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ARTICLE

Ruminal in vitro gas production, dry matter digestibility, methane abatement potential, and fatty acid biohydrogenation of six species of microalgae

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Abstract: This study evaluated the composition, digestibility [dry matter digestibility (DMD)], CH_4 abatement potential, and fatty acid biohydrogenation of six species of microalgae. Lipid content ranged from 115 g kg^{-1} dry matter (DM) (*Scenedesmus* sp. AMDD) to 361 g kg^{-1} DM (*Tetracystis* sp.), while *Scenedesmus* sp. AMDD had the highest carbohydrate (364 g kg^{-1} DM) and fibre content (277 g kg^{-1} DM). Gas production was highest ($P < 0.001$) for *Micractinium reisseri* and *Chlorella vulgaris*. In vitro DMD ranged from 654 g kg^{-1} for *Scenedesmus* sp. AMDD to 797 g kg^{-1} for *Nannochloris bacillaris*. Total CH_4 differed ($P < 0.001$) among microalgae, ranging from 1.76 mL g^{-1} DM for *Tetracystis* sp. to 4.07 mL g^{-1} DM for *M. reisseri*. *Nannochloropsis granulata* (marine) had higher myristic, palmitoleic, and eicosapentaenoic acid levels than freshwater microalgae. Levels of α-linolenic acid were higher in *Scenedesmus* sp. AMDD than all other microalgae. CH_4 production negatively correlated ($P < 0.05$) with levels of total carbohydrate, oleic, and α-linolenic acid. Despite having a lower lipid content, CH_4 reductions with *Scenedesmus* sp. AMDD were comparable to *Tetracystis* sp. and *N. bacillaris*. Reductions in CH_4 with *Tetracystis* sp. and *N. bacillaris* occurred without a decline in DMD, suggesting that overall microbial activity was not inhibited.

Key words: biohydrogenation, batch culture, lipid, methane, microalgae.

Résumé : Cette étude avait pour but d'évaluer la composition, la digestibilité (DMD — « dry matter digestibility »), le potentiel de réduction du CH_4 et la biohydrogénation des acides gras de six espèces de microalgues. Le contenu lipidique variait de 115 g kg^{-1} de matières sèches (DM — « dry matter ») (*Scenedesmus* sp. AMDD) à 361 g kg^{-1} DM (*Tetracystis* sp.), tandis que *Scenedesmus* sp. AMDD avait la plus grande teneur en hydrates de carbone (364 g kg^{-1} DM) et en fibres (277 g kg^{-1} DM). La production de gaz était la plus élevée ($P < 0{,}001$) chez *Micractinium reisseri* et *Chlorella vulgaris*. La DMD in vitro variait de 654 g kg^{-1} chez *Scenedesmus* sp. AMDD à 797 g kg^{-1} chez *Nannochloris bacillaris*. Le CH_4 total différait ($P < 0{,}001$) parmi les microalgues, variant de 1,76 mL g^{-1} DM chez *Tetracystis* sp. à 4,07 mL g^{-1} DM chez *M. reisseri*. *Nannochloropsis granulata* (une espèce marine) avait des niveaux d'acides myristique, palmitoléique et éicosapentaénoique que les microalgues d'eau douce. Les niveaux d'acide α-linolénique étaient plus élevés chez *Scenedesmus* sp. AMDD que chez toutes les autres espèces de microalgues. Il y avait une corrélation négative ($P < 0{,}05$) entre la production de CH_4 et les niveaux totaux d'hydrates de carbone et d'acides oléique et α-linolénique. Malgré une plus faible teneur en lipides, les réductions de productions de CH_4 chez *Scenedesmus* sp. AMDD étaient comparables à celles de *Tetracystis* sp. et *N. bacillaris*. Les réductions de production de CH_4 chez *Tetracystis* sp. et *N. bacillaris* avaient lieu sans réduction de DMD, ce qui suggère que l'activité microbienne totale n'a pas été inhibée. [Traduit par la Rédaction]

Mots-clés : biohydrogénation, culture en batch, lipide, méthane, microalgue.

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Abbreviations: **BSCFA**, branched short-chain fatty acid; **CP**, crude protein; **DM**, dry matter; **DMD**, dry matter digestibility; **GE**, gross energy; **GP**, gas production; **MUFA**, monounsaturated fatty acids; **PUFA**, polyunsaturated fatty acids; **SCFA**, short-chain fatty acids; **SFA**, saturated fatty acids; **TCHO**, total carbohydrate.

