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

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Short title: Effect of microalgae on *in vitro* rumen fermentation

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ABSTRACT

This study evaluated the ruminal gas production, dry matter digestibility (DMD), methane (CH_4) abatement potential and fatty acid biohydrogenation of six species of microalgae using an *in vitro* batch culture technique. Lipid content ranged from 115 g/kg DM in *Scenedesmus* sp. AMDD to 361 g/kg DM in *Tetracystis* sp., while protein content varied from 132 to 350 g/kg DM in *Tetracystis* sp. and *Nannochloropsis granulata*, respectively. *Scenedesmus* sp. AMDD had the highest total carbohydrate (364 g/kg DM) and fibre content (277 g/kg DM); whereas its protein content was intermediate (285 g/kg DM). Asymptotic gas production was higher ($P<0.001$) for fermentation cultures containing *Micractinium reisseri* and *Chlorella vulgaris* (92-94 mL/g DM) than the other species. *In vitro* DMD ranged from 654 g/kg for *Scenedesmus* sp. AMDD to 797 g/kg for *Nannochloris bacillaris*. Total CH_4 production varied ($P<0.001$) among the fermentation cultures containing microalgae, ranging from 1.76 mL/g DM for *Tetracystis* sp. to 4.07 mL/g DM for *M. reisseri*. Greater molar proportion of propionic acid in *Tetracystis* sp. and *N. bacillaris* resulted in the lowest acetic acid:propionic acid ratio for these two samples. Pre-*in vitro* fermentation fatty acid profiles showed higher myristic acid (C14:0), palmitoleic acid (C16:1*n*−7) and eicosapentaenoic acid (20:5*n*–3) in the marine microalga *N. granulata* compared to the freshwater microalgae species. A higher level of α-linolenic acid (C18:3*n*-3) was found in *Scenedesmus* sp. AMDD than all other microalgae species. Both monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid concentrations were generally reduced after *in vitro* fermentation with concomitant increases in saturated fatty acid (SFA) concentrations. In contrast, concentrations of stearic acid (C18:0) and eicosapentaenoic acid (20:5*n*–3) in *N. granulata* increased after *in vitro* fermentation. CH_4 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). Interestingly,

even though *Scenedesmus* sp. AMDD had the lowest lipid content, CH_4 reduction with this species was comparable to *Tetracystis* sp. and *N. bacillaris*. Reductions in CH_4 with *Tetracystis* sp. and *N. bacillaris* were achieved without a decline in DMD, suggesting that this reduction was achieved without inhibiting overall microbial activity.

Keywords: Biohydrogenation; *In vitro* batch culture; Lipids; Methane; Microalgae

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

1. Introduction

Microalgae are prokaryotic or 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 (PUFA) 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 towards increasing the *n*-3 fatty acid composition of ruminant meat and milk as these PUFA 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); a response that has also been confirmed to occur in ruminants *in vivo* (Boeckaert et al., 2007; Calsamiglia et al., 2007; Jouany and Morgavi, 2007). This response is

thought to mainly arise due to the toxicity of PUFA to methanogenic bacteria in the rumen (Dohme et al., 2001), but the diversion of some H_2 away from CH_4 towards 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).

Depending on down-stream processing methods, lipid-extracted microalgae biomass remaining after oil 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). 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., 2015a,b). 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, wastewater) and, as such, was suitable for use as animal feed as it is unlikely to contain accumulated toxicants as a result of the production process. However, there is a need to assess the value of microalgae as a feed source, particularly from the perspective of fermentation as there appears to be good potential to use this biomass as cattle feed in Canada.

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.

2. Materials and methods

2.1. 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). The methods used to isolate, confirm species, cultivate and harvest these microalgae have been previously described by Tibbetts et al. (2015a,b,c). With the exception of *N. granulata* (which is a marine microalgae), the remaining microalgae were all freshwater species. Samples were obtained from pooled composites of duplicate biomass harvests with the exception of *Scenedesmus* sp. AMDD, which was a pooled composite of triplicate biomass harvests.

2.2. In vitro incubations

Approximately 0.5 g of algal biomass sample was weighed into triplicate 500 mL Ankom gas production modules (RF1; Ankom Technology, Macedon, NY, USA). Ruminal fluid was collected 2 h after feeding (0900 h) from three ruminally-fistulated beef heifers (650 kg body weight) provided (DM basis) *ad libitum* access to a diet consisting of whole crop barley silage (700 g/kg), dry-rolled barley grain (270 g/kg), and a vitamin and mineral supplement (30 g/kg). 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 3 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 re-strained through four layers of cheesecloth to remove any contaminating 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^{o}C at 125 rpm for 24 h. The entire process was repeated twice, generating six analytical replicates (*i.e.* three per run) for each treatment. In addition, 3 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 head-space volume in the bottle in L, 'T' is temperature in Kelvin and 'R' the gas constant. Gas data obtained were fitted to the exponential model (Ørskov and McDonald, 1979) as:

$$y = B(1-\exp -c\times[t-lag]),$$

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).

