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Effects of *Schizochytrium* microalgae and sunflower oil as sources of unsaturated fatty acids for the sustainable mitigation of ruminal biogases methane and carbon dioxide

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ABSTRACT

Mitigation of methane (CH_4) and carbon dioxide (CO_2) emissions as well as ruminal fermentation parameters of a total mixed ration in the presence of *Schizochytrium* microalgae (SA) and sunflower oil (SO) or their mixture (SASO) as unsaturated fatty acid sources was investigated. Rumen liquor from two rumen cannulated Holstein steers and two rumen cannulated Creole goats was used as inoculum. Interactions between inoculum source × additive type, and inoculum source × additive type × dose were observed for gas, CH_4 and CO_2 production and fermentation parameters. Additives affected the fermentation parameters in a dose-dependent manner. With goats' inoculum, the inclusion of SO (1, 2, 4, 5%), SA (2, 3, 5%) and SASO (1, 3%) increased gas production (GP) and decreased the rate of GP, while with the steer inoculum, SO at 1 and 4% increased GP and the rate of GP. All levels of SA and SASO decreased the asymptotic GP and increased the rate of GP. The goat inoculum decreased CH_4 at different doses of SO, SA and SASO whereas the steer inoculum decreased CH_4 production. At all doses, additives decreased fermentation pH, protozoal counts, and increased ammonia-N, DM degradability and total bacterial counts. Sunflower oil (i.e., SO) at 1–3%, SA at 1–2%, and SASO at 1–2% were the most efficacious in the nutrition of goats, compared with SO at 1 to 2 in steers. The results suggest that *Schizochytrium* microalgae and sunflower oil could be a valuable means of sustainably mitigating CH_4 and CO_2 emissions for improved environmental conditions.

1. Introduction

Biogases produced during ruminant production needs to be reduced. The Food and Agriculture Organization (FAO) estimated CH_4 production from livestock to contribute about 18% of all greenhouse gas emissions, while carbon dioxide (CO2) accounted for about 9% of the emission (FAO, 2006). Besides, these gases including CH_4, CO_2, and H_2 are produced during ruminal fermentation and cause losses amounting to 2–12% of dietary energy in ruminants (Johnson and Johnson, 1995). Furthermore, these emissions have been implicated in causing climate change. Yeast, organic acids salt, exogenous enzymes, and essential oils have been used as new strategies to mitigate the production of ruminal methane from ruminants (Elghandour et al., 2016, 2017; Hernandez et al., 2017).

The biogases production from ruminants could be reduced with inclusion of lipids in ruminant diets (Hook et al., 2010), which has been attributed to a reduction of ruminal protozoal concentrations in the rumen (Abubakr et al., 2013). Dietary lipids rich in docosahexaenoic acid (DHA) have been reported to enhance the nutritive value of the product of ruminant production (*e.g.,* milk and meat), and improve animal performance (Kholif et al., 2016).

Vegetable oils and microalgae are rich sources of unsaturated fatty acids (UFA) including DHA and conjugated linoleic acid (CLA, Kelly et al., 2003). Addition of vegetable oils to the lactating animal

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rations has been used as a strategy to alter the proportion of saturated (SFA) and UFA in animal products through the extensive ruminal biohydrogenation (Kholif et al., 2016). Linoleic acid is the main compound in sunflower oil (*i.e.,* SO), which may increase CLA in the animal product (Morsy et al., 2015). Furthermore, feeding vegetable oils to lactating goats modified the FA profile without any negative effects on ruminal fermentation or nutrients digestibility (Morsy et al., 2015; Kholif et al., 2016).

Microalgae (*Schizochytrium* spp.), is a microalgae rich in dietary FA and protein content making it a potential feed supplement to improve feed utilization and animal performance (Burnett et al., 2017). *Schizochytrium* microalgae is a rich source of long-chain PUFA including DHA and eicosapentaenoic acid (EPA), therefore, it can be used to inhibit the *in vitro* biohydrogenation of FA, resulting in reduction in SFA amount of SFA and an increase in UFA (Boeckaert et al., 2007). *Schizochytrium* microalgae have also been shown to increase the concentration of DHA in the milk of dairy animals (Moate et al., 2013).

Improving feed utilization and animal performance is the main goal for nutritionists; however, social and environmental obligations to reduce biogases emission are crucial for the sustainable intensification of livestock production. The present experiment aimed to study the effect of including SO and/or SA as UFA sources on *in vitro* ruminal fermentation for the sustainable mitigation of CH_4 and CO_2 emissions using rumen inoculums from steers and goats fed the same diet. The hypothesis was that differences among ruminant species and different sources of dietary fats would alter ruminal microflora resulting in improved dietary nutritive value and reduced biogases production.

2. Materials and methods

2.1. Preparation of total mixed ration and treatments

The composition of total mixed ration prepared as a substrate is shown in Table 1. The total mixed ration without additive was considered as the control treatment. *Schizochytrium* microalgae (Xuhuang Bio-Tech Co., Ltd., Shaanxi, China) and SO from a local supplier, were individually or their mixture at 1:1 DM basis (SASO) added to the total mixed ration at levels of 1, 2, 3, 4, and 5% on DM basis. The chemical composition of the SA is shown in Table 2. The individual fatty acids (g/100 g total fatty acids) of the oil were 5.4 g, C16:0; 4.6 g, C18:0; 21.0 g, C18:1 and 69.0 g, C18:2.