Introduction

Microalgae are eukaryotic photosynthetic microorganisms that convert sunlight and carbon dioxide into biomass rich in lipids, proteins, and carbohydrates (Chisti 2007). Some microalgae are considered rich sources of *n*-3 polyunsaturated fatty acids (PUFAs) such as α-linolenic acid (C18:3*n*-3), eicosapentaenoic acid (C20:5*n*-3), and docosahexaenoic acid (C22:6*n*-3). A great deal of effort has been directed toward increasing the *n*-3 fatty acid composition of ruminant meat and milk, as these PUFAs have been associated with a reduced risk of coronary heart disease in humans (Gatrell et al. 2014). Like fish oil (Fievez et al. 2003), some microalgae have been shown to reduce ruminal methane (CH_4) production in vitro (Fievez et al. 2007), likely in part due to the biohydrogenation of fatty acids (Boeckaert et al. 2007).Studies have yet to be conducted to confirm if microalgae lower in vivo CH_4 emissions, but based on the levels of oils in some microalgae, a reduction could be anticipated. When other oils are fed, the reduction in emissions is thought to mainly arise due to the toxicity of PUFA to rumen methanogens (Dohme et al. 2001). However, the diversion of some H_2 away from CH_4 toward biohydrogenation of PUFA may also play a minor role (Johnson and Johnson 1995). Considering that microalgae lack lignin (Chen et al. 2013), they are able to sequester more CO_2 into digestible biomass (carbohydrate, protein, or lipids) than terrestrial plants (Walker 2009). Consequently, they have been proposed as potential strategy for carbon capture from fossil fuel manufacturing facilities (Sayre 2010).

Depending on downstream processing methods, the microalgae biomass remaining after lipid extraction (e.g., for renewable energy production) may contain high levels of residual oil that is suitable as an energy-dense feed as well as a source of essential fatty acids for livestock (Tibbetts et al. 2015*b*). The six microalgae species used in this study were previously identified as promising candidates for carbon sequestration and biomass production in Northern climates (Bjornsson et al. 2012; McGinn et al. 2012; Park et al. 2012; Bhatti et al. 2014; Tibbetts et al. 2014, 2015*a*). As such, they were mass cultivated in illuminated 1000-L enclosed photobioreactors to produce sufficient biomass for evaluation. Biomass was cultivated without the use of waste streams (e.g., flue gas and wastewater) so it would not accumulate toxins during production and would be suitable for animal feed. As a large portion of this produced biomass would likely be fed to cattle as a lipid supplement, there is a need to assess the rumen fermentation of these microalgae.

The objective of this study was to compare the in vitro ruminal gas production, dry matter digestibility, CH_4 abatement potential, and fatty acid biohydrogenation of whole, freeze-dried biomass produced from six microalgae species.

Materials and Methods

Microalgal biomass

Microalgae species used in this study included *Scenedesmus* sp. AMDD (SK-1), *Chlorella vulgaris* (AB02-C-U-BBM), *Nannochloris bacillaris* (AB03-C-F-PLM), *Tetracystis* sp. (AB04-C-F-PLM02), *Micractinium reisseri* (AB05-C-U-BBM02), and *Nannochloropsis granulata* (CCMP 535). *Scenedesmus* sp. was isolated from a soil sample in Saskatoon, Saskatchewan; *Tetracystis* sp., *C. vulgaris*, *M. reisseri*, and *N. bacillaris* were isolated from Sylvan Lake, Athabasca River, Pigeon Lake, and Greogoire Lake in Alberta, respectively; *N. granulata* was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, USA. The methods used to isolate, confirm species, cultivate, and harvest these microalgae have been previously described by Tibbetts et al. (2014, 2015*a*, 2015*b*). With the exception of *N. granulata* (which is a marine microalgae), the remaining microalgae were all freshwater species. Samples were obtained by pooling harvested biomass from duplicate cultures with the exception of *Scenedesmus* sp. AMDD, which was pooled from triplicate biomass harvests.

In vitro incubations

Approximately 0.5 g of algal biomass sample (used as the sole substrate) was weighed into triplicate 500 mL Ankom gas production modules (RF1; Ankom Technology, Macedon, NY, USA). Ruminal fluid was collected 2 h after feeding from three ruminally fistulated beef heifers (650 kg body weight) provided ad libitum access to a diet consisting of whole crop barley silage (700 g kg^{-1}), dry-rolled barley grain (270 g kg^{-1}), and a vitamin and mineral supplement (30 g kg^{-1}). All animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (2009). Whole ruminal contents were obtained from four locations within the rumen of the three animals, mixed together, and squeezed through four layers of cheesecloth. The pH of ruminal fluid was measured immediately (B20PI, SympHony Benchtop Meters; VWR, Edmonton, AB, Canada) and ranged from 5.60 to 6.03 throughout the study. The strained ruminal fluid was immediately transferred to the laboratory in an insulated, air-tight container and held at 39 °C in a water bath. Rumen fluid was restrained through four layers of cheesecloth to remove any particles that could interfere with dispensing rumen fluid into serum bottles. Each Ankom gas production module received 45 mL of McDougall's buffer (McDougall 1948) and 15 mL of strained ruminal fluid (3:1 ratio), after which each module was flushed with oxygen-free CO_2 and sealed. Modules were incubated on an oscillating shaker at 39 °C at 125 rev min^{-1} for 24 h. The entire process was repeated twice, generating six analytical replicates (i.e., three per run) for each treatment. In addition, three blanks containing 60 mL of medium only were included

to correct for gas production that was not associated with substrate digestion.

