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In vitro digestion of microalgal biomass from freshwater species isolated in Alberta, Canada for monogastric and ruminant animal feed applications

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ABSTRACT

In vitro digestion studies were conducted to examine the potential nutritional value of whole (WAB) and lipid-extracted biomass (LEB) from freshwater microalgae from Alberta, Canada. For WAB, protein solubility (PS) was statistically highest and the same ($P = 0.109$) for *Chlorella vulgaris* at 84% and *Micractinium reisseri* at 78%, lowest ($P < 0.001$) for *Nannochloris bacillaris* at 64% and intermediate for *Tetracystis* sp. at 73%. Dilute pepsin digestibility (DPD) was highest ($P < 0.001$) for *C. vulgaris* at 80% and lowest ($P < 0.001$) for *N. bacillaris* and *Tetracystis* sp. at 60–64%, which were the same ($P = 0.075$) and *M. reisseri* was intermediate at 72%. Two-phase gastric/pancreatic digestibility of protein ($GPD_{Protein}$) and energy (GPD_{Energy}) were highest ($P < 0.001$) for *M. reisseri* at 78 and 57%, respectively, lowest ($P < 0.001$) for *N. bacillaris* and *Tetracystis* sp. at 49–52 and 41–43%, respectively, which were the same ($P = 0.101$ and 0.058, respectively) and *C. vulgaris* was intermediate at 69 and 52%, respectively. For LEB, PS was highest ($P < 0.001$) and the same ($P = 0.088$) for *C. vulgaris* and *M. reisseri* at 72–76%; which were higher ($P < 0.001$) than *N. bacillaris* and *Tetracystis* sp. at 60–62%, which were the same ($P = 0.405$). Similarly, DPD was highest ($P < 0.001$) and the same ($P = 1.000$) for *C. vulgaris* and *M. reisseri* both at 69%; which were higher ($P < 0.001$) than *N. bacillaris* and *Tetracystis* sp. at 58–62%, which were the same ($P = 0.083$). $GPD_{Protein}$ was highest ($P < 0.001$) and the same ($P = 0.944$) for *M. reisseri* and *C. vulgaris* at 79–80%, lowest ($P < 0.001$) for *N. bacillaris* at 50% and intermediate for *Tetracystis* sp. at 55%. GPD_{Energy} was highest ($P < 0.001$) for *C. vulgaris* at 69%, followed by *M. reisseri* at 61%, *Tetracystis* sp. at 48% and lowest ($P = 0.006$) for *N. bacillaris* at 45%. Organic matter digestibility (OMD) of a ruminant control diet was 45% and not significantly affected ($P \geq 0.071$) by dietary algal supplementation with an average OMD of 36% when incorporated at 50% forage replacement (equivalent to 25–43% of the diet); except *Tetracystis* sp. LEB which decreased ($P = 0.020$) OMD to 28%. Dietary inclusion of all biomass at 100% forage replacement (equivalent to 51–85% of the diet) decreased ($P \leq 0.026$) OMD to an average of 28%; except *M. reisseri* LEB which did not significantly affect ($P = 0.921$) OMD at 41%. Apparent metabolizable energy (aME) content of the control diet was 3.7 MJ kg^{-1} and was not affected ($P \geq 0.179$) by algal supplementation at an average of 3.1 MJ kg^{-1}, although a general trend of decreased aME with increased dietary levels was noted. Methane production from 48 h *in vitro* fermentation of diets with varying levels of WAB was 2.8–3.3 mol^{-10} and was the same ($P \geq 0.429$) as the control diet at 2.9 mol^{-10}. However, LEB at all levels decreased ($P < 0.001$) methane production by about 50% to 0.9–1.2 mol^{-10}, which suggests the potential for abating enteric methane emissions from ruminants by feeding microalgae, unrelated to its lipid content.

1. Introduction

Microalgae are one of the most efficient organisms at converting solar energy, carbon dioxide and inorganic elements into nutrient-rich biomass [1], which represents a potential source of renewable fuel and animal feed. Although algal oil for third generation biodiesel production has been the subject of much research and a major driver for technological innovations in recent years, by most assessments it is still not economically viable [2–4]. Consequently, algal products/co-products resulting from biofuel applications have been identified in Canada and elsewhere as a priority for investigation as valuable commodities for revenue generation and sustainable replacement of terrestrial livestock and aquaculture feed inputs [4–6]. Four freshwater species isolated in Northern Alberta, Canada have been identified as promising candidates for industrial carbon conversion in Northern climates; including *Chlorella vulgaris*, *Nannochloris bacillaris*, *Tetracystis* sp. and *Micractinium reisseri* [7] and have been mass cultivated in

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enclosed photobioreactors [8]. The first in a series of studies with this algal biomass showed them to have similar growth characteristics, proximate compositions, favorable essential amino acid and fatty acid profiles, attractive minerals and trace element compositions and were devoid of contaminating heavy metals [9]. However, there were notable differences in their carbohydrate compositions with respect to starch and fiber, which could greatly affect their extent of digestion and ultimately their overall nutritional value when fed to target animal species. Bioavailability of nutrients from novel ingredients varies between animal species due to the differences in feeding habits and digestive physiologies found in the livestock sectors broadly classified as monogastric (e.g., fish, poultry, swine) or ruminant (e.g., cattle, sheep, goats). The differences in their digestion, absorption and metabolism can be vast and, in particular, when associated with high levels of dietary fiber, which is utilized at varying degrees by farmed animals species. As a result, nutritional evaluation methods for monogastric animals can generate highly valuable data for the broad class of monogastric livestock but are likely of little value for ruminant livestock and *vice versa*. Beyond biochemical composition, digestibility is generally one of the most important aspects defining the nutritional quality of novel feed ingredients and is largely dependent upon their solubility and the extent of their chemical hydrolysis and enzymatic digestion in the gut; which can be affected by processing treatment(s) [10–13].