2.2. Collecting of inoculum

Two rumen cannulated Holstein steers (450 ± 20 kg LW), and two rumen cannulated Creole goats (50 ± 2 kg body weight), were used as the source of the inoculum. The animals were housed in individual pens and fed a diet consisting of oat hay and concentrate (PURINA®, Toluca, Mexico) at 60:40 ratio *ad libitum*, with free access to water. The animals were fed twice at 08:00 and 16:00 h daily and managed under stipulated conditions (Official Mexican Standard of technical specifications for the production, care and use of laboratory animals, NOM-062-ZOO-1999). Plastic thermos flasks preheated at 39 °C and flushed with CO_2 were used to collect the rumen contents and then transported to the laboratory. The rumen contents were mixed and strained through four layers of cheesecloth into a flask with O_2-free headspace. Subsequently, the rumen contents were maintained at a temperature of 39 °C with a continuous flow of CO_2.

Table 2
Chemical composition of *Schizochytrium* microalgae.

Nutrients	g/kg dry matter
Proximate composition	
Dry matter	950
Organic matter	880
Crude fat	470
Crude protein	120
Carbohydrates	180
Crude fiber	50
Fatty acids profile[a]	
C14:0	81
C16:0	256
C20:4 ARA	23
C20:5 EPA	23
C22:5 DPA	168
C22:6 DHA	400
Others	49
Amino acids profile[a]	
Alanine	54
Arginine	90
Aspartic acid	94
Cystine	15
Glutamic acid	270
Glycine	44
Histidine	16
Isoleucine	27
Leucine	52
Lysine	32
Methionine	17
Phenylalanine	32
Proline	35
Serine	43
Threonine	40
Tyrosine	21
Valine	57
Others	61

[a] Provided by the manufacture, Xuhuang Bio-Tech Co., Ltd., Shaanxi, China.

2.3. In vitro incubation process

The medium used for the incubations contained buffer, macromineral, micromineral, resarzurin solutions and distilled water (Goering and Van Soest, 1970). All the contents were mixed in a volumetric flask using a magnetic stirrer set at 39 °C. Consequently, the ruminal inoculum and the reducing solution were mixed at the ratio of 1:4 (v/v). 50 mL of the prepared rumen liquor plus buffer were poured over 0.5 g of substrate in 120-mL serum bottles with

Table 1
The composition of total mixed ration.

Ingredient		Chemical composition	
Material	g/kg dry matter	Composition	g/kg dry matter
Alfalfa hay (*Medicago sativa*)	400	Dry matter	880 (wet weight basis)
Crushed yellow corn	250	Organic matter	934
Soybean meal	250	Crude protein	218
Wheat bran	100	Neutral detergent fiber	219
		Acid detergent fiber	201

appropriate addition of the additives (*i.e.,* SA, SO or a mixture of SA and SO)/g DM. Bottles were maintained at constant CO_2 flow for 30 s, capped with neoprene plugs and sealed with aluminum rings. The vials were placed in an incubator (Riossa®, F-51 D, Mexico State, Mexico) at 39 °C for 48 h. Additionally, three bottles as blanks (rumen fluid only) were incubated for 48 h. Three incubation runs were performed in three weeks.

2.4. Measurement of biogas production

The readings of biogas production (GP) were recorded at 2, 4, 6, 8, 10, 12, 24 and 48 h of incubation using a water displacement apparatus (Fedorak and Hrudey, 1983). The apparatus was designed with a universal support, with a conical funnel, a 100 mL burette and two latex hoses of 0.5 (1 m in length and 3/8-inch diameter). The vials were punctured with a 16-gauge needle placed at the end of the hose and the volume of GP (mL) was measured.

After 48 h of incubation, 5 ml of gas was taken and stored in vials with saturated saline solution prepared with 400 g of NaCl in 1 L of distilled water and the pH set at 2. Subsequently, 5 mL of 20% methyl orange was added as indicator for CH_4 and CO_2 concentration determinations. The previously prepared saturated saline solution was stored in 60 mL serological vials without headspace and neoprene plugs placed and sealed with aluminum rings, and stored away from light.

To determine the CH_4 and CO_2, a sample of gas phase (10 μL) was taken from the vials with saturated saline and injected into a PerkinElmer, Claurus 500 gas chromatograph (Mexico City, Mexico) with a flame ionization detection and helium as the carrier gas. A thermal conductivity detector was used with the oven, column and TCD temperatures programmed at 80 °C, 170 °C and 130 °C, respectively. Retention times were 0.73 min and 1.05 min for CH_4 and CO_2, respectively.

At the end of incubation at 48 h, the fermentation process was stopped by swirling the bottles in ice for 5 min and then the bottles uncapped and the pH measured immediately using a pH meter (Thermo Scientific, Orion Star™ A121, Beverly, MA, USA). The bottles' contents were filtered in to Ankom® Technologies F57 bags (at constant weight) using a filtration system connected to a vacuum pump. Hot water was used to rinse the bottles three times to ensure recovery of all the residue of fermentation and then the bags dried in a forced air oven (55 °C for 48 h). Dry matter (DM) degradation was calculated by difference between the initial weight of the dried substrate and the weight of the dried residue.

After the pH measurement and filtration, 4 mL of the medium was mixed with 1 mL of metaphosphoric acid (25%), and another 4 mL mixed with 1 mL of formaldehyde (10%), shaken slightly and placed in a refrigerator at 4 °C until analysis of ammonia-N concentration and bacterial and protozoal count, respectively.

2.5. Counting of total bacteria and protozoa

A Petroff-Hausser counting chamber (Hausser Scientific®, 3900, Horsham, PA) and a phase contrast microscope (100x, Olympus®, BX51, Mexico City, Mexico) were used to quantify the concentration of total bacteria after 48 h of incubation. 0.5 mL of 10% formaldehyde fixed medium was taken and diluted in 4.5 mL of distilled water. The bacterial concentration per mL was determined as the average number of bacteria observed in each grid, multiplied by the dilution factor and the chamber factor (2×10^7), according to the following formula:

$$\text{Bacterial number number/mL} = \mu \times FD1 \times FD2 \times 2^7$$

where: μ is the average number of bacteria in each grid per treatment, FD1 is the first dilution factor (1.25) and FD2 is the second dilution factor (10).