The modules were programmed to measure gas pressure every 30 min and valves were set to release at 3 psi (21 kPa). Data were sent wirelessly to a software-controlled base unit (Base Coordinator (RF2); Ankom Technology, Macedon, NY, USA) attached to a desktop computer. Pressure data were converted to moles of gas produced using the "Ideal" gas law [$n = p(V/RT)$] and then converted to mL of gas produced using Avogadro's law (gas in mL $= n \times 22.4 \times 1000$), where "$n$" is gas produced in moles, "p" is pressure in kPa, "V" is headspace volume in the bottle in L, "T" is temperature in Kelvin, and "R" is the gas constant. Gas data obtained were fitted to the exponential model (Ørskov and McDonald 1979) as:

$$y = B[1 - \exp -c \times (t - \text{lag})] \quad (1)$$

where "y" is the cumulative volume of gas produced at time "t" (h), "B" is the asymptotic gas volume, "c" is the rate constant, and "lag" is the time (h) between inoculation and commencement of gas production (GP). Initial GP rate (Abs_g) was calculated as the product of asymptotic cumulative gas volume and rate of fermentation (Larbi et al. 1996). Methane gas was estimated after 24 h by sampling gas from each module. Using a 20-mL syringe, about 20 mL of headspace gas was sampled through the septum of each module and forced into a 5-mL exetainer (Labco Ltd., High Wycombe, England). Methane was measured using a Varian 4900 GC (Agilent Technologies Canada Inc., Mississauga, ON). This chromatograph was equipped with a thermal conductivity detector and a 10 m PPU^H column (Supelco Inc., Oakville, ON, Canada). Column temperature was 36 °C, and the injector temperature was 70 °C with methane being resolved at 0.77 min. Helium was used as the carrier gas. Methane was expressed as mL CH_4 g^{-1} DM.

After 24 h of incubation, bottles were placed on ice to impede fermentation, and the pH (B20PI, SympHony Benchtop Meters; VWR, Edmonton, AB, Canada) of the incubation fluid was measured. Dry matter digestibility was determined by capturing residues by high-speed centrifugation (20 000 × g) at 4 °C for 30 min (Blümmel and Lebzien 2001). Blanks were also centrifuged and the pellet weight was used to correct for residues from the ruminal inoculum. In vitro apparent-degraded DM coefficients were calculated as

$$\text{Substrate DM incubated} - (\text{substrate pellet DM} - \text{blank pellet DM})/\text{substrate DM incubated} \quad (2)$$

A sample of the fermentation liquid (5 mL) was obtained from each bottle and preserved in 1 mL 0.25% (w/v) HPO_3 and stored at −20 °C until analyzed for short chain fatty acids (SCFA). The SCFA production after 24 h of incubation was calculated by subtracting initial (blanks) SCFA from the final estimates.

Chemical analyses

Samples were analyzed according to the standard methods of AOAC (1995) for DM (976.63). Nitrogen (N) content was determined by elemental analysis (950 °C furnace) using a Leco N determinator (model FP-528, Leco Corporation, St. Joseph, MI, USA) with ultrahigh purity oxygen as the combustion gas and ultrahigh purity helium as the carrier gas. Protein content was calculated using a nitrogen-to-protein conversion factor of N × 4.78 (Lourenço et al. 2004). Lipids were extracted by methanolic HCl in situ transesterification as described by McGinn et al. (2012). For fatty acid analyses, lipid extraction and methylation were as described by He et al. (2012). Fatty acid methyl esters were quantified using a gas chromatograph (Hewlett-Packard GC System 6890; Hewlett-Packard, Mississauga, ON, Canada) equipped with a flame ionization detector and SP-2560 fused silica capillary column (75 m × 0.18 mm × 0.14 μm; Supelco Inc.). To obtain fatty acid profiles, hexane extracts (1 μL) were injected using a 20:1 split. The initial oven temperature (55 °C) was held for 5 min, increased by 15 °C min^{-1} to 155 °C, held for 56 min, and increased at 10 °C min^{-1} to 240 °C at which point it was allowed to stand for an additional 15 min. Hydrogen was used as the carrier gas (head pressure 112.4 kPa and flow rate of 0.3 mL min^{-1}), and helium was used as the makeup gas (10 mL min^{-1}). Peaks in chromatograms were identified and quantified using pure methyl ester standards (Sigma-Aldrich Inc., Bellefonte, PA, USA). Total carbohydrate (TCHO) levels were determined by colorimetry using phenol and sulfuric acid as described by Dubois et al. (1956), following acid hydrolysis (2.5 M HCl at 80–90 °C for 3 h; Sukenik et al. 1993). Final results were determined against a dextrose standard curve (0–100 μg mL^{-1}; D-glucose, solid, 99% pure, Sigma Product No. G5400). Starch content was determined by the α-amylase and amyloglucosidase method (Fernandes et al. 2012) using a total starch assay kit (K-TSTA, Megazyme International Ireland Ltd., Wicklow, Ireland) according to AOAC (Official Method 996.11) and AACC (Method 76.13). Fibre content was estimated by difference (fibre = total carbohydrate − starch). Caloric content was measured as gross energy (MJ kg^{-1}) using an oxygen bomb calorimeter (model 6200, Parr Instrument Company, Moline, IL, USA) equipped with a Parr 6510 water handling system for closed-loop operation.