Evaluation of nutritional quality *in vivo* is time-consuming and costly, while *in vitro* assays that involve simulated digestion of test ingredient suspensions with purified digestive enzymes or ruminal fluid allow screening of large numbers of samples with minimal use of animals [14]. While not fully definitive of whole animal response, these methods can complement biochemical composition data as they are relatively inexpensive, results are rapidly obtained using small sample sizes, they side-step animal palatability issues and they are generally regarded as effective tools for making predictions of potential nutritional quality for research and industrial use [15]. Due to the difficulties in extrapolating nutritional value results between monogastric and ruminant animals, separate *in vitro* digestion techniques are required, however, both can provide valuable data on bioavailability of dietary protein and energy from novel feed ingredients and may also provide a preliminary selection of treatments prior to undertaking costly *in vivo* feeding trials. The present study is the second in a series of projects designed to evaluate the nutritional value of four freshwater chlorophytic microalgae species isolated in Northern Alberta, Canada for animal feed applications [9]. The main objective was to generate novel *in vitro* digestion data of whole algal biomass (WAB) and lipid-extracted biomass (LEB) for both monogastric and ruminant livestock including protein solubility (PS), dilute pepsin digestibility (DPD), two-phase gastric/pancreatic protein digestibility ($GPD_{Protein}$) and energy digestibility (GPD_{Energy}), ruminal organic matter digestibility (OMD), apparent metabolizable energy (aME) content and methane production.

2. Materials and methods

2.1. Microalgal biomass

Microalgae species used in this study included *C. vulgaris* (AB02-C-U-BBM), *N. bacillaris* (AB03-C-F-PLM), *Tetracystis* sp. (AB04-C-F-PLM02) and *M. reisseri* (AB05-C-U-BBM02). Isolation conditions, 18S gene sequence identification, screening criteria, mass cultivation, harvesting and processing and biochemical characterization are fully described in Tibbetts et al. [9]. Proximate and caloric content of whole and lipid-extracted biomass are presented in Table 1.

Table 1
Proximate composition and caloric content of whole algal biomass (WAB) and lipid-extracted biomass (LEB) used for *in vitro* digestion studies (DW basis)[a].

	C. vulgaris	*M. reisseri*	*N. bacillaris*	*Tetracystis* sp.
WAB				
Ash (%)	2.4	2.4	1.9	1.8
Crude protein (%)	14.8	14.8	14.9	14.7
Esterifiable lipid (%)	34.8	32.3	35.4	36.1
Carbohydrate (%)	29.8	30.0	27.2	27.7
Starch (%)	15.4	19.3	1.3	1.5
Fiber (%)	14.4	10.7	25.9	26.2
Gross energy (MJ kg^{-1})	26.9	26.3	28.0	28.3
LEB				
Ash (%)	2.7	2.6	2.8	2.7
Crude protein (%)	18.8	18.2	23.3	24.3
Esterifiable lipid (%)	31.8	27.7	6.1	9.4
Carbohydrate (%)	33.6	35.7	43.9	43.2
Starch (%)	20.1	24.3	2.5	3.0
Fiber (%)	13.5	11.4	41.3	40.2
Gross energy (MJ kg^{-1})	23.9	24.5	20.7	21.2

[a] From Tibbetts et al. [9].

2.2. In vitro digestion

2.2.1. Monogastric assays

Protein solubility was measured by incubation of 250 mg of WAB or LEB in 0.2% potassium hydroxide (0.036 N KOH, pH 13) for 20 min at 22 °C according to Araba and Dale [16] and Parsons et al. [17]. Dilute pepsin digestibility was measured by incubation of 200 mg of WAB or LEB in 0.0002% porcine pepsin (P7000, Sigma-Aldrich) enzyme solution (1:10,000 *w/v* in 0.075 N HCl; pH 1.5) for 16 h at 39 °C according to AOAC [18] and Komaki et al. [19]. Two-phase gastric/pancreatic digestibility was measured by incubation of 250 mg of WAB or LEB in porcine pepsin (P7000, Sigma-Aldrich) enzyme solution (25 mg mL^{-1} *w/v* in 0.2 N HCl, pH 1) for 2 h at 39 °C (gastric phase) and then subsequent incubation in porcine pancreatin, containing amylase, lipase and protease (P1750, Sigma-Aldrich) enzyme solution (100 mg mL^{-1} *w/v* in 0.05 M Tris, 0.0115 M $CaCl_2$ buffer; pH 7) for 4 h at the same temperature (pancreatic phase) according to Yegani et al. [20]. For all *in vitro* assays, three 5 mm glass beads (Cat. 11-312C, Thermo Fisher Scientific, Waltham, MA, USA) were included to aid sample dispersion. Due to the small particle size of microalgae (generally 2–20 μm) all *in vitro* assays had a minor modification with regard to filtering. After termination of chemical/enzymatic digestion, hydrolyzed contents were passed through a Whatman GF/A filter (1.6 μm porosity) rather than a Whatman no. 54 filter (20–25 μm porosity). Additionally, a microalgae-appropriate nitrogen-to-protein conversion factor (N × 4.78) [21] was used instead of the conventional N × 6.25. All reagents and enzyme cocktails were prepared fresh on a weekly basis and kept refrigerated (4 °C); with the exception of KOH, which was kept at room temperature. *In vitro* digestibility (IVD) of protein and energy were calculated as: IVD (%) = ([{Protein or Energy in initial sample − Protein or Energy in dry residue} ÷ {Protein or Energy in initial sample}] × 100%). All *in vitro* digestion assays were conducted with five replicates and procedural blanks were run in parallel to correct final IVD calculations.

2.2.2. Ruminant assays

Organic matter digestibility and apparent metabolizable energy content of diets containing varying levels of WAB and LEB were estimated using a modified batch-culture *in vitro* ruminal fermentation system with total gas capture [22]. Twenty-five isonitrogenous (12.4% crude protein; CP, DM basis) dietary treatments (Table 2) were formulated using a constant inclusion level of medium grind corn (15% of diet; 10% of total CP) and three inclusion levels of WAB or LEB (Low, 23% of total CP; Medium, 45% of total CP; High, 90% of total CP) replacing grass/legume forage; 1 mm grind (Low, 67% of total CP; Medium, 45% of total CP; High, 0% of total CP) and nitrogen-free cellulose. These levels represented dietary *as-fed* ratios of forage and algae corresponding to Control (100Forage:0Algae), Low (75Forage:25Algae), Medium (50Forage:50Algae) and High (0Forage:100Algae). Mixed rumen fluid was obtained from two ruminally-fistulated mid-lactation Holstein-Friesian dairy cows that were housed and cared for in accordance

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Table 2
Composition of dietary treatments used for *in vitro* ruminant digestion studies of whole algal biomass (WAB) and lipid-extracted biomass (LEB) (DW basis).