For the protozoal count, 1 mL of the 10% formaldehyde fixed sample was diluted in 1 mL of distilled water, then 0.5 mL of the mixture was taken with a Pasteur pipette (BRAND, 7712, Wertheim, Germany) and deposited into a Neubauer chamber (BRAND, 7178-10, Wertheim, Germany), and subsequently observed under a contrast microscope (400×, Carl Zeiss®, Axiostar, Mexico City, Mexico). The protozoa count was made in eight quadrants (4 of each grid), taking as viable protozoa those that maintained their morphological integrity. The concentration of protozoa per mL of culture medium was estimated as the average number of protozoa observed in each grid, multiplied by the dilution factor and the chamber factor (1×10^4), according to the formula:

$$\text{Protozoal number} = \mu \times FD1 \times FD2 \times 10^4$$

where: = μ is the average number of protozoa in each grid per treatment, FD1 is the first dilution factor (5), and FD2 is the second dilution factor (3).

2.6. Chemical analyses

Table 3 presents the chemical analysis methods used for the total mixed ration and microalgae. The fatty and amino acid content of SA were determined according to the Chinese national standard methods (National Standards of People's Republic of China 2010) using the analysis methods (method ID: GB 5413.27–2010) and (method ID: GB/T 5009·124–2003), respectively. The SO fatty acid contents were analyzed according to AOAC (1997) using fatty acids methyl esters prepared by base-catalyzed methanolysis of the glycerides as provided by Xuhuang Bio-Tech Co., Ltd., Shaanxi, China. The concentration of ruminal ammonia-N was determined according to Broderick and Kang (1980) method.

2.7. Calculations

For the estimation of GP, gas volumes (mL/g DM) were fitted using the NLIN procedure of SAS (2002) according to France et al. (2000) model as:

$$y = b \times \left[1 - e^{-c(t-Lag)}\right]$$

where y is the volume of GP at time t (h); b is the asymptotic GP (mL/g DM); c is the fractional rate of fermentation (/h), and L (h) is the discrete lag time prior to any gas is released.

Metabolizable energy (ME, MJ/kg DM) and *in vitro* organic matter digestibility (OMD, g/kg DM) were estimated according to Menke et al. (1979) as:

$$ME = 2.20 + 0.136GP + 0.057CP$$

where GP is gas production (mL/0.5 g DM) and CP is crude protein

Table 3
Chemical analyses methods used for analyzing the total mixed ration.

Chemical parameter	Method
Dry matter content	(AOAC, 1997; #934.01)
Ash content	(AOAC, 1997; #942.05)
Nitrogen content	(AOAC, 1997; #954.01)
Ether extract	(AOAC, 1997; #920.39)
Neutral detergent fiber	(Van Soest et al., 1991)
Acid detergent fiber	(AOAC, 1997; #973.18)
Acid detergent lignin	(AOAC, 1997; #973.18)

(g/kg DM)

$$OMD = 148.8 + 8.89GP + 4.5CP + 0.651A$$

where GP is net GP in mL from 200 mg of dry sample after 24 h of incubation, CP is crude protein (g/kg DM) and A is ash (g/kg DM).

The partitioning factor at 24 h of incubation (PF_{24}; a measure of fermentation efficiency) was calculated as the ratio of DM degradability *in vitro* (DMD, mg) to the volume (mL) of GP at 24 h (i.e., DMD/total GP (GP_{24})) according to Blümmel et al. (1997). Gas yield (GY_{24}) was calculated as the volume of gas (V, mL gas/g DM) produced after 24 h of incubation divided by the amount of DMD (m, g) as:

$$GY_{24} = \frac{V}{m}$$

Short chain fatty acid concentrations (SCFA, mmol/200 mg DM) were calculated according to Getachew et al. (2002) as:

$$SCFA = 0.0222GP - 0.00425$$

where GP is the 24 h net GP (mL/200 mg DM).

Microbial biomass production (MCP, mg/g DM) was calculated (Blümmel et al., 1997) as:

$$MCP = m - (gas \times 2.2)$$

where the 2.2 (mg/mL) is a stoichiometric factor that expresses mg of C, H and O required for the SCFA gas associated with production of 1 mL of gas (Blümmel et al., 1997) and *gas* is gas (mL).

2.8. Statistical analyses

The effects of additive type, inoculum source and additive dose were analyzed using a 3 × 4 factorial design with 3 replicates in a randomized complete block design. Data were analyzed using the GLM procedure (SAS, 2002) using the model: $Y_{ijkl} = \mu + A_i + R_j + D_k + (A \times R)_{ij} + (A \times D)_{ik} + (R \times D)_{jk} + (A \times R \times D)_{ijk} + \varepsilon_{ijkl}$ where: Y_{ijkl} is the observation, μ is the population mean, A_i is the additive type effect, R_j is the inoculum source effect, D_k is the additive dose effect, $(A \times R)_{ij}$ is the interaction between additive type and inoculum source, $(A \times R \times D)_{ijk}$ is the interaction between additive type, inoculum source and additive dose, and ε_{ijkl} is the residual error. Tukey test was used to separate means.

3. Results and discussion

The occurrence of interactions between inoculum source × additive type, and inoculum source × additive type × dose are evidences that the effect of each additive was inoculum and dose-dependent. Therefore, the discussion will be based on the effect of each feed additive at different doses with both rumen inoculum sources.