In vitro SCFA concentrations were separated and quantified using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA, USA) equipped with a capillary column (30 m × 0.32 mm i.d., 1-μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA, USA) and flame ionization detection. Crotonic acid (*trans*-2-butenoic acid) was used as an internal standard, and helium was used as the carrier gas (28.5 cm s^{-1}). For

Table 1. Chemical composition (g kg^{-1}, DM basis) of microalgal biomass samples.

Samples	DM	CP	Ash	Lipid	TCHO	Fibre	GE
Scenedesmus sp. AMDD[a]	918	285	30	115	364	277	23.2
Tetracystis sp.[b]	958	132	19	361	277	262	28.3
Chlorella vulgaris[b]	957	138	24	348	298	144	26.9
Micractinium reisseri[b]	956	146	24	323	301	107	26.3
Nannochloris bacillaris[b]	960	138	19	354	272	259	28.0
Nannochloropsis granulata[b]	952	350	78	285	149	ND	23.4

Note: DM, dry matter; CP, crude protein; TCHO, total carbohydrate; Fibre was estimated as TCHO–starch; GE, gross energy.
[a]Freshwater microalgae species.
[b]Marine water microalgae species.

SCFA determination, 1 μL was injected using a split ratio of 50:1. The injector temperature was set at 225 °C, and the column temperature was held at 150 °C for 1 min followed by a 5 °C min^{-1} increase in temperature until reaching 195 °C, after which the temperature was held for 5 min. The detector temperature was held constant at 250 °C.

Statistical analyses

Data from the in vitro study were subjected to analysis of variance (ANOVA) using the mixed model procedure of SAS version 9.3 (SAS Institute, Inc., Cary NC, USA) in a completely randomized design. Differences among sample means with $P < 0.05$ were accepted as statistically significant. Correlation analysis was used to establish relationships between variables using PROC CORR of SAS.

Results

Chemical composition

There were marked differences in the chemical composition of the microalgae samples (Table 1). Crude protein, lipid, and TCHO contents of the microalgae varied from 132–350, 115–361, and 149–364 g kg^{-1} DM, respectively. The same was true for ash, fibre, and gross energy contents at 19–78 g kg^{-1} DM, 107–277 g kg^{-1} DM, and 23–28 MJ kg^{-1} DM, respectively.

In vitro gas production

Gas production kinetics of the microalgae are shown in Table 2. Only the change in asymptotic gas production (b) was significant, being greater ($P < 0.001$) in *M. reisseri* and *C. vulgaris* (92–94 mL g^{-1} DM) compared with the other microalgae species (60–88 mL g^{-1} DM).

In vitro DMD, pH, and CH_4 production

In vitro DMD ranged from 654 g kg^{-1} for *Scenedesmus* sp. AMDD to 797 g kg^{-1} for *N. bacillaris* (Table 3). Total CH_4 production differed ($P < 0.001$) among the microalgae, ranging from 1.76 mL g^{-1} DM for *Tetracystis* sp. to 4.07 mL g^{-1} DM for *M. reisseri*. When corrected for CH_4 produced from the rumen fluid, the results showed a tendency for *Tetracystis* sp. to reduce ($P = 0.079$) CH_4 production.

Concentrations of short- and long-chain fatty acids

No differences were noted in the butyric, valeric, or branched short-chain fatty acids' concentrations among the samples (Table 4). Total SCFA varied from 48.9 mmol L^{-1} in *Tetracystis* sp. to 57.5 mmol L^{-1} in *M. reisseri*. Greater molar proportion of propionic acid in *Tetracystis* sp. and *N. bacillaris* resulted in the lowest acetate:propionate ratio for these samples.