Dietary treatment	Contribution to dietary treatment (% of diet)[a]				Contribution to dietary crude protein (CP) (% of dietary CP)		
	Corn[b]	Forage[c]	Cellulose[d]	Algae[e]	Corn	Forage	Algae
Control (100F:0A)	15.0	75.0	10.0	–	9.73	90.27	–
WAB							
C. vulgaris							
Low (75F:25A)	15.0	56.25	8.375	20.375	9.70	67.47	22.84
Medium (50F:50A)	15.0	37.5	6.75	40.75	9.66	44.82	45.52
High (0F:100A)	15.0	–	3.475	81.525	9.59	–	90.41
M. reisseri							
Low (75F:25A)	15.0	56.25	9.475	19.275	9.69	67.42	22.89
Medium (50F:50A)	15.0	37.5	8.975	38.525	9.65	44.78	45.57
High (0F:100A)	15.0	–	7.95	77.05	9.58	–	90.42
N. bacillaris							
Low (75F:25A)	15.0	56.25	8.375	20.375	9.69	67.41	22.90
Medium (50F:50A)	15.0	37.5	6.75	40.75	9.65	44.74	45.61
High (0F:100A)	15.0	–	3.475	81.525	9.56	–	90.44
Tetracystis sp.							
Low (75F:25A)	15.0	56.25	7.45	21.3	9.69	67.40	22.91
Medium (50F:50A)	15.0	37.5	4.875	42.625	9.64	44.72	45.64
High (0F:100A)	15.0	–	–	85.0	9.58	–	90.42
LEB							
C. vulgaris							
Low (75F:25A)	15.0	56.25	12.5	16.25	9.69	67.45	22.85
Medium (50F:50A)	15.0	37.5	14.975	32.525	9.66	44.79	45.56
High (0F:100A)	15.0	–	19.975	65.025	9.59	–	90.41
M. reisseri							
Low (75F:25A)	15.0	56.25	12.850	15.9	9.69	67.42	22.89
Medium (50F:50A)	15.0	37.5	15.725	31.775	9.65	44.78	45.56
High (0F:100A)	15.0	–	21.45	63.55	9.58	–	90.42
N. bacillaris							
Low (75F:25A)	15.0	56.25	16.075	12.675	9.69	67.41	22.90
Medium (50F:50A)	15.0	37.5	22.15	25.35	9.65	44.75	45.60
High (0F:100A)	15.0	–	34.325	50.675	9.57	–	90.43
Tetracystis sp.							
Low (75F:25A)	15.0	56.25	15.9	12.85	9.69	67.41	22.90
Medium (50F:50A)	15.0	37.5	21.825	25.675	9.65	44.77	45.58
High (0F:100A)	15.0	–	33.625	51.375	9.57	–	90.43

[a] Total CP of all dietary treatments was 12.4 ± 0.1% of DW.
[b] Corn, medium grind (7.95% CP).
[c] Grass/legume forage, 1 mm grind (14.75% CP).
[d] Nitrogen-free pure cellulose (CP-free).
[e] WAB and LEB (CP according to Table 1).

with the Canadian Council on Animal Care [23] and fed a complete ration containing a 60:40 blend of grass/legume forage and a concentrate composed of barley grain (40.0%), solvent-extracted canola meal (21.1%), soybean meal (20.9%), medium grind corn (9.3%) and vitamin/mineral supplement (8.7%). Rumen fluid (pH 5.8 ± 0.4) was collected by hand sampling various locations of the rumens, mixed and coarsely filtered to remove large particles before transporting to the laboratory in a warmed insulated container where it was further filtered through 3 layers of nylon followed by 16 layers of cheesecloth into an Erlenmeyer flask (purged with nitrogen gas to maintain anaerobiosis) in a heated water bath (39 °C). For each treatment, 0.4 g of test diet, 30 mL of warm (39 °C) simulated saliva ($NaHCO_3$, 4.6 g L^{-1}; $NaH_2PO_4 \cdot H_2O$, 4.29 g L^{-1}; NaCl, 0.28 g L^{-1}; KCl, 0.358 g L^{-1}; $CaCl_2 \cdot 2H_2O$, 0.0176 g L^{-1}; $MgCl_2 \cdot 6H_2O$, 0.0365 g L^{-1}; $NH_2CONH_2CH_4N_2O$, 0.3 g L^{-1} in distilled water [24]) and 10 mL of filtered rumen fluid (39 °C) were sequentially added to a capped 150 mL Luer lock syringe (5 replicates per treatment) and lightly lubricated plungers were inserted to provide expandable gas collection capacity. Headspace air was eliminated from the syringes, the cap was tightly secured and syringes were placed randomly in a 39 °C water bath with periodic manual mixing. After 48 h of incubation, volume of headspace gas was measured and a sample was transferred to exutainers. Syringes were submersed in a crushed ice water bath to terminate fermentation and contents were transferred into 100 mL glass beakers, partially dried at 70 °C and then fully dried for 12 h at 105 °C. Dried residues were stored at −80 °C for subsequent analysis. Procedural blanks were included to correct for unfiltered digestible material in the rumen fluid.

OMD (%) was calculated as: ([g of OM in initial sample − g of OM in residue dry matter] ÷ [g of OM in initial sample] × 100%).

Table 3
Protein solubility (PS) and dilute pepsin digestibility (DPD) of whole algal biomass (WAB) and lipid-extracted biomass (LEB) (n = 5)[a].

	C. vulgaris	*M. reisseri*	*N. bacillaris*	*Tetracystis* sp.
WAB				
PS (%)	83.7 ± 3.4[a]	77.6 ± 4.5[ab]	64.0 ± 3.3[c]	73.0 ± 4.5[b]
DPD (%)	80.0 ± 2.9[a]	72.3 ± 3.0[b]	64.3 ± 1.7[c]	60.3 ± 1.7[c]
LEB				
PS (%)	75.9 ± 2.2[A]	71.9 ± 3.2[A]	62.2 ± 1.9[B]	59.7 ± 2.3[B]
DPD (%)	69.4 ± 2.6[A]	69.2 ± 2.8[A]	57.9 ± 2.9[B]	62.2 ± 2.2[B]
Within microalgae species comparisons[b] (P-value)				
PS	0.002	0.049	0.334	<0.001
DPD	<0.001	0.128	0.003	0.165

[a] Values within the same row having different superscript letters are significantly different ($P < 0.05$).
[b] Indicates the significance levels within the same microalgae species (e.g., WAB vs LEB).