After 24 h of incubation, bottles were placed in ice to impede fermentation. Dry matter digestibility was determined by high-speed centrifugation (20,000×g) of incubation residues 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:

Substrate DM incubated−[substrate pellet DM−blank pellet DM]/substrate DM incubated.

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 acid (SCFA) concentrations. The SCFA production at the end of 24 h of incubation was calculated by subtracting initial (blanks) SCFA from the final estimates.

2.3. 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 ultra-high purity oxygen as the combustion gas and ultra-high 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., Oakville, ON, Canada). 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 to 155°C, held for 56 min, and increased at 10°C/min 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) and helium was used as the make-up gas (10 mL/min). 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; D-glucose, solid, 99% pure, Sigma Product # 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). Fiber contents were estimated by difference (fiber = total carbohydrate - starch). Caloric content was measured as gross energy (MJ/kg) 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). For 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 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.

2.4. Statistical analyses

Data from the *in vitro* study were subjected to analysis of variance (ANOVA) using the mixed model procedure of SAS (2002) 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 (2002).

3. Results

3.1. 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 to 350, 115 to 361 and 149 to 364 g/kg DM, respectively. The same was true for ash, fibre and gross energy contents at 19 to 78, 107 to 277 g/kg DM and 23 to 28 MJ/kg DM, respectively.

3.2. In vitro gas production

Gas production kinetics of the microalgae are shown in Table 2. Only the change in asymptotic gas production (b) were significant; being greater (P<0.001) in *M. reisseri* and *C. vulgaris* (92 to 94 mL/g DM) compared to the other microalgae species (60 to 88 mL/g DM).

3.2. In vitro DMD, pH and CH_4 production

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

3.3. 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 in *Tetracystis* sp. to 57.5 mmol/L 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 composition 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 5 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.

3.4. Relationship between selected variables

CH_4 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 the 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.

4. Discussion

4.1. Chemical composition

The greater CP content of *N. granulata* and *Scenedesmus* sp. AMDD make these species very attractive dietary supplements for livestock feed as ingredients with high CP content typically command higher market value. Despite having a high fibre content (0.36–0.95 of TCHO), the fibre in microalgae contains no lignin and is low in hemicellulose (Tibbetts et al., 2015). 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 post-extraction algae residue (CP; 179 g/kg DM) in steers fed oat straw (CP; 45 g/kg DM). Similarly, the greater lipid content of *Tetracystis* sp., *N. bacillaris* and *C. vulgaris* is advantageous as it is well documented that supplementation of diets with certain lipids frequently reduces enteric CH_4 emissions from ruminant animals (Boadi et al., 2004; Beauchemin et al., 2007).

4.2. In vitro gas production

Gas production is a reflection of differences in the chemical composition of feedstuffs and may have 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 substrate carbohydrates and proteins are fermented to acetate, butyrate and ammonia (Getachew et al., 1998). One can only speculate that the greater lipid content in *Tetracystis* sp. may have been responsible for the reduced gas production compared to 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 had numerically the lowest concentrations of acetate and butyrate and the highest concentration of propionate, compared to the other microalgae species; which also exhibited the lowest asymptotic gas values (60.5 and 72.2 mL/g DM).

4.3. In vitro DMD, pH and CH_4 *production*

The relatively high DMD values (654 to 797 g/kg) observed for the microalgae suggests 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 due to the level of potentially digestible materials. 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.

CH_4 production followed a trend similar as asymptotic gas volume with greater CH_4 produced by *M. reisseri* and *C. vulgaris* as compared to 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 can decrease CH_4 production by lowering the quantity of organic matter fermented in the rumen and to a very minor extent through biohydrogenation of unsaturated fatty acids. Similarly, Dohme et al. (2001) reported that increased lipid content in feed is thought to decrease 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, but without knowing exactly how *Scenedesmus* sp. AMDD was able to reduce CH_4, one can only speculate that this particular species may possess secondary compounds such as phenolic acids that may have elicited the response. Additionally, lower DMD noted for *Scenedesmus* sp. AMDD *versus* other microalgae could be responsible for this reduction in CH_4 production.

4.4. 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, we can only speculate because we did not measure microbial mass in the present study. 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 generated (*i.e.* indirect gas production) and that molar production of CO_2 equals the molar SCFA production.

