (2016b), that used only 34.5 μg $CHBr_3$/g OM supplied through freeze-dried algae biomass. This difference could suggest that freeze-dried biomass would be more effective than bromoil, as it reduced CH_4 by 95%. However, the same team recently compared the effects of *A. taxiformis* biomass and bromoil on in vitro ruminal CH_4 production and concluded that bromoil was more effective than the biomass (Kinley et al., 2022). In fact, algae biomass supplying 95 μg/g DM of $CHBr_3$, a similar dose to the ones tested in the present experiment, was ineffective in inhibiting CH_4 production, which occurred only for higher dosages (i.e., 191 and 286 μg/g DM; Kinley et al., 2022), further suggesting the effectiveness of bromoil compared with the biomass.

Considering the inconsistency of previous reports regarding the effective dosages of $CHBr_3$ to inhibit in vitro ruminal CH_4 production, it is conceivable that differences between *A. taxiformis* bromoil and biomass might occur due to the higher or lower immediacy of exposure of ruminal microbiota to $CHBr_3$. Bromoform is produced and stored in specialized gland cells of the alga (Paul et al., 2006) so, when *A. taxiformis* biomass is used as a supplement, $CHBr_3$ release in the rumen is likely to be slower than when bromoil is used. More research is needed to decipher how the $CHBr_3$ release pattern in the rumen modulates the antimethanogenic response.

The present experiment also allowed to discriminate the effect of $CHBr_3$ from its oil carrier. Thus, the reduced OM degradability was likely due to the carrier (i.e., sunflower oil), whereas the decreased VFA was likely due to the $CHBr_3$. However, as bromoil would supply both $CHBr_3$ and sunflower oil, both negative effects on ruminal fermentation should be expected. At the highest $CHBr_3$ dosage (150 μg/g substrate DM), adverse effects on OM degradability and total VFA concentration (20% reductions) were present. It was noted that $CHBr_3$ changed the molar % of the main VFA in a dose dependent fashion. Similar results were reported by Kinley et al. (2016) which reported that OM degradability of Rhodes grass was depressed only when *A. taxiformis* was included at levels higher than 5%. Still, in that report, there is no reference to $CHBr_3$ concentration in the *A. taxiformis* used. More recently, Andreen et al. (2023) using an in vitro continuous cultures system were able to inhibited almost completely the ruminal CH_4 production with dosages of 0.5% or 1.5% of *A. taxiformis* (corresponding to approximately 35 μg and 106 μg $CHBr_3$/g substrate DM) whereas the decrease in OM true digestibility reported were only less than 16%.

Volatile FA are often the primary source of energy for ruminants and the adverse effects of the inclusion of 150 μg/g substrate DM on OMD and total VFA

production suggests that despite the strong inhibition of methanogenesis, the microbial activity, and feed utilization efficiency in vitro were hampered. As energy losses due to digestive CH_4 emissions in ruminants can comprise up to 12% of gross energy intake (Johnson and Johnson, 1995), achieving its inhibition without major depression of ruminal fermentative activity would be desirable. This should increase or at least maintain the animal productivity as some in vivo results suggest (Kinley et al., 2020), which was not observed in the present in vitro experiment.

Moreover, the observed decrease of acetate: propionate ratio in the rumen when the levels of $CHBr_3$ increased, also observed by Machado et al. (2016a,b) and Andreen et al. (2023), suggests an increase of the energy efficiency for the host anabolic processes. The possible reduction of archaea activity due to $CHBr_3$ supplementation is expected to increase the H_2 pressure in the rumen, which may justify the shift from acetate to propionate synthesis, as an alternative H_2 sink (Lourenço et al., 2010). In this case, the disruption of electron flow to methanogenesis might have been compensated by disposing of excess reducing equivalents as described by Ungerfeld (2020), favoring the production of more reduced VFA (propionate) and less production of acetate, as observed in this present study.

The BC-SFA, odd-SFA, and DMA are present in cell membrane phospholipids and can be interpreted as markers of microbial biomass (Vlaeminck et al., 2006; Bessa et al., 2009; Alves et al., 2013). They all decreased with sunflower oil supplementation but were not affected by $CHBr_3$ level in the diet. Thus, the maintenance of BC-SFA and odd-SFA content in the oil-supplemented fermentation bottles with increasing $CHBr_3$ dosage suggests that the markers of microbial biomass were not negatively affected by $CHBr_3$ supplied in the bromoil. In fact, the VFA decrease together with the relatively stability of OM degradability and the markers of microbial biomass with increasing $CHBr_3$ dosage, are consistent with a higher recover of C in microbial biomass and thus greater efficiency in microbial growth (Preston and Leng, 1987). Despite that the structure of ruminal microbiota might be profoundly affected as observed by others, prominent fiber-degrading and VFA-producing bacteria (*Fibrobacter* and *Ruminococcus*) were inhibited by *A. taxiformis* supplementation, while other groups of bacteria have been observed to increase with *A. taxiformis*, such as *Succinivibrio* and *Prevotella* (O'Hara et al., 2023).