3.1. Biogases production

Significant effects ($P < 0.05$) of inoculum source × additive type, and inoculum source × additive type × dose interactions were observed for GP, CH_4 and CO_2 production (Table 4). The goat inoculum at levels 1, 2, 4, and 5% of SO, the doses 2, 3 and 5% of SA and the doses 1 and 3% of SASO increased ($P < 0.001$) the asymptotic GP, and GP, while decreasing ($P < 0.05$) the rate of GP compared with the control treatment. The steer inoculum at doses 1 and 4% of SO increased ($P < 0.001$) the asymptotic GP and the rate of GP, while all levels of SA and SASO treatments decreased the asymptotic GP and increased the rate of GP. All doses of additives linearly decreased ($P < 0.001$) lag time of GP with the steers inoculum whereas the goat inoculum had no effect on lag time of GP.

At the levels of 3% of SO, 1 and 4% of SA and 2, 4 and 5% of SASO, the goats' inoculum decreased CH_4 production ($P < 0.001$), while the other levels of additives increased it (Table 4). All doses of SO decreased ($P < 0.005$) proportional CH_4 production, and increased proportional CO_2 production. Without affecting the proportional CH_4 production, SO at 2, 3 and 5%, and all levels of both SA and SASO decreased CH_4 production ($P = 0.001$) with the steers inoculum. The levels 3 and 5% of SO increased the production of CO_2 without affecting the proportional CO_2 production and CO_2 production.

It has been previously reported that using rumen fluid from different ruminant species to inoculate the *in vitro* incubation cultures is a useful tool to examine possible differences in the ruminal microbial population and the digestive capacity of each ruminant species (Salem, 2005; Weimer, 2015). The lack of effect on asymptotic GP with cattle and goat inoculums is in agreement with earlier works by Aderinboye et al. (2016) who reported similarity in the estimated total GP among inoculums collected from cattle, sheep and goats. In the present experiment, the rate of GP was higher for goat than steer inoculum. In contrast, Aderinboye et al. (2016) observed higher rate of GP with cattle compared with goat inoculum, which might be due to the varied diets fed to rumen liquor donors. Cone et al. (2000) reported a well-correlated total GP from inoculum of sheep and cattle, but poor correlation of rates of GP with the two inoculums. At the same time, the lack of effect on lag time between steer and goat inoculum in the current study reveals that the time taken for microbes to adhere to the substrates, and microbial attachment to insoluble substrate was similar.

Sunflower oil at all levels produced higher GP with increasing rate of GP with goat inoculum whereas with the steer inoculum, SO only caused the same effect at two levels (*i.e.*, 1 and 4%) - Fig. 1. The reasons for these difference between inoculum from goats and steers are unclear, however, the differences in lipid metabolism among ruminant species (Chilliard et al., 2003) is a probable reason. It was expected that increasing the level of SO would disturb fermentation and digestion activities of the total mixed ration due to the anti-microbial effect of UFA of the oil. Additionally, the inclusion of SO up to 4 and 5% of the diet did not negatively affect ruminal fermentation. In agreement with results of the current study, Narimani-Rad et al. (2011) observed a positive effect with the inclusion SO at 2.5% to a concentrate-based diet containing alfalfa forage and barley grain at 40:60, and a negative effect on GP at 5% of SO inclusion. Furthermore, Narimani-Rad et al. (2012) observed lack of effect on GP or rate of GP with the inclusion of SO at the same levels with forage-based diets. This variation might be due to the different substrates used. Sunflower oil inclusion to a forage-based diet may coated feed particles and decreased attachment of ruminal microbes resulting in similar fermentation patterns (Narimani-Rad et al., 2012).

The *in vitro* results of micro-algae supplementation appear promising, in agreement with Kholif et al. (2017), however, further *in vivo* studies are required to elucidate the optimum dose in ruminants in terms of effects on feed nutritive value and CH_4 production. *Schizochytrium* microalgae alone or with SO (*i.e.*, SASO treatment) negatively affected GP with steer inoculum revealing negative effects on steers' nutrition; however, some doses increased GP with goat inoculum. More research is required to elucidate the effect of SA on feed utilization between goats and steers. This difference is another indicator about different microbial profile between goats and steers. It is well documented that most species of microalgae contain some antioxidative, antimicrobial or compounds with cytotoxic effects (Scholz and Liebezeit, 2012), which might be negatively affected GP. Furthermore, the contents

Table 4
In vitro biogas production (mL/g DM) of a total mixed ration incubated with inoculum from goats and steers in the presence of sunflower oil, *Schizochytrium* microalgae or their mixture at 1:1 DM basis.