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Table 4
Two-phase gastric/pancreatic digestibility (GPD) of whole algal biomass (WAB) and lipid-extracted biomass (LEB) (n = 5).[a]

	C. vulgaris	*M. reisseri*	*N. bacillaris*	*Tetracystis* sp.
WAB				
$GPD_{Protein}$ (%)	69.0 ± 2.4^{b}	77.8 ± 2.0^{a}	49.3 ± 1.8^{c}	52.4 ± 1.5^{c}
GPD_{Energy} (%)	52.2 ± 0.2^{b}	57.1 ± 1.0^{a}	43.0 ± 0.3^{c}	41.4 ± 0.5^{c}
DP[b] (%)	10.2 ± 0.35^{b}	11.5 ± 0.29^{c}	7.3 ± 0.26^{a}	7.7 ± 0.22^{a}
DE[c] (MJ kg^{-1})	14.0 ± 0.06^{b}	15.0 ± 0.27^{c}	12.0 ± 0.10^{a}	11.7 ± 0.14^{a}
LEB				
$GPD_{Protein}$ (%)	79.0 ± 1.8^{A}	79.8 ± 2.9^{A}	49.9 ± 1.8^{C}	55.2 ± 2.5^{B}
GPD_{Energy} (%)	68.9 ± 1.0^{A}	61.1 ± 1.0^{B}	44.8 ± 0.4^{D}	48.3 ± 1.0^{C}
DP (%)	14.9 ± 0.34^{C}	14.5 ± 0.52^{C}	11.6 ± 0.43^{A}	13.4 ± 0.61^{B}
DE (MJ kg^{-1})	16.4 ± 0.24^{D}	15.0 ± 0.24^{C}	9.3 ± 0.09^{A}	10.2 ± 0.22^{B}
Within microalgae species comparisons[d] (P-value)				
$GPD_{Protein}$	<0.001	0.235	0.621	0.066
GPD_{Energy}	<0.001	0.008	0.006	<0.001
DP	<0.001	<0.001	<0.001	<0.001
DE	<0.001	0.817	<0.001	<0.001

[a] Values within the same row having different superscript letters are significantly different ($P < 0.05$).
[b] Digestible protein
[c] Digestible energy
[d] Indicates the significance levels within the same microalgae species (e.g., WAB vs LEB).

2.3. Analytical techniques

Proximate composition and caloric content of WAB and LEB used for *in vitro* digestion studies is described in Tibbetts et al. [9]. Methane content of headspace gas samples obtained from *in vitro* ruminal fermentation studies was determined according to Burton et al. [25] using gas chromatography (model Star 3800, Varian, Mississauga, ON) equipped with TCD, ECD and FID detectors in series and a Combi-PAL autosampler. Briefly, a 0.5 mL sample was injected into a Haysep N 80/100 mesh (0.32 cm diameter × 50 cm length) pre-column followed by a Porapak QS 80/100 mesh (0.32 cm diameter × 200 cm length) column with pure helium carrier gas at 20 psi maintained at 70 °C and the FID was operated at 250 °C. Operational conditions and data handling employed Varian Star software. In each analytical run of 147 samples, a single replicate of three concentrations of standard gas was included for quality control.

aME (MJ kg^{-1}) was calculated as: (MJ kg^{-1} in initial sample − MJ kg^{-1} in residue dry matter − MJ kg^{-1} enteric gas) ÷ (g forage dry matter fed).

2.4. Statistical methods

Data are reported as mean ± standard deviation. Statistical analyses were performed using one-way analysis of variance, ANOVA (SigmaStat® v.3.5) with a 5% level of probability ($P < 0.05$) selected in advance to sufficiently demonstrate a statistically significant difference. Where significant differences were observed, treatment means were differentiated using pairwise comparisons using the Tukey test. Correlations between response variables were calculated by Pearson correlation analysis (r) using SigmaStat® v.3.5. Raw data was checked for normality and equal variance using the Kolmogorov–Smirnov test (SigmaStat® v.3.5).

3. Results

3.1. Monogastric in vitro digestion

PS and DPD of WAB and LEB are shown in Table 3. With regard to WAB, PS was highest and statistically the same ($P = 0.109$) for *C. vulgaris* (84%) and *M. reisseri* (78%), significantly lowest ($P < 0.001$)

Table 5
Organic matter digestibility (% OMD) of diets containing varying levels of whole algal biomass (WAB) and lipid-extracted biomass (LEB) (n = 5).[a]

	Dietary inclusion level (% of forage replacement)			
	Low (25%)	Medium (50%)	High (100%)	Pooled data
WAB				
Control diet	44.8 ± 7.1^{ns}	44.8 ± 7.1^{ns}	44.8 ± 7.1^{b}	44.8 ± 7.1^{b}
C. vulgaris	43.6 ± 6.5	33.2 ± 9.1	29.8 ± 6.1^{a}	35.5 ± 3.3^{ab}
M. reisseri	34.0 ± 6.3	31.3 ± 4.5	29.4 ± 9.1^{a}	31.2 ± 5.5^{a}
N. bacillaris	35.8 ± 4.7	36.0 ± 8.8	22.2 ± 7.5^{a}	31.3 ± 5.7^{a}
Tetracystis sp.	38.7 ± 7.5	29.8 ± 8.3	24.0 ± 5.4^{a}	30.8 ± 6.1^{a}
LEB				
Control diet	44.8 ± 7.1^{NS}	44.8 ± 7.1^{B}	44.8 ± 7.1^{C}	44.8 ± 7.1^{B}
C. vulgaris	34.0 ± 9.2	37.4 ± 3.3^{AB}	31.4 ± 8.0^{AB}	34.3 ± 5.2^{AB}
M. reisseri	34.0 ± 6.7	44.6 ± 9.8^{B}	41.5 ± 5.6^{BC}	40.0 ± 6.8^{AB}
N. bacillaris	44.7 ± 7.1	30.7 ± 9.4^{AB}	26.7 ± 4.8^{A}	34.1 ± 5.1^{AB}
Tetracystis sp.	33.0 ± 4.9	27.6 ± 8.3^{A}	33.7 ± 5.6^{AB}	31.4 ± 4.4^{A}
Within microalgae species comparisons[b] (P-value)				
C. vulgaris	0.092	0.358	0.733	0.661
M. reisseri	1.000	0.075	0.035	0.053
N. bacillaris	0.047	0.391	0.298	0.448
Tetracystis sp.	0.191	0.686	0.024	0.862

[a] Values within the same column having different superscript letters are significantly different ($P < 0.05$).
[b] Indicates the significance levels within the same microalgae species (e.g., WAB vs LEB).