Biohydrogenation of unsaturated fatty acids constitute 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 concentrations (0.78 of linoleic acid (18:2n–6), 0.94 of α-linolenic acid (C18:3*n*–3) and 1.0 of eicosapentaenoic acid (C20:5*n*−3) was greatest for the 3 microalgae (*Scenedesmus* sp. AMDD, *Tetracystis* sp. and *N. bacillaris*) that 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 275 g/kg total FA after 24 h of incubation. One can only speculate on what led to the increase in eicosapentaenoic acid (C20:5*n*−3; EPA) concentration of *N. granulata* which led to a concomitant increase in its 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 0.94 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 saturation often being incomplete, 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, 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 0.70–0.95 of linoleic acid (18:2n–6) and 0.85–1.00 of α-linolenic acid (C18:3*n*–3) were biohydrogenated in the rumen.

4.5. Relationship between selected variables

The negative relationship observed for CH_4 production with oleic (C18:1*n*–9) and α-linolenic acid (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).

5. 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* are correlated with resulted in 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 although 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 ability of *Scenedesmus* sp. AMDD to exhibit similar CH_4 abatement potential is quite promising but without knowing exactly how *Scenedesmus* sp. AMDD was able to reduce CH_4 production, one can only speculate that a form of synergistic effect of low lipid and high CHO may be responsible. Additionally, lower DMD noted for *Scenedesmus* sp. AMDD *versus* other microalgae could also be responsible for this reduction.

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Table 1. Chemical composition (g/kg, DM basis) of microalgal biomass samples.

Samples	DM[1]	CP	Ash	Lipid	TCHO	Fibre	GE
Scenedesmus sp. AMDD[2]	918	285	30	115	364	277	23.2
Tetracystis sp.[3]	958	132	19	361	277	262	28.3
Chlorella vulgaris[3]	957	138	24	348	298	144	26.9
Micractinium reisseri[3]	956	146	24	323	301	107	26.3
Nannochloris bacillaris[3]	960	138	19	354	272	259	28.0
Nannochloropsis granulata[4]	952	350	78	285	149	ND	23.4

[1]DM, dry matter; CP, crude protein; TCHO, total carbohydrate; Fibre was estimated as TCHO – starch; GE, gross energy.

[2]Freshwater strain of *Scenedesmus* sp. AMDD isolated from a soil sample in Saskatoon, Saskatchewan.

[3]Samples are all similar freshwater species isolated from Sylvan Lake, Athabasca River, Pigeon Lake and Gregoire Lake in Alberta.

[4]Whole biomass of marine species of *N. granulata* (originally purchased from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, USA).

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

Samples	M[1] (mL/g DM)	k (mL/h)	Lag (h)	Abs_g (mL/g DM)
Scenedesmus sp. AMDD[2]	87.9[b]	4.58	-5.04	4.03
Tetracystis sp.[3]	60.5[d]	4.66	-2.62	2.82
Chlorella vulgaris[3]	92.1[a]	6.13	-2.99	5.65
Micractinium reisseri[3]	93.6[a]	4.19	-5.41	3.92
Nannochloris bacillaris[3]	72.2[c]	3.02	-2.97	2.18
Nannochloropsis granulata[4]	72.3[c]	4.05	-5.56	2.93
SEM	2.97	1.152	0.966	0.317
P value	<0.001	0.778	0.362	0.514

Means within a column with different superscripts differ (P<0.05).

[1]M, asymptotic gas volume; k, specific rate of gas production; Lag, lag time; Abs_g, absolute initial gas production during the first hour (mL/g DM).

[2]Freshwater strain of *Scenedesmus* sp. AMDD isolated from a soil sample in Saskatoon, Saskatchewan.

[3]Samples are all similar freshwater species isolated from Sylvan Lake, Athabasca River, Pigeon Lake and Gregoire Lake in Alberta.

[4]Whole biomass of marine species of *N. granulata* (originally purchased from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, USA).

Table 3. Dry matter digestibility (g/kg), pH, total and net CH_4 production (mL/g DM) of microalgal biomass samples during *in vitro* incubation.

Samples	DMD[1]	pH	Total CH_4	Net CH_4[2]
Scenedesmus sp. AMDD[3]	654^{d}	6.25^{b}	2.96^{b}	-0.45
Tetracystis sp.[4]	769^{a}	6.25^{b}	1.76^{c}	-1.30
Chlorella vulgaris[4]	763^{ab}	6.21^{c}	3.99^{a}	0.13
Micractinium reisseri[4]	690^{cd}	6.18^{c}	4.07^{a}	0.35
Nannochloris bacillaris[4]	797^{a}	6.26^{b}	2.53^{bc}	-0.70
Nannochloropsis granulata[5]	719^{bc}	6.31^{a}	3.24^{b}	0.43
SEM	12.3	0.011	0.211	0.439
P value	<0.001	<0.001	<0.001	0.079

Means within column with different superscripts differ (P<0.05).