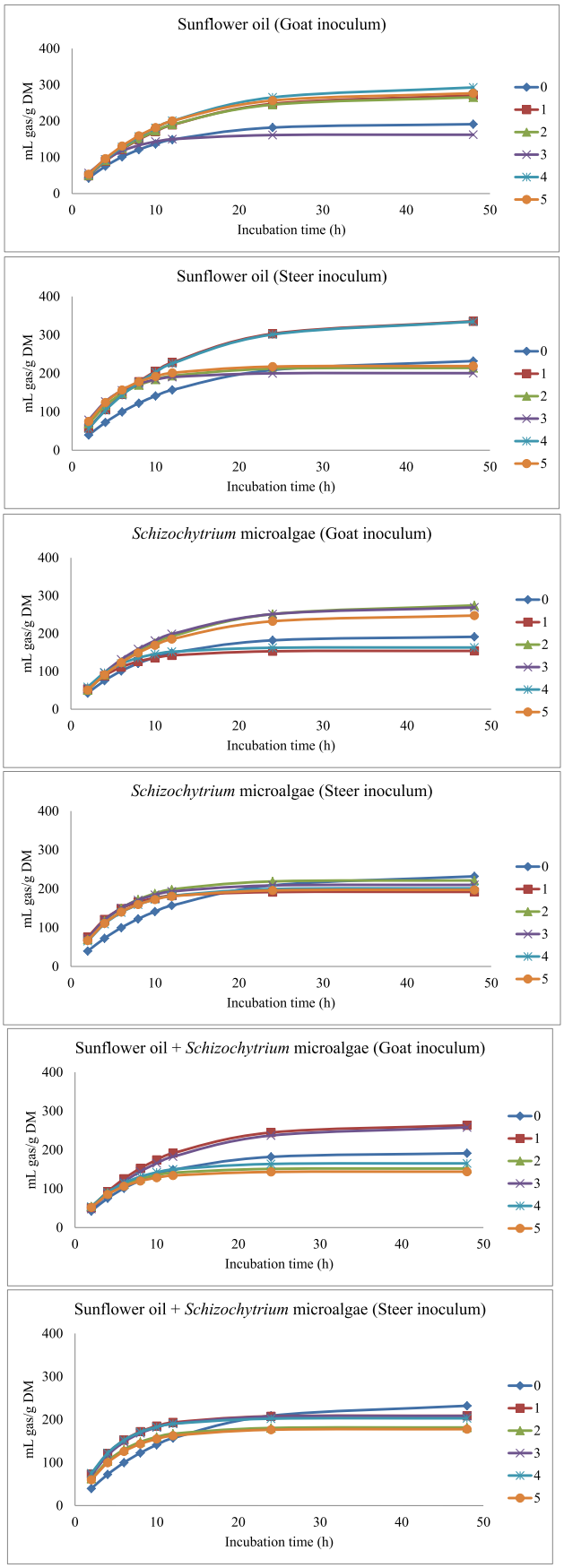
Inoculum	Additive	Dose (% of ration DM)	Gas production parameters[a]				CO_2 production at 48 h of incubation			CH_4 production at 48 h of incubation		
			b	*c*	*Lag*	mL gas/g degraded DM	CO_2 production	Proportional CO_2 production	mL CO_2/g degraded DM	CH_4 production	Proportional CH_4 production	mL CH_4/g degraded DM
Goat	Control	0	192	0.125	2.31	288	170	89.0	257	21.1	11.0	31.8
	Sunflower oil	1	273	0.099	1.87	360	232	85.8	309	38.4	14.2	51.2
		2	267	0.104	2.15	355	223	84.1	299	42.2	15.9	56.6
		3	162	0.215	2.14	215	135	83.5	180	26.8	16.5	35.5
		4	296	0.095	1.31	388	248	84.9	329	44.5	15.1	59.0
		5	278	0.106	2.04	363	224	80.9	294	52.9	19.1	69.4
	Schizochytrium microalgae	1	154	0.214	2.44	198	132	85.5	170	22.4	14.5	28.8
		2	277	0.100	2.10	353	236	86.0	304	38.5	14.0	49.5
		3	270	0.111	1.98	359	231	86.1	309	37.4	13.9	50.0
		4	163	0.223	2.84	213	143	87.8	187	19.8	12.2	26.0
		5	248	0.114	2.08	328	212	85.6	281	35.6	14.4	47.1
	Sunflower + *Schizochytrium* microalgae	1	265	0.108	1.96	349	232	88.3	308	31.0	11.8	41.0
		2	152	0.222	2.29	202	132	86.6	175	20.4	13.4	27.0
		3	260	0.101	1.96	338	224	87.0	294	33.7	13.0	44.0
		4	165	0.197	2.12	220	143	86.3	190	22.7	13.7	30.2
		5	144	0.224	2.74	193	125	87.0	168	18.7	13.0	25.2
	SEM		4.5	0.0039	0.148	7.5	4.5	1.07	7.3	2.71	1.07	3.63
	Additive		<0.001	<0.001	0.001	<0.001	<0.001	0.538	<0.001	0.004	0.547	0.004
	Linear		<0.001	<0.001	0.299	0.018	<0.001	0.001	0.008	0.892	0.001	0.033
	Quadratic		<0.001	0.022	0.148	0.229	<0.001	0.005	0.016	0.168	0.006	0.015
Steers	Control	0	235	0.093	2.12	340	204	87.9	299	28.0	12.1	41.0
	Sunflower oil	1	339	0.093	1.51	437	286	85.1	373	49.8	14.9	64.8
		2	214	0.196	1.75	281	181	84.7	238	32.7	15.3	43.0
		3	201	0.250	1.84	271	167	83.4	226	33.2	16.6	44.9
		4	339	0.091	1.79	433	272	81.4	353	62.1	18.6	80.5
		5	219	0.210	1.79	289	176	80.3	232	43.0	19.7	56.9
	Schizochytrium microalgae	1	192	0.249	1.84	257	168	87.4	225	24.1	12.6	32.1
		2	221	0.189	1.73	290	189	85.6	248	31.9	14.4	41.7
		3	210	0.210	1.77	285	180	85.9	245	29.7	14.1	40.3
		4	201	0.197	1.64	269	173	86.1	231	28.0	13.9	37.6
		5	197	0.207	1.79	254	169	85.9	218	27.9	14.1	36.0
	Sunflower + *Schizochytrium* microalgae	1	209	0.217	1.81	274	180	86.0	235	29.2	14.0	38.3
		2	182	0.211	1.51	240	156	86.0	206	25.5	14.0	33.8
		3	208	0.206	1.80	271	178	85.6	232	30.0	14.4	39.0
		4	203	0.231	1.83	264	178	87.9	232	24.7	12.1	32.2
		5	178	0.207	1.45	232	156	87.7	204	21.7	12.3	28.4
	SEM		8.0	0.0047	0.137	12.2	7.3	1.166	11.5	3.05	1.16	4.05
	Additive		<0.001	<0.001	<0.001	<0.001	<0.001	0.443	<0.001	0.004	0.443	0.005
	Linear		<0.001	<0.001	0.067	<0.001	<0.001	0.122	<0.001	0.537	0.224	0.521
	Quadratic		<0.001	<0.001	<0.001	0.053	0.001	0.743	0.104	0.045	0.758	0.208
Pooled SEM[b]			6.5	0.0043	0.143	10.10	6.06	1.12	9.61	2.88	1.12	3.85
P value												
Inoculum			0.090	<0.001	0.387	0.260	0.019	0.035	0.082	0.282	0.034	0.161
Additive			<0.001	<0.001	<0.001	<0.001	<0.001	0.326	<0.001	<0.001	0.324	<0.001
Dose												
Linear			0.156	<0.001	0.615	<0.001	0.101	0.561	<0.001	0.709	0.549	0.054
Quadratic			<0.001	<0.001	<0.001	0.022	<0.001	0.080	0.007	0.535	0.082	0.455
Inoculum × additive			0.007	0.001	0.004	0.038	0.002	0.713	0.052	0.466	0.726	0.629
Inoculum × additive × dose			<0.001	<0.001	<0.001	<0.001	<0.001	0.483	<0.001	0.040	0.475	0.071