Fatty acid compositions of the microalgae pre- and post-in vitro fermentation are shown in Tables 5 and 6, respectively. Pre-in vitro fermentation fatty acid results showed greater ($P < 0.001$) myristic acid (C14:0), palmitoleic acid (C16:1*n*-7), and eicosapentaenoic acid (20:5*n*-3) in *N. granulata* (the only marine microalgae used in the study) compared with the other microalgae. Among the five freshwater microalgae, heptadecenoic acid (C17:1) was only detected in *Scenedesmus* sp. AMDD. In addition, greater ($P < 0.001$) α-linolenic acid (C18:3*n*-3) was noted in *Scenedesmus* sp. AMDD compared with other microalgae. As a proportion of the lipid remaining after in vitro fermentation, stearic acid (C18:0) and eicosapentaenoic acid (20:5*n*-3) concentrations of *N. granulata* increased.

Correlations to CH_4 production

Methane production was negatively correlated ($P < 0.05$) with microalgae levels of total carbohydrate, oleic acid (C18:1*n*-9), and α-linolenic acid (C18:3*n*-3) (Table 7). Results showed that asymptotic gas production was negatively correlated ($P < 0.01$) with final pH and fibre content of microalgae. Increasing levels of TCHO and α-linolenic acid (C18:3*n*-3) in the microalgae resulted in decreasing DMD, whereas both lipid and oleic acid (C18:1*n*-9) contents of the microalgae were positively correlated ($P < 0.05$) with DMD.

Discussion

Chemical composition

Seldom does the proximate analysis of microalgae total 100%, with mass balances ranging from 630 to 1170 g kg^{-1} (Volkman and Brown 2009). The short fall in mass balance in our analysis may be related to unaccounted soluble carbohydrates, chlorophyll, nonprotein nitrogen, and other solubles such as B vitamins. The

Table 2. In vitro gas production kinetics of microalgal biomass samples.

Samples	M (mL g^{-1} DM)	k (mL h^{-1})	Abs_g (mL g^{-1} DM)
Scenedesmus sp. AMDD[a]	87.9b	4.58	4.03
Tetracystis sp.[b]	60.5d	4.66	2.82
Chlorella vulgaris[b]	92.1a	6.13	5.65
Micractinium reisseri[b]	93.6a	4.19	3.92
Nannochloris bacillaris[b]	72.2c	3.02	2.18
Nannochloropsis granulata[b]	72.3c	4.05	2.93
SEM	2.97	1.152	0.317
P value	<0.001	0.778	0.514

Note: M, asymptotic gas volume; k, specific rate of gas production; Abs_g, absolute initial gas production during the first hour (mL g^{-1} DM). No lag time was apparent. Means within a column with lowercased letters differ ($P < 0.05$).

[a]Freshwater microalgae species.

[b]Marine water microalgae species.

Table 3. Dry matter digestibility (g kg^{-1}), pH, total and net CH_4 production (mL g^{-1} DM) of microalgal biomass samples during in vitro incubation.

Samples	DMD[a]	pH[b]	Total CH_4	Net CH_4[c]
Scenedesmus sp. AMDD[d]	654d	6.25b	2.96b	−0.45
Tetracystis sp.[e]	769a	6.25b	1.76c	−1.30
Chlorella vulgaris[e]	763ab	6.21c	3.99a	0.13
Micractinium reisseri[e]	690cd	6.18c	4.07a	0.35
Nannochloris bacillaris[e]	797a	6.26b	2.53bc	−0.70
Nannochloropsis granulata[e]	719bc	6.31a	3.24b	0.43
SEM	12.3	0.011	0.211	0.439
P value	<0.001	<0.001	<0.001	0.079

Note: Means within column with lowercased letters differ ($P < 0.05$).

[a]DMD, dry matter digestibility.

[b]pH of the rumen fluid after 24 h of in vitro incubation.

[c]Net CH_4 was estimated as CH_4 produced in samples−CH_4 produced in blanks.

[d]Freshwater microalgae species.

[e]Marine water microalgae species.

Table 4. Concentration and molar proportion of individual short-chain fatty acids (SCFA, mmol L^{-1}) of the microalgal biomass samples after 24 h of in vitro incubation.

Samples	C_2	C_3	C_4	C_5	BSCFA	C_2:C_3	TSCFA
Scenedesmus sp. AMDD[a]	0.488b	0.170bc	0.183	0.058	0.089	2.91bc	56.1ab
Tetracystis sp.[b]	0.468b	0.197ab	0.175	0.063	0.085	2.56c	48.9c
Chlorella vulgaris[b]	0.510a	0.132d	0.206	0.056	0.087	3.90a	53.5abc
Micractinium reisseri[b]	0.499ab	0.149cd	0.207	0.055	0.081	3.35ab	57.5a
Nannochloris bacillaris[b]	0.485b	0.203a	0.164	0.062	0.078	2.59c	52.5bc
Nannochloropsis granulata[b]	0.509ab	0.139d	0.195	0.063	0.085	3.66a	53.8ab
SEM	0.0083	0.0119	0.0110	0.0028	0.0034	0.193	1.53
P value	0.013	<0.001	0.069	0.243	0.135	<0.001	0.003

Note: C_2, acetic; C_3, propionic; C_4, butyric; C_5, valeric; BSCFA, branched short-chain fatty acids; C_2:C_3, acetic to propionic ratio; TSCFA, total short-chain fatty acids. Means within a column with lowercased letters differ ($P < 0.05$).