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Table 6
Apparent metabolizable energy (MJ kg^{-1} aME) content of diets containing varying levels of whole algal biomass (WAB) and lipid-extracted biomass (LEB) (n = 5)[a].

	Dietary inclusion level (% of forage replacement)			
	Low (25%)	Medium (50%)	High (100%)	Pooled data
WAB				
Control diet	3.7 ± 0.4^{ns}	3.7 ± 0.4^{ns}	3.7 ± 0.4^{ns}	3.7 ± 0.4^{ns}
C. vulgaris	4.1 ± 0.9	3.0 ± 0.8	3.1 ± 1.1	3.4 ± 0.6
M. reisseri	3.1 ± 0.3	2.9 ± 0.4	3.0 ± 0.9	3.0 ± 0.4
N. bacillaris	3.3 ± 0.8	3.4 ± 0.8	2.3 ± 0.8	3.0 ± 0.7
Tetracystis sp.	3.7 ± 0.8	2.8 ± 0.6	2.5 ± 0.5	3.1 ± 0.5
LEB				
Control diet	3.7 ± 0.4^{AB}	3.7 ± 0.4^{NS}	3.7 ± 0.4^{NS}	3.7 ± 0.4^{NS}
C. vulgaris	3.1 ± 0.8^{A}	2.7 ± 1.4	2.5 ± 0.8	3.1 ± 0.6
M. reisseri	4.4 ± 1.0^{AB}	3.4 ± 0.8	3.6 ± 0.6	3.8 ± 0.7
N. bacillaris	4.5 ± 0.8^{B}	2.6 ± 1.2	3.0 ± 1.0	3.4 ± 0.6
Tetracystis sp.	3.0 ± 0.3^{A}	2.7 ± 1.1	3.1 ± 1.3	3.2 ± 0.5
Within microalgae species comparisons[b] (P-value)				
C. vulgaris	0.119	0.651	0.355	0.432
M. reisseri	0.027	0.232	0.232	0.063
N. bacillaris	0.046	0.281	0.247	0.383
Tetracystis sp.	0.092	0.890	0.431	0.589

[a] Values within the same column having different superscript letters are significantly different ($P < 0.05$).
[b] Indicates the significance levels within the same microalgae species (e.g., WAB vs LEB).

for *N. bacillaris* (64%) and intermediate for *Tetracystis* sp. (73%). DPD was highest ($P < 0.001$) for *C. vulgaris* (80%) and lowest ($P < 0.001$) for *N. bacillaris* and *Tetracystis* sp. (60–64%), which were statistically the same ($P = 0.075$) and *M. reisseri* was intermediate (72%). With regards to LEB, PS was highest ($P < 0.001$) and statistically the same ($P = 0.088$) for *C. vulgaris* and *M. reisseri* (72–76%); which were significantly higher ($P < 0.001$) than *N. bacillaris* and *Tetracystis* sp. (60–62%), which were statistically the same ($P = 0.405$). Similarly, DPD was highest ($P < 0.001$) and statistically the same ($P = 1.000$) for *C. vulgaris* and *M. reisseri* (69%); which were significantly higher ($P < 0.001$) than *N. bacillaris* and *Tetracystis* sp. (58–62%), which were statistically the same ($P = 0.083$). Two-phase GPD and estimated digestible protein (DP) and digestible energy (DE) content of WAB and LEB are shown in Table 4. For WAB, $GPD_{Protein}$ and GPD_{Energy} were statistically highest ($P < 0.001$) for *M. reisseri* (78 and 57%, respectively), lowest ($P < 0.001$) for *N. bacillaris* and *Tetracystis* sp. (49–52 and 41–43%, respectively), which were statistically the same ($P = 0.101$ and 0.058, respectively) and *C. vulgaris* was intermediate (69 and 52%, respectively). As a result, DP and DE levels were highest for *M. reisseri* (11% and 15 MJ kg^{-1}), lowest for *N. bacillaris* and *Tetracystis* sp. (7–8% and 12 MJ kg^{-1}), which were statistically the same ($P = 0.278$ and 0.155, respectively) and *C. vulgaris* was intermediate (10% and 14 MJ kg^{-1}). For LEB, $GPD_{Protein}$ was highest ($P < 0.001$) and statistically the same ($P = 0.944$) for *M. reisseri* and *C. vulgaris* (79–80%), lowest ($P < 0.001$) for *N. bacillaris* (50%) and intermediate for *Tetracystis* sp. (55%). Two-phase GPD of energy was statistically highest ($P < 0.001$) for *C. vulgaris* (69%), followed by *M. reisseri* (61%), *Tetracystis* sp. (48%) and lowest ($P = 0.006$) for *N. bacillaris* (45%). DP levels were highest and statistically the same ($P = 0.651$) for *C. vulgaris* and *M. reisseri* (14–15%), lowest for *N. bacillaris* (12%) and intermediate for *Tetracystis* sp. (13%). DE levels were highest for *C. vulgaris* (16 MJ kg^{-1}), followed by *M. reisseri* (15 MJ kg^{-1}), *Tetracystis* sp. (10 MJ kg^{-1}) and lowest for *N. bacillaris* (9 MJ kg^{-1}). PS data correlated reasonably well with monogastric DPD ($r = 0.73$; $P < 0.001$) and $GPD_{Protein}$ ($r = 0.64$; $P < 0.001$) while DPD correlated well with $GPD_{Protein}$ ($r = 0.67$; $P < 0.001$) but only weakly with GPD_{Energy} ($r = 0.53$; $P = 0.008$), which were highly correlated to each other ($r = 0.91$; $P < 0.001$).