[a] *b* is the asymptotic gas production (mL/g DM); *c* is the rate of gas production (/h); *Lag* is the initial delay before gas production begins (h).
[b] SEM, standard error of the mean.

of long chain fatty acids in SA may have a negative effect on feed digestion and fermentation (Beauchemin et al., 2007; Burnett et al., 2017).

The different effects of rumen inoculum on CH_4 and fermentation parameters could be a result of different bacterial and protozoal populations and microbial activity in goats and steers (Aderinboye et al., 2016). Hook et al. (2010) reported that CH_4 production varies based on the ruminant species because the protozoal population varies from animal-to-animal despite the feeding of the same diets (Boeckaert et al., 2007). Ruminal microbial populations depend mainly on the type of diet fed, and since both of steers and goats were maintained on the same diet, microbial species were not expected to vary (Mould et al., 2005). Other factors such as host animal effects, sampling time and source, sample preparation and inoculation could have caused some variations in inoculum (Mould et al., 2005). Additionally, differences in dentition, chewing/eating behavior, gut physiology, compartment dimensions and retention time would influence gut microflora (Salem et al., 2013). Thus, variations in CH_4 production and fermentation parameters between steer and goat rumen fluid indicates that one species cannot be used to predict CH_4 production and fermentation profile of feeds (Aderinboye et al., 2016), and wherever possible that the inoculum should be obtained from an appropriate host animal.

Sustainable mitigation of CH_4 emissions is not only environmentally beneficial, but would also increase feed energy-use



efficiency in ruminants. Based on the energy balances reported by Nkrumah et al. (2006), the reduction in CH_4 emission could potentially increase body weight gain of growing cattle by 75 g/d and milk production in dairy cows by approximately 1 L/d. The inclusion of dietary lipids is considered as one of the most effective strategy of depressing ruminal methanogenesis (Martin et al., 2010). However, although high levels of dietary lipids may reduce CH_4 production up to 40% (Jordan et al., 2006), increasing the level of dietary lipids beyond 8% can reduce feed intake. Therefore, the level of lipids in ruminant diets must be limited to 6–8% DM to avoid negative impacts on feed intake and fiber digestion and to reduce CH_4 production by up to 10–25% (Beauchemin et al., 2008). In the present experiment, SO, SA and SASO decreased proportional CH_4. This result was expected based on the well documented theory that dietary lipids can be used as an option to reduce methanogenic archaea, due to their ability to inhibit ruminal protozoa (Hook et al., 2010). Diets that high in UFA undergo ruminal biohydrogenation, which forms an alternative H_2 sink to methanogenesis (Johnson and Johnson, 1995). Reduction of H_2 accumulation in the rumen by addition of UFA seems to be a promising procedure to reduce rumen CH_4 production. Fatty acids can bind to the cell membrane and interrupt membrane transport (Dohme et al., 2001). Suppression of ruminal methanogenesis with dietary lipids depends mainly on the degree of unsaturation of the fatty acids, where sources rich in long chain fatty acids inhibit ruminal cellulolytic microbes to a greater degree than SCFA (Meale et al., 2012). This may explain the varied responses between SO and SA.

Microalgae with high concentrations of EPA and DHA fatty acids have been shown to shift ruminal fermentation towards increased propionate production and decreased CH_4 production (Johnson and Johnson, 1995). Fievez et al. (2007) reported a reduction on *in vitro* CH_4 production up to 80% with the addition of DHA-enriched microalgae.

The varied responses to SO and SA inclusion between goat and steer inoculum were expected because many factors including the ruminant species, experimental diet, and the type of lipid used accounted for varying effects of lipids on methane abatement (Hook et al., 2010). Beauchemin et al. (2007) reported an 11.5–22.0% reduction in methanogenesis with the inclusion of SO in the diet of cows.

3.2. Ruminal bacteria and protozoa count

There were no significant statistical differences ($P>0.05$) with the inoculum source × additive type or inoculum source × additive type × dose interactions for bacterial and protozoal counts (Table 5). Both inoculum sources and additives at all doses quadratically decreased ($P = 0.003$) total protozoal counts and linearly increased ($P = 0.002$) total bacterial counts.

Different effects on ruminal protozoa with different dietary fat sources have been reported (Wanapat and Khampa, 2006; Abubakr et al., 2013). The lower protozoal counts with SO and SA can be explained based on the toxic effect of oils at high levels (Abubakr et al., 2013) and unsaturated C18 fatty acids (*i.e.*, SO; Newbold and Chamberlain, 1988) to rumen ciliate protozoa. High level of dietary lipid is toxic to rumen protozoa due to the limited ability of protozoa to absorb and transform lipids (Williams, 1989), resulting in rupture of the protozoa cells (Girard and Hawke, 1978). Protozoa has a low ability to perform ruminal fat biohydrogenation compared with bacteria, thus the inclusion of dietary lipids in the

Fig. 1. *In vitro* biogas production (mL/g incubated DM) of a total mixed ration incubated with inoculum from goats and steers in the presence of sunflower oil, *Schizochytrium* microalgae or their mixture (1:1 DM basis) at five levels.