[a]Freshwater microalgae species.

[b]Marine water microalgae species.

Table 5. Fatty acid composition (g kg^{-1} of total fatty acid) of microalgae before in vitro fermentation.

Fatty acid	*Scenedesmus* sp. AMDD[a]	*Tetracystis* sp.[b]	*Chlorella vulgaris*[b]	*Micractinium reisseri*[b]	*Nannochloris bacillaris*[b]	*Nannochloropsis. Granulata*[b]
14:0	3.89b	2.58c	2.68c	3.09c	2.42c	62.8a
16:0	111e	208b	136d	183c	204b	264a
18:0	15.7c	46.9b	13.4d	4.93f	48.9a	8.50e
16:1*n*-7	4.71c	5.56c	2.09d	8.79b	4.91c	298a
17:1	36.1a	nd	nd	nd	nd	2.52b
18:1*n*-9	326c	449ab	493a	404b	445ab	200d
18:1*n*-11	58.6bc	62.3ab	53.5d	54.9cd	63.2a	4.78e
18:2*n*-6	123c	122c	209b	267a	126c	23.8d
18:3*n*-3	222a	86.7b	72.7bc	62.4c	91.9b	0.90d
20:5*n*-3	nd	0.46b	0.73b	0.93b	0.95b	84.0a
ΣSFA	184c	265b	157d	194c	262b	353a
ΣMUFA	427e	520bc	554a	470d	516c	532b
ΣPUFA	389a	215d	289c	336b	222d	115e
Lipid (g kg^{-1}DM)	115d	361a	348a	323b	354a	285c

Note: Means within a row with lowercased letters differ ($P < 0.05$).
[a]Freshwater microalgae species.
[b]Marine water microalgae species.

Table 6. Fatty acid composition (g kg^{-1} of total fatty acid) of microalgae after in vitro fermentation.

Fatty acid	*Scenedesmus* sp. AMDD[a]	*Tetracystis* sp.[b]	*Chlorella vulgaris*[b]	*Micractinium reisseri*[b]	*Nannochloris bacillaris*[b]	*Nannochloropsis granulata*[b]
14:0	4.39c	4.60bc	7.23b	6.04bc	4.36c	83.9a
16:0	152d	266b	179cd	217c	280b	357a
18:0	45.7b	78.7a	91.5a	46.7b	94.2a	40.1b
16:1*n*-7	8.82a	5.38ab	3.71b	6.84ab	6.09ab	nd
17:1	20.5a	nd	nd	nd	nd	2.07b
18:1*n*-9	352a	398a	401a	408a	374a	242b
18:1*n*-11	21.9b	24.8ab	30.9a	15.4c	23.8b	5.69d
18:2*n*-6	19.6b	29.2b	141a	144a	34.3b	27.6b
18:3*n*-3	7.69c	5.39d	43.3a	32.1b	6.99cd	1.61e
20:5*n*-3	nd	nd	nd	nd	nd	144
ΣSFA	484a	495a	315b	321b	517a	533a
ΣMUFA	475a	462a	479a	482a	433a	275b
ΣPUFA	39.9b	43.1b	206a	197a	50.1b	192a

Note: Means within a row with lowercased letters differ ($P < 0.05$).
[a]Freshwater microalgae species.
[b]Marine water microalgae species.

greater CP contents of *N. granulata* and *Scenedesmus* sp. AMDD make these species very attractive as livestock feed as ingredients with high CP content typically command higher market value. Despite having a high fibre content (36%–95% of TCHO), the fibre in microalgae contains no lignin and is low in hemicellulose (Tibbetts et al. 2014). This raises the likelihood that the cell wall fraction in microalgae is highly digestible and that the protein will also be readily available as it will not be complexed with lignin (Moore and Jung 2001). Drewery et al. (2014) reported an increase in OM digestibility with increasing supplementation of postextraction algae residue (CP; 179 g kg^{-1} DM) in steers fed oat straw (CP; 45 g kg^{-1} DM). Similarly, the greater lipid content of *Tetracystis* sp., *N. bacillaris*, and *C. vulgaris* is advantageous as it would increase the energy density of the diet. It is well documented that lipids frequently reduce enteric CH_4 emissions from ruminants (Boadi et al. 2004; Beauchemin et al. 2007).

In vitro gas production

Gas production is a reflection of differences in the chemical composition of feedstuffs and has application in predicting their nutritional value. It is also a reflection of the formation of SCFA and the synthesis of microbial biomass (Getachew et al. 1998). Gas is produced when

Table 7. Correlation coefficients of gas kinetics, DMD, pH, methane, chemical and fatty acid composition of microalgal biomass samples.