Table 7
Methane production (mol^{-10}) from 48 h *in vitro* fermentation of diets containing varying levels of whole algal biomass (WAB) and lipid-extracted biomass (LEB) (n = 5)[a].

	Dietary inclusion level (% of forage replacement)			
	Low (25%)	Medium (50%)	High (100%)	Pooled data
WAB				
Control diet	2.9 ± 0.7^{ns}	2.9 ± 0.7^{ns}	2.9 ± 0.7^{ns}	2.9 ± 0.7^{ns}
C. vulgaris	3.1 ± 0.4	3.0 ± 0.5	3.2 ± 0.3	3.1 ± 0.4
M. reisseri	3.3 ± 0.3	2.8 ± 0.7	3.1 ± 0.4	3.1 ± 0.5
N. bacillaris	3.2 ± 0.4	3.1 ± 0.2	3.0 ± 0.3	3.1 ± 0.3
Tetracystis sp.	3.3 ± 0.3	3.2 ± 0.3	3.1 ± 0.4	3.2 ± 0.3
LEB				
Control diet	2.9 ± 0.7^{A}	2.9 ± 0.7^{A}	2.9 ± 0.7^{A}	2.9 ± 0.7^{A}
C. vulgaris	1.1 ± 0.2^{B}	1.0 ± 0.2^{B}	1.2 ± 0.1^{B}	1.1 ± 0.2^{B}
M. reisseri	1.1 ± 0.1^{B}	1.0 ± 0.1^{B}	1.1 ± 0.3^{B}	1.1 ± 0.2^{B}
N. bacillaris	1.1 ± 0.2^{B}	1.0 ± 0.3^{B}	1.1 ± 0.2^{B}	1.1 ± 0.2^{B}
Tetracystis sp.	1.0 ± 0.1^{B}	0.9 ± 0.5^{B}	1.1 ± 0.1^{B}	1.0 ± 0.3^{B}
Within microalgae species comparisons[b] (P-value)				
C. vulgaris	<0.001	<0.001	<0.001	<0.001
M. reisseri	<0.001	<0.001	<0.001	<0.001
N. bacillaris	<0.001	<0.001	<0.001	<0.001
Tetracystis sp.	<0.001	<0.001	<0.001	<0.001

[a] Values within the same column having different superscript letters are significantly different ($P < 0.05$).
[b] Indicates the significance levels within the same microalgae species (e.g., WAB vs LEB).

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3.2. Ruminant in vitro digestion

OMD of diets supplemented with varying levels of WAB and LEB is shown in Table 5. OMD of the control diet was generally low (44.8%) compared with that expected *in vivo* [40]. Supplementation with both WAB and LEB decreased average OMD to 32 and 35%, respectively. The greatest reduction in OMD by WAB occurred at the 100% forage replacement level. At the 25% replacement level, neither WAB nor LEB significantly affected ($P = 0.078$) OMD averaging 38% (WAB) and 36% (LEB). The same was generally true at the 50% replacement level with average OMD of 33% (WAB) and 38% (LEB), with exception of *Tetracystis* sp. LEB (28%); which was significantly decreased ($P = 0.007$) compared to the control diet. At the 100% replacement level, OMD significantly decreased ($P < 0.001$) in nearly all cases with average OMD of 26% (WAB) and 31% (LEB), with exception of *M. reisseri* LEB (41%); which was statistically the same ($P = 0.921$) as the control diet. Overall, supplementation with WAB produced from *M. reisseri*, *N. bacillaris* and *Tetracystis* sp. significantly decreased ($P = 0.004$) dietary OMD to an average of 31% (range 30.8–31.3%), while that of *C. vulgaris* WAB (36%) was statistical similar ($P = 0.112$) to the control diet. On the other hand, supplementation with LEB produced from *C. vulgaris*, *M. reisseri* and *N. bacillaris* did not significantly affect dietary OMD ($P = 0.646$) with an average of 36% (range 34.1–40.0%); while supplementation with *Tetracystis* sp. LEB significantly decreased ($P = 0.013$) dietary OMD to 31%. aME content of diets supplemented with varying levels of WAB and LEB is shown in Table 6. Overall, aME content of the control diet, which was 3.7 MJ kg^{-1}, was high for a standard dairy cattle diet [40] and supplementation with algal biomass had no significant effect ($P = 0.447$) on diet aME content. At the 25% forage replacement level, algal biomass did not significantly affect ($P = 0.662$) dietary aME content with average aME values of 3.5 MJ kg^{-1} (WAB) and 3.7 MJ kg^{-1} (LEB). The same was true at the 50% ($P = 0.338$) and 100% ($P = 0.160$) forage replacement levels with average aME values of 2.9 MJ kg^{-1} for both WAB and LEB. However, there was a general decreasing trend with increasing dietary levels of algal biomass with the exception of diets supplemented with *M. reisseri* LEB that had an average aME content of 3.8 MJ kg^{-1} (range 3.4–4.4 MJ kg^{-1}), similar to the control diet at 3.7 MJ kg^{-1}. Enteric production levels of methane from diets supplemented with varying levels of WAB and LEB are shown in Table 7. Feeding WAB did not significantly affect production of methane ($P \geq 0.429$) compared with the control diet, which was 2.9 mol^{-10}, at all forage replacement levels; averaging 3.1 mol^{-10} (range, 2.8–3.3 mol^{-10}). Conversely, feeding LEB of all microalgae species and forage replacement levels significantly ($P < 0.001$) reduced methane production to an average of 1.1 mol^{-10} (range, 0.9–1.2 mol^{-10}).