Table 5
In vitro fermentation parameters[a] of a total mixed ration incubated with inoculum from goats and steers in the presence of sunflower oil, *Schizochytrium* microalgae or their mixture at 1:1 DM basis.

Inoculum	Additive	Dose (% of ration DM)	pH	SCFA	NH_3-N	DMD	OMD	ME	PF_{24}	GY_{24}	MCP	Total bacteria × 10^8	Total protozoa × 10^5
Goat	Control	0	6.91	4.02	55.7	664	575	8.39	5.59	179	616	4.88	6.82
	Sunflower oil	1	6.49	5.47	66.4	753	691	10.17	5.18	193	739	9.73	4.36
		2	6.49	5.42	61.9	747	687	10.11	5.19	192	734	10.58	3.75
		3	6.44	3.56	60.3	755	538	7.83	5.78	173	578	11.97	3.09
		4	6.46	5.86	61.7	755	723	10.66	5.11	196	772	11.72	3.72
		5	6.46	5.67	61.6	762	707	10.42	5.14	194	756	9.60	4.49
	Schizochytrium microalgae	1	6.45	3.38	61.5	776	524	7.61	5.88	170	562	4.98	4.39
		2	6.47	5.56	61.3	776	698	10.28	5.17	194	746	8.92	4.30
		3	6.47	5.55	60.4	749	698	10.28	5.17	193	746	8.95	3.74
		4	6.48	3.58	61.7	765	540	7.86	5.77	173	580	7.28	4.22
		5	6.48	5.14	69.7	754	664	9.76	5.26	190	711	9.48	4.13
	Sunflower + *Schizochytrium* microalgae	1	6.46	5.41	56.3	755	686	10.10	5.20	192	734	9.05	3.78
		2	6.40	3.33	56.4	754	520	7.55	5.90	170	559	11.17	3.54
		3	6.48	5.24	58.7	764	673	9.89	5.23	191	719	12.55	3.81
		4	6.43	3.62	58.1	750	543	7.90	5.75	174	582	8.97	3.41
		5	6.38	3.16	60.1	744	506	7.34	6.01	167	544	10.30	3.79
	SEM		0.009	0.091	0.91	9.9	7.3	0.111	0.038	1.1	7.6	1.626	0.661
	Additive		<0.001	<0.001	<0.001	0.023	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	0.048
	Linear		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.930	0.006
	Quadratic		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0005	<0.001	0.041	0.007
Steers	Control	0	6.96	4.61	55.7	683	622	9.12	5.40	185	666	4.26	6.23
	Sunflower oil	1	6.52	6.71	62.3	767	790	11.69	4.98	201	843	12.22	3.05
		2	6.51	4.69	61.3	761	628	9.21	5.37	186	673	11.82	4.20
		3	6.47	4.42	65.6	740	607	8.88	5.45	183	650	10.72	3.24
		4	6.47	6.66	62.9	772	786	11.63	4.99	200	839	7.57	4.35
		5	6.47	4.81	62.2	757	638	9.36	5.34	187	683	8.95	4.06
	Schizochytrium microalgae	1	6.47	4.23	63.0	748	592	8.65	5.52	181	634	7.45	2.28
		2	6.47	4.84	61.7	764	641	9.40	5.33	188	686	6.13	4.76
		3	6.50	4.61	61.8	737	622	9.12	5.39	185	666	8.10	2.95
		4	6.49	4.40	70.0	749	606	8.86	5.46	183	649	8.13	4.71
		5	6.48	4.32	65.5	776	599	8.77	5.48	182	642	9.88	2.54
	Sunflower + *Schizochytrium* microalgae	1	6.47	4.59	57.9	763	620	9.09	5.40	185	664	7.22	3.08
		2	6.42	3.98	59.0	756	572	8.35	5.61	178	613	11.05	3.30
		3	6.49	4.57	58.5	768	619	9.07	5.41	185	663	9.00	3.15
		4	6.45	4.46	61.5	767	610	8.94	5.44	184	654	11.50	3.53
		5	6.41	3.89	60.6	766	565	8.24	5.64	177	606	10.08	4.38
	SEM		0.016	0.139	1.03	9.6	11.1	0.170	0.039	1.3	11.7	1.718	0.541
	Additive		0.002	<0.001	<0.001	0.038	<0.001	<0.001	<0.001	<0.001	<0.001	0.006	0.049
	Linear		<0.001	0.338	<0.001	<0.001	0.340	0.347	0.231	0.244	0.339	0.561	0.854
	Quadratic		<0.001	<0.001	0.003	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	0.023	0.003
Pooled SEM[b]			0.013	0.117	0.973	9.7	9.4	0.144	0.039	1.2	9.9	1.672	0.604
P value													
Inoculum			<0.001	<0.001	0.1452	0.1413	<0.001	<0.001	<0.001	<0.001	<0.001	0.022	0.037
Additive			<0.001	<0.001	<0.001	0.8433	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.027
Dose													
Linear			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.008	<0.001	0.716	0.003
Quadratic			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.881
Inoculum × additive			0.715	0.001	0.001	0.103	0.001	0.001	0.021	0.0197	0.001	0.223	0.174
Inoculum × additive × dose			0.981	<0.001	<0.001	0.453	<0.001	<0.001	<0.001	<0.001	<0.001	0.677	0.535