	M	*k*	DMD	pH	Methane	TCHO	Fibre	Lipid	18:1*n*-9	18:3*n*-3
M	1.00	0.13	−0.36†	−0.59**	0.40†	0.29	−0.58**	−0.15	0.16	0.10
k		1.00	0.01	−0.34	0.08	0.13	−0.09	−0.07	0.07	0.12
DMD			1.00	−0.10	0.08	−0.55**	−0.18	0.79**	0.50*	−0.60**
pH				1.00	−0.14	−0.36†	0.73***	−0.22	−0.64*	−0.08
Methane					1.00	−0.38*	−0.41†	0.06	−0.36*	−0.42*
TCHO						1.00	0.43†	−0.66***	0.13	0.87***
Fibre							1.00	−0.55*	−0.47*	0.69***
Lipid								1.00	0.63**	−0.89***
18:1*n*-9									1.00	0.58*
18:3*n*-3										1.00

Note: *M*, asymptotic gas volume; *k*, specific rate of gas production; Abs_g, absolute initial gas production during the first hour (mL g^{-1} DM); DMD, dry matter disappearance. †, $0.05 < P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

substrate carbohydrates and proteins are fermented to acetate, butyrate, and ammonia (Getachew et al. 1998). One can speculate that the greater lipid content in *Tetracystis* sp. may have been responsible for the reduced gas production compared with other microalgae. Greater asymptotic gas volume noted for *M. reisseri* and *C. vulgaris* (despite their high lipid content) is contrary to this assumption. Getachew et al. (1998) reported that greater gas production is associated with acetate and butyrate production while propionate production is associated with lower gas production. Consistent with their results, both *Tetracystis* sp. and *N. bacillaris*, which exhibited the lowest asymptotic gas values (60.5 and 72.2 mL g^{-1} DM), had the lowest concentrations of acetate and butyrate and the highest concentration of propionate as compared with other microalgae.

In vitro DMD, pH, and CH_4 production

The relatively high DMD values (654–797 g kg^{-1}) observed for the microalgae suggest that they could be easily digested and utilized by ruminants. Observed differences in DMD among microalgae may be due to differences in the rate of fermentation as well as to the level of potentially digestible DM. Unlike *C. vulgaris*, the greater asymptotic gas volume of *M. reisseri* did not result in increased DMD. We expected increased asymptotic gas volume to result in greater DMD, as gas is produced when carbohydrates are fermented to acetate and butyrate (Getachew et al. 1998). In addition, there was no correlation between asymptotic gas and DMD. This is illustrated with *Tetracystis* sp. which had the lowest asymptotic gas estimate, but a DMD that was comparable to the other species of microalgae.

Methane production followed a trend similar to asymptotic gas volume with greater CH_4 produced by *M. reisseri* and *C. vulgaris* as compared with other species. Reduction of CH_4 by *Tetracystis* sp. without a concomitant decrease in DMD suggests that the microbial activity involved in the digestion of this species was not inhibited. This is contrary to a previous report by Johnson and Johnson (1995) who noted that lipid supplementation frequently decreases CH_4 production by lowering the quantity of organic matter fermented in the rumen and to a minor extent, through biohydrogenation of unsaturated fatty acids. Similarly, Dohme et al. (2001) reported that increased lipid content in feed decreases methanogenesis through inhibition of protozoa, increased production of propionic acid, and by biohydrogenation of unsaturated fatty acids. Other fatty acids (C12:0, C14:0, and C18:2) are thought to inhibit methanogens directly through binding to the cell membrane and interrupting membrane transport (Dohme et al. 2001).

Interestingly, even though *Scenedesmus* sp. AMDD possessed the lowest lipid content, it reduced CH_4 in a manner similar to the high lipid containing species, *Tetracystis* sp. and *N. bacillaris*. The ability of *Scenedesmus* sp. AMDD to exhibit similar CH_4 abatement potential is rather promising, and although the compositional components responsible for this reduction are unknown, one can speculate that this species may possess secondary compounds such as phenolic acids that may elicit this response. Additionally, lower DMD noted for *Scenedesmus* sp. AMDD *versus* other microalgae could be responsible for this reduction in CH_4 production. It will be interesting to see how these three microalgae species alter CH_4 emissions in whole animal studies.

Concentrations of short- and long-chain fatty acids

Contrary to expectations, greater DMD noted in *Tetracystis* sp. and *N. bacillaris* did not result in greater SCFA concentration for these samples. A biological explanation could be that these samples partitioned more nutrients into microbial mass *versus* SCFA. Although this explanation is consistent with Hungate (1966), who reported that microbial mass and SCFA are inversely related, this is only speculation as we did not measure microbial biomass. Differences in

SCFA concentrations are consistent with differences noted in asymptotic gas volume. Blümmel et al. (1997) reported that in addition to CO_2 and CH_4 produced as a result of fermentation (i.e., direct gas production), CO_2 is also produced upon buffering of SCFA (i.e., indirect gas production) and that molar production of CO_2 equals the molar SCFA production.