4. Discussion

4.1. Monogastric animals

Nutritional data of microalgae fed to most monogastric animals is inconclusive. As a general rule, it appears that the upper dietary inclusion limit of algae co-products in feeds for most commercially-relevant monogastric animals such as rodents and farmed poultry, swine and fish is ~7–15% of the diet. The limitations appears mostly related to low digestibility associated with rigid cells walls of some microalgae and reduced feed intake associated with poor palatability of diets supplemented with microalgae [1,26]; both of which may be overcome through advanced feed processing and rational diet formulation. Protein digestibility determined *in vitro* with variable digestive enzyme cocktails, assays conditions and sample preparation methods has been highly variable; ranging from 27 to 97% [14]. The present study examined *in vitro* protein digestion using three assays with increasing sophistication from a simple chemical-only digestion assay to one consisting of chemical, gastric and pancreatic digestions. A comparison of the general conclusions from each *in vitro* assay used should be useful because, as previously mentioned, so much of the previous literature is inconclusive as a result of the *in vitro* assay conditions being so highly variable between studies. Having results from a wider range of commonly used *in vitro* assays should lead us to draw stronger conclusions about these particular microalgal samples based upon the complexity of the assay and also assess the correlation, or lack-thereof, between the results obtained from the various assays. Protein solubility in KOH is a test developed by Araba and Dale [16] for rapidly screening the protein quality of soybean meal (e.g., thermal damage, disulfide bonding, presence of anti-nutrients) by exposing it briefly to high pH (12–13). The test is based on the principle that, although proteins are comprised of chains of covalently linked amino acids, their three-dimensional structure (which dictates their function) is only weakly held together by hydrogen bonds; which can be easily broken [17]. However, if test ingredient proteins are of poor nutritional quality as a result of thermal damage or protein binding, the degree to which these hydrogen bonds are broken is reduced in a manner proportional to their solubility. This property is generally consistent for many other types of protein sources used in animals feeds and can be applied to microalgae as well. Pioneering work in this area by Chronakis [27] with *Spirulina* showed that the three-dimensional structure and subsequent functions of algal proteins are also highly regulated by strong hydrophobic protein–protein bonds, disulfide bonds and weak hydrogen bonds. As such, the influence of brief exposures of these proteins to high pH should also serve as a measure of their gelation behavior and solubility. Thus, PS can provide a quick and simple initial indication of protein quality and potential for nutritional value for those working on algal nutritional evaluation in the lab or under an industrial setting, if they are proven to be predictive of true potential for digestion. With regard to PS, some differences might be expected between various conventional plant proteins and algal proteins based on the relative hydrophobicity of their amino acids. Kumar et al. [28] reported that non-polar (hydrophobic) amino acids make up 35% of total amino acids in soy proteins while Borgen [29] found that hydrophobic amino acids are higher (44%) for *Cladophora* sp. algal proteins. As such, it is possible that solubility of algal protein, and its subsequent digestibility, may inherently be lower than other ingredients under similar levels of processing and under the same assay conditions. PS was highest for *C. vulgaris* WAB at 84%, moderate for *C. vulgaris* LEB at 76%, *M. reisseri* WAB and LEB at 72–78% and *Tetracystis* sp. WAB at 73% and was low for *N. bacillaris* WAB and LEB at 62–64% and *Tetracystis* sp. LEB at 60%. Interestingly, PS for LEB which averaged 72% was consistently lower than WAB which averaged 75%. This was surprising since it is well known that mild heating, such as that used in the present defatting process, generally increases protein digestibility of feed ingredients [30]. In fact, we previously demonstrated this effect with similar microalgal biomass that was defatted in the same manner and processing temperature of 150 °C [14]. In that study; IVD of WAB using pancreatic digestive enzymes was either the same or higher in LEB and this was likely related to minor protein damage associated with thermal processing. Mild heating of native proteins causes disruption of secondary and tertiary structures, which allows proteins to unfold, permitting for more efficient enzymatic digestion. Although the amino acid profiles of microalgal proteins are generally high relative to other common plant proteins [9,14,31], the functional properties of algal proteins are poorly understood. Chronakis [27] demonstrated that *Spirulina* proteins are highly viscous at acidic pH but become less viscous and more highly soluble at higher pH levels and this could affect the results from the various *in vitro* protein digestion assays used. Unlike the aforementioned study, the PS assay used in this study does not involve an enzymatic digestion phase and is strictly a chemical digestion (KOH) assay, so this may be the reason for the conflicting result. However, the PS results were also reflected in the DPD results where digestibility was also lower for LEB at an average of 65% than for WAB at an average of 69%. Since the DPD assay involves both chemical digestion (HCl) and gastric enzymatic (<pH 2) digestion but not pancreatic enzyme (pH 8) digestion like the

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study of Tibbetts et al. [14], it may be that it is the alkaline pancreatic enzyme digestion phase that is the culprit. Indeed, the results from GPD assays in this study confirm this hypothesis, where $GPD_{Protein}$ of LEB which averaged 66% was higher than WAB which averaged 62%, which seems more logical and consistent with expected *in vivo* digestion. DPD was high for *C. vulgaris* WAB at 80%, moderate for *M. reisseri* WAB at 72% and low for all other samples at 58–69%. At the present time, we are not aware of any literature data on the *in vitro* digestibility of *M. reisseri*, *N. bacillaris* or *Tetracystis* sp. or products produced from them. This paucity of knowledge for most of the species examined in the present study limits our comparisons to *in vitro* results with commercially-established species like *C. vulgaris* and certain other similar algae species. In this regard, it is encouraging that the *in vitro* DPD value of 80 ± 2.9% observed for *C. vulgaris* WAB in this study is consistent with those of 80.4–81.2% reported for *C. vulgaris* WAB by Komaki et al. [19]; demonstrating the standardization and consistency with this particular *in vitro* assay. GPD of protein was high for *M. reisseri* LEB at 80%, moderate for *M. reisseri* WAB and *C. vulgaris* LEB both at 79% and low for all other samples at 49–69%. Since native proteins tend to fold into globular masses; reducing their surface area and generally making them hydrophobic, they essentially protect themselves from interaction with solvents [32]. It seems that algal proteins defatted in the manner used in this study and with the chloroform/methanol solvent system may be protected to some degree despite the thermal treatment and that a gastric digestion phase followed by a pancreatic digestion phase (as is the case *in vivo*) is required to better reflect the potential for monogastric algal protein digestion. While we are aware that PS is a simplistic test that does not simulate monogastric gastric and pancreatic digestion, we nevertheless determined it in order to learn how well or poor the results might correlate with these more advanced *in vitro* enzymatic digestion assays that more closely simulate gastric digestion (the DPD assay) and two-phase gastric/pancreatic digestibility (the GPD assay). The fact that PS results correlated with DPD and $GPD_{Protein}$ data suggests that the rapid and simple PS assay may be a useful tool for initial assessment of algal biomass protein quality. The major digestive protease enzyme in the stomach of most farmed monogastric animals is pepsin which is activated from its zymogen pepsinogen by gastric HCl. Together, HCl at low pH (1–2.5) and pepsin at typical monogastric stomach concentrations (0.0002%) are effective at breaking down cell walls and hydrolyzing the released proteins. As such, the *in vitro* DPD assay has been used extensively to provide a preliminary estimation of a novel feeds ingredient's potential for digestion in the rest of the digestive tract. In fact, it is the DPD assay that has been used previously to assess the protein quality of *C. vulgaris* biomass [19] and is most commonly used for product labeling on nutritional supplements produced from *Spirulina* and *Chlorella*. It is highly encouraging that DPD results correlated well with $GPD_{Protein}$ but not overly surprising that the correlation was rather weak with GPD_{Energy}, although $GPD_{Protein}$ and GPD_{Energy} were highly correlated to each other. To our knowledge, this is the first study to report the *in vitro* energy digestibility of algal biomass using monogastric-derived digestive enzymes under controlled assay conditions that simulate both gastric-phase and pancreatic-phase digestion [20] and the results are highly encouraging. Using analyzed gross energy content data and the results from the GPD_{Energy} assay, the calculated *in vitro* DE value of 15 MJ kg^{-1} (range 14.0–16.4 MJ kg^{-1}) we have reported for *C. vulgaris* is consistent with DE value of 15 MJ kg^{-1} (range 14.7–15.1 MJ kg^{-1}) reported for *C. vulgaris* measured directly through *in vivo* digestibility studies with laboratory rats housed in metabolism chambers and fed experimental diets [19].

4.2. Ruminant animals

Interest in the effects of dietary algal biomass supplementation on OMD and aME of ruminant diets is related in part to its lipid content. Research *in vivo* showed no effects on feed intake or production performance of Arcott lambs when polyunsaturated fatty acids (PUFA) in the form of 0–3% WAB produced from *Schizochytrium* spp. replaced flaxseed oil and barley grain, across all inclusion levels [33]. Dib [34] fed isonitrogenous diets containing 0–20% *Chlorella* spp. LEB, replacing soybean and rice meals, to male crossbred goats in a 28 day feeding trial and reported no effects on feed intake, diet digestibility, blood chemistry, organ weights, growth and carcass characteristics and fatty acid profile of muscle, however, broad effects on mineral metabolism were noted. Intake and excretion of key macrominerals (e.g., Ca, Mg, K and Na) and trace elements (Cu, Fe, Zn, Mn, Mo and Co) were altered. Drewery et al. [35] observed that Angus steers fed low-quality forage (oat straw) and isonitrogenous levels of conventional cottonseed meal or *Chlorella* spp. LEB ruminally-infused over the range of 0–150 mg N kg body $weight^{-1}$ increased OMD and N balance at 50–100 mg N kg body $weight^{-1}$. These results could be related to impacts on rumen microflora. McCann et al. [36] examined the effects of *Chlorella* spp. LEB on the ruminal microbiome. The beneficial effects of LEB on dietary forage utilization were a result of stimulation in the relative abundance of the Bacteroidetes and Firmicutes phyla. Changes in rumen microbiome with dietary microalgae supplementation could have practical environmental benefits. Certain lipid-rich biomass has been shown to reduce enteric methane emissions from ruminants by inhibiting the activity of ruminal methanogens [37–42]. This may help to substantially reduce a major source of agricultural greenhouse gas emissions [43]. Others have shown that long-chain PUFA found in marine organisms and WAB inhibit methanogenesis by 30 to 80% with no significant reduction in diet digestibility [39,44,45]. Interest in the algal effects on methane production has largely been focussed either upon biogas production [46] or the reduction of enteric greenhouse gas emissions [47]. In the present study, mitigation of enteric methane was evaluated. The observed suppression of methane production by algal biomass was however, not entirely related to lipid content as observed previously [47]. The LEB, which contained 6 to 32% esterifiable lipid, reduced methane production by over 60% compared with WAB, containing 32 to 36% esterifiable lipid, which did not suppress methane production relative to the control diet. Tartakovsky et al. [46] observed that *Scenedesmus* sp. AMDD, in the presence of food waste sludge, promoted growth of sulfate-reducing bacteria which correlated with higher hydrogen sulfide and lower methane production in continuous flow anaerobic bioreactors. Based on these findings; WAB, with its higher lipid content, was expected to depress methane emissions to a greater degree than LEB; however, the opposite was observed. The WAB did not suppress methane emissions to any appreciable extent and LEB caused a substantial reduction, which was accompanied by reduced digestibility, but that was not much different from that caused by WAB. Lodge-Ivey et al. [48] used continuous flow artificial rumen fermentation systems to assess the effect of complete replacement of soybean meal (7–15% of the diet) in forage and concentrate-based diets with *Chlorella* spp. LEB (3 products) and *N. salina* LEB (3 products) on IVD, rumen fermentation and N metabolism. They found variable results both between algae species and within algal products of the same species. Similar to the present study, LEB either had no effects or slightly increased digestibility depending upon diet and type of algal product used. *Chlorella* spp. LEB increased microbial fermentation efficiency in some cases (particularly in forage-based diets) but depressed it in others, while *N. salina* LEB consistently reduced microbial efficiency. The authors concluded that LEB is a potential protein supplement for ruminants.

Based on our *in vitro* evaluation of the effects of freshwater algal species, WAB and especially LEB are suitable as feed supplements for ruminants. In the present study, diet OMD was not significantly affected with up to 50% forage replacement (equivalent to 38–43% of the diet) with all WAB at an average of 35%; however, 100% forage replacement (equivalent to 77–85% of the diet) significantly reduced OMD to an average of 26%. Similarly, OMD of the control diet was 45% and not significantly affected with up to 50% forage replacement (equivalent to 25–32% of the diet) with LEB at an average of 37%, with the exception of *Tetracystis* sp.

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which was only 28%. Again, 100% forage replacement with most LEB (equivalent to 51–65% of the diet) significantly reduced OMD to an average of 31% but this was not the case for *M. reisseri*; having and OMD of 41% which was similar to that of the control diet. As a feed for ruminants, some algae compare favorably with conventional plant-based feedstuffs. In a recent study, an unspecified algal meal was shown to be more digestible than soy hulls and hay during 24 h incubations, but was similar at 48 h [49]. The meal was subsequently fed to growing steers without performance effects up to 45% of the diet, suggesting that the algae meal can provide