[1]DMD, dry matter digestibility.

[2]Net CH_4 was estimated as: CH_4 produced in samples – CH_4 produced in blanks.

[3]Freshwater strain of *Scenedesmus* sp. AMDD isolated from a soil sample in Saskatoon, Saskatchewan.

[4]Samples are all similar freshwater species isolated from Sylvan Lake, Athabasca River, Pigeon Lake and Gregoire Lake in Alberta.

[5]Whole biomass of marine species of *N. granulata* (originally purchased from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, USA).

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

Samples	C_2[1]	C_3	C_4	C_5	BSCFA	C_2:C_3	TSCFA
Scenedesmus sp. AMDD[2]	0.488^{b}	0.170^{bc}	0.183	0.058	0.089	2.91^{bc}	56.1^{ab}
Tetracystis sp.[3]	0.468^{b}	0.197^{ab}	0.175	0.063	0.085	2.56^{c}	48.9^{c}
Chlorella vulgaris[3]	0.510^{a}	0.132^{d}	0.206	0.056	0.087	3.90^{a}	53.5^{abc}
Micractinium reisseri[3]	0.499^{ab}	0.149^{cd}	0.207	0.055	0.081	3.35^{ab}	57.5^{a}
Nannochloris bacillaris[3]	0.485^{b}	0.203^{a}	0.164	0.062	0.078	2.59^{c}	52.5^{bc}
Nannochloropsis granulata[4]	0.509^{ab}	0.139^{d}	0.195	0.063	0.085	3.66^{a}	53.8^{ab}
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

Means within a column with different superscripts differ (P<0.05).

[1]C_2, acetic; C_3, propionic; C_4, butyric; C_5, valeric; BSCFA, branched short-chain fatty acids; C2:C3, acetic to propionic ratio; TSCFA, total short-chain fatty acids.

[2]Freshwater strain of *Scenedesmus* sp. AMDD isolated from a soil sample in Saskatoon, Saskatchewan.

[3]Samples are all similar freshwater species isolated from Sylvan Lake, Athabasca River, Pigeon Lake and Gregoire Lake in Alberta.

[4]Whole biomass of marine species of *N. granulata* (originally purchased from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, USA).

Table 5. Fatty acid composition (g/kg of total fatty acid) of microalgae before *in vitro* fermentation.

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

Means within a row with different superscripts differ (P<0.05).

[1]Freshwater strain of *Scenedesmus* sp. AMDD isolated from a soil sample in Saskatoon, Saskatchewan.

[2]Samples are all similar freshwater species isolated from Sylvan Lake, Athabasca River, Pigeon Lake and Gregoire Lake in Alberta.

[3]Whole biomass of marine species of *Nannochloropsis granulata* (originally purchased from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, USA).

Table 6. Fatty acid composition (g/kg of total fatty acid) of microalgae after *in vitro* fermentation.

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

Means within a row with different superscripts differ (P<0.05).

[1]Freshwater strain of *Scenedesmus* sp. AMDD isolated from a soil sample in Saskatoon, Saskatchewan.

[2] Samples are all similar freshwater species isolated from Sylvan Lake, Athabasca River, Pigeon Lake and Gregoire Lake in Alberta.

[3]Whole biomass of marine species of *Nannochloropsis granulata* (originally purchased from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, USA).

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

	M[1]	k	DMD	pH	Methane	TCHO	Fibre	Lipid	18:1n–9	18:3n–3
M	1.00	0.13	-0.36[T]	-0.59**	0.40[T]	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[T]	0.73***	-0.22	-0.64*	-0.08
Methane					1.00	-0.38*	-0.41[T]	0.06	-0.36*	-0.42*
TCHO						1.00	0.43[T]	-0.66***	0.13	0.87***
Fibre							1.00	-0.55*	-0.47*	0.69***
Lipid								1.00	0.63**	-0.89***
18:1n–9									1.00	0.58*
18:3n–3										1.00

[1]M, asymptotic gas volume; k, specific rate of gas production; Lag, lag time; Abs_g, absolute initial gas production during the first hour (mL/g DM); DMD, dry matter disappearance. [T], 0.05 <P<0.1; *, P<0.05; **, P<0.01; ***, P<0.001.