Biohydrogenation of unsaturated fatty acids constitutes an alternative pathway for hydrogen disposal, thereby reducing the amount of hydrogen available for CH_4 production (Johnson and Johnson 1995). We noted that biohydrogenation of PUFA: 78% of linoleic acid (18:2*n*-6), 94% of α-linolenic acid (C18:3*n*-3), and 100% of eicosapentaenoic acid (C20:5*n*-3) was greatest for *Scenedesmus* sp. AMDD, *Tetracystis* sp., and *N. bacillaris*, which had negative net CH_4 production. Contrary to the reduction (as a result of biohydrogenation) noted in PUFA concentrations of the microalgae after in vitro fermentation, PUFA concentration of *N. granulata* increased from 115 to 192 g kg^{-1} total FA after 24 h of incubation. It is unclear what led to the increase in eicosapentaenoic acid (C20:5*n*-3; EPA) concentration of *N. granulata*, which contributed to the increase in PUFA concentration. There have been some discrepancies in results from previous in vitro and in vivo studies regarding the extent to which EPA is biohydrogenated in the rumen. Gulati et al. (1999) reported that biohydrogenation of EPA was minimal when fish oil was incubated with rumen contents for 24 h. However, AbuGhazaleh and Jenkins (2004) noted that up to 94% of EPA disappeared when this free fatty acid was incubated in cultures of mixed rumen organisms. However, Chilliard et al. (2000) reported that EPA is biohydrogenated to a lesser extent than typically observed for other 18-carbon PUFA, with incomplete saturation often resulting in the formation of *trans* isomer mixtures as intermediates. Palmitoleic acid (C16:1*n*-7) was completely biohydrogenated in *N. granulata* samples as it was not detected after in vitro incubation, resulting in a greater concentration of palmitic acid (C16:0) in these samples.

The slight increase in α-linolenic acid (C18:3*n*-3) concentration of *N. granulata* may have also contributed to greater PUFA concentration associated with this species. Carriquiry et al. (2008) reported that biohydrogenation of unsaturated fatty acids is variable and is affected by the nature of the unsaturated fatty acid itself and the relative proportions of different unsaturates in the mixture and temperature. In a previous study (Doreau and Ferlay 1994), in which several relatively common diets were examined, the authors found that 70%–95% of linoleic acid (18:2*n*-6) and 85%–100% of α-linolenic acid (C18:3*n*-3) were biohydrogenated in the rumen.

Relationship between selected variables

The negative relationship observed for CH_4 production with oleic (C18:1*n*-9) and α-linolenic acids (C18:3*n*-3) can be partly attributed to biohydrogenation. Previous studies have shown that biohydrogenation of PUFA provides an alternative hydrogen sink to methanogenesis (Dohme et al. 2001; Boeckaert et al. 2007; Fievez et al. 2007). Similarly, Fievez et al. (2007) reported that in addition to the inhibitory effect of microalgae on CH_4 production, a shift in the fermentation pattern results in a decrease of acetate and butyrate and an increase in propionate production. This was true for some but not all of the microalgae evaluated in the present study. We were not surprised that CH_4 was negatively correlated with TCHO as previous studies have shown that high grain-based diets (high in TCHO) reduced enteric CH_4 emissions (Johnson and Johnson 1995; Grainger and Beauchemin 2011). Carbohydrate fermentation favours propionate production over acetate which creates an alternative hydrogen sink to methanogenesis, thereby reducing enteric CH_4 emissions (Murphy et al. 1982). Soluble carbohydrates may also negatively influence enteric CH_4 emissions by promoting shorter residence times in the rumen (Grainger and Beauchemin 2011).

Conclusion

Overall, both MUFA and PUFA concentrations were reduced after in vitro fermentation with concomitant increases in SFA concentration. Greater lipid contents of *Tetracystis* sp. and *N. bacillaris* were correlated with reduced CH_4 production. In addition, reduction of CH_4 production by *Tetracystis* sp. and *N. bacillaris* without a concomitant decrease in DMD suggests that microbial activity was not inhibited, even though total SCFA concentration was slightly reduced. Interestingly, *Scenedesmus* sp. AMDD (with the least lipid) was able to reduce CH_4 in a manner similar to that achieved with the higher lipid containing microalgae, *Tetracystis* sp. and *N. bacillaris*. The lower DMD noted for *Scenedesmus* sp. AMDD *versus* other microalgae could also be responsible for this reduction. Further studies with sheep are planned to assess the feed value and evaluate the potential of these promising microalgae to reduce enteric methane emissions.

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