The DMA are the product of methylation of vinyl ether chain of microbial membrane plasmalogens (Alves et al., 2013). Plasmalogens are more rigid than their correspondent phospholipids thus decreasing the permeability and fluidity of membranes (Goldfine, 2010). Reducing membrane permeability is a common stress response in bacteria (Keweloh and Heipieper, 1996). Thus, DMA increase with increasing $CHBr_3$ concentration observed in this study might be an adaptive response of ruminal microbiota that allows the pursuit of fermentative activity.

Biohydrogenation of UFA in the rumen is an extensive metabolism that isomerizes and hydrogenates the dietary UFA and thus it also serves as sink for the H_2 generated during fermentation. Despite that, the relevance of BH as a H_2 sink is minor compared with methanogenesis (Harfoot and Hazlewood, 1997). The primary function of BH in the rumen had been proposed to be a protective response against membrane target UFA toxicity by reducing PUFA abundance (Maia et al., 2010) or a more general adaptive response to increase the availability of *trans*-18:1 in response to environmental stress stimuli, by reducing membrane fluidity (Bessa et al., 2000). Thus, the increase on *trans*-18:1 observed in the present experiment is consistent with the putative role of BH as a general adaptive response to environmental stress, particularly considering the inhibition of methanogenesis and the consequent need for alternative H_2 sink pathways.

The potent inhibition of methanogenesis observed with the highest $CHBr_3$ concentration is coupled with an increase of H_2 in the rumen, as demonstrated by others (Machado et al., 2018). Thus, we expected a high H_2 availability in $CHBr_3$ supplemented diets, and that such conditions would promote the BH of UFA. Despite that, no evidence supports that BH of UFA, in particular that of 18:2n-6 (the major FA in bromoil) has been stimulated. However, the decreased percentage of 18:0, the end product of 18:2n-6 BH when the concentration of $CHBr_3$ increased, coupled with the increase of some BH intermediates (mainly 18:1*t*10 and 18:1*t*11), suggests that the $CHBr_3$ decreases BH completeness of 18:2n-6, yielding more *trans* intermediate products.

The reduction of BH completeness by inhibition of the last reductive step of BH is often observed with high dietary intake of PUFA, in particular of n-3 LCFA, but also in the presence of high dietary tannins or high grain diets that allow low ruminal pH conditions (Bessa et al., 2000; Frutos et al., 2020). The incomplete BH due to the inhibition of the last step of BH that hydrogenate the 18:1 isomers into 18:0 has been classically explained by the specific inhibition of the specialized hydrogenating bacteria, which are highly susceptible to PUFA toxicity when cultured (Maia et al., 2010). Despite that, in vivo data failed to relate the abundance of these bacteria with 18:0 and *trans*-18:1 (Kim et al., 2008; Lourenço et al., 2010). Alternatively, the

incomplete BH with *trans*-18:1 accumulation can be an adaptive response of the microbiota to the putative stress induced by increased $CHBr_3$ concentration as observed for tannins (Costa et al., 2018). This modulatory effect of $CHBr_3$ on BH can be desirable if the inhibition of CH_4 production occurs simultaneously with the increase in synthesis and transfer to the milk of health promoting FA, such as vaccenic acid (18:1*t*11) and rumenic acid (18:2*c*9,*t*11) (Bessa et al., 2015). This effect depends on the basal diet, and the large increase of 18:1*t*10 observed in this experiment at the highest $CHBr_3$ incorporation is a matter of concern and needs to be confirmed in future studies. The accumulation of 18:1*t*10 instead of 18:1*t*11 is a result of an ill-known dysbiosis linked to the etiology of dietary-induced milk fat depression (Bauman et al., 2011; Dewanckele et al., 2020) and that worsens the nutritional value of ruminant products (Alves et al., 2021).

CONCLUSIONS

The oils containing $CHBr_3$ obtained from the immersion of *A. taxiformis* biomass in sunflower oil decreased the in vitro ruminal production of CH_4. Methane production decreased with $CHBr_3$ concentration in the diet and became expressive at concentrations equal to or above 75 μg/g diet DM, with 33%, 47%, and 87% reductions for B75, B100, and B150, respectively. The effect on VFA concentration led to a decrease of about 20% in $CHBr_3$-containing treatments compared with B0, although without any dose-response pattern. Moreover, increasing the dosage of $CHBr_3$ reduced the BH completeness promoting the accumulation of *trans*-18:1 isomers and the reduction of 18:0. These results provide evidence that oil immersions of *A. taxiformis* might be useful in inhibiting CH_4 production in vitro and simultaneously modulating the BH. Nevertheless, more research is needed to confirm these data, mainly in vivo feeding experiments, and to ensure the safety for animals, animal product and practical feasibility.

ACKNOWLEDGMENTS

The authors thank Fundação para a Ciência e Tecnologia (FCT; Lisbon, Portugal) for funding through PhD research grants to authors FS (UI/BD/152817/2022) and DMS (202 2.13385.BDANA) and the research contract to SPA (DL 57/2016/CP1438/CT0008), as well as through PTDC/CAL-ZOO/29654/2017, UIDB/00276/2020 (CIISA), and LA/P/0059/2020 (AL4AnimalS) grants. Funding from the projects GEEBovMit (PRR-C05-i03-I-000027 GEEBovMit) Greenbeef (POCI-01-0247-FEDER-047050/LISBOA-01-FEDER-047050) are acknowledged. The authors have not stated any conflicts of interest.

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