[a] DMD is dry matter degradability (mg/g DM), GY_{24} is gas yield at 24 h (mL gas/g DMD), MCP is microbial protein production (mg/g DM), ME is metabolizable energy (MJ/kg DM), NH_3-N (g/L) is ammonia-N, OMD is *in vitro* organic matter digestibility (g/kg DM), PF_{24} is partitioning factor at 24 h of incubation (mg DMD/mL gas), pH is ruminal pH, SCFA is short-chain fatty acids (mmol/g DM).
[b] SEM, standard error of the mean.

diet decreased the protozoal growth. Boeckaert et al. (2007) showed a decreased importance of *Isotricha prostoma* and *Isotricha intestinalis* and some species of *Epidinium caudatum* ciliates in the rumen of SA-fed cows. Protozoa engulfs rumen bacteria cells up to 20,000 cells per hour (Dehority, 2003). This pronounced effect of protozoa on the bacterial activities in the rumen (Williams and Coleman, 2012) may explain the increased bacterial count with the inclusion of SO, SA, and SASO additives.

Sunflower oil and SA increased total bacterial counts and MCP production suggesting an increase in the availability of energy for microbial growth. Previously, it was found that feeding SA shifted ruminal bacteria toward cellulolytic population instead of amylolytic bacteria in the rumen of SA-fed dairy cow (Boeckaert et al., 2007). While the decreased protozoa can be a reason for increased bacterial number because protozoa engulf rumen bacteria cell up to 20,000 cells per hour (Dehority, 2003), therefore, bacterial cell number could be multiplied after protozoal decline.

3.3. Fermentation parameters

Inoculum source × additive type and inoculum source × additive type × dose interactions were observed for fermentation SCFA, ammonia-N, OMD, ME, PF_{24}, GY_{24}, and MCP (Table 5). Inoculum source altered the pH, SCFA, OMD, ME, PF_{24}, GY_{24}, and MCP of the incubation medium. Both inoculum sources and additives at all doses significantly decreased ($P < 0.001$) fermentation pH and increased ammonia-N ($P < 0.001$), as well as DMD ($P < 0.05$). The goat inoculum at levels 1, 2, 4 and 5% of SO,

levels 2, 3 and 5% of SA and the levels 1 and 3% of SASO significantly increased ($P < 0.001$) SCFA, OMD, ME, GY_{24}, and MCP but decreased PF_{24} (Table 5). The inoculum from steers at levels 1, 4 and 5% of SO and 2% of SA quadratically increased ($P < 0.003$) SCFA, OMD, ME, GY_{24}, and MCP but decreased ($P = 0.001$) PF_{24}. All levels of SASO quadratically decreased ($P = 0.003$) SCFA, OMD, ME, GY_{24}, and MCP but increased ($P = 0.001$) PF_{24}.

The feed additives improved fermentation parameters in a dose-dependent manner. Both of SO and SA decreased fermentation pH. However, the reported values of fermentation pH were within the range of 6.38–6.49, which is above the values that might negatively affected rumen function (de Veth and Kolver, 2001). The reported values in Table 5 indicate a normal ruminal fermentation environment for the culture medium. The lower ruminal pH may be related to the increased concentration of ammonia and total SCFA observed with the additive supplementation (Sucu et al., 2017), or due to increased energy density (Morsy et al., 2015) in the diets. The increased total SCFA production and decreased pH reflect a higher extent of fermentation with the inclusion of SO and SA. The greater SCFA and ME were a result of improved fermentation and OMD with the additives, and may be due to improved synchronization between energy and N release in the ruminal medium. Morsy et al. (2015) observed a decline in ruminal pH and an increase in SCFA concentration with the inclusion of SO at 20 mL/d in the diet of goats.

Sunflower oil and SA increased ammonia-N, which may be because of increased bacterial count with the inclusion of SO and SA, and with the presence of soybean meal as the main protein source in the total mixed ration. The enhanced microbial activity and nutrients degradation due to increased total ruminal bacterial numbers in response to the inclusion of SO and SA in the diet has been linked to an increase in SCFA production (Morsy et al., 2015; Sucu et al., 2017). Ueda et al. (2003) observed that the inclusion of flaxseed oil increased ruminal ammonia concentration in dairy cows due to increasing bacterial N flow with SO.

Sunflower oil and SA enhanced total mixed ration degradability because of increasing total bacterial count and MCP production. Narimani-Rad et al. (2012) observed that the inclusion of SO at 0, 2.5 or 5% of a forage-based diet did not affect ME, OMD, and net energy of diets. Sucu et al. (2017) observed that feeding lambs on diets supplemented with SA at 5 g/d increased ruminal SCFA concentration and decreased rumen pH, without affecting ruminal ammonia-N concentration.

These improved fermentation kinetics as OMD, ME, GY_{24}, and MCP in steer compared with goat inoculum do not support the often-stated superiority of goats over cattle in terms of nutrients digestibility (Domingue et al., 1991).

4. Conclusion

The tested feed additives could be used to improve feed utilization, and to reduce biogases production for sustainable livestock production and the improvement of environmental conditions. The optimal levels of feed additives for sunflower oil were at 1–3%, *Schizochytrium* microalgae at 1–2%, and their mixture at 1–2%. The reduced diet's nutritive value with some levels, and increases with other levels, suggests it may be possible to prepare appropriate doses and mixtures of algae and sunflower oil to obtain both methane reduction, and maintain or improve feed utilization. These observations imply that the *in vitro* evaluation of feeds using different inoculums could be recommended to examine differences between animal species. More research is however desirable to determine whether *Schizochytrium* microalgae and sunflower oil or their mixture could be used as feed additives for improving the environmental conditions and affect feed utilization and methane production in dairy and beef cattle, goats and steers.

Conflict of interest

All authors declare that there are no present or potential conflicts of interest among the authors and other people or organizations that could inappropriately bias their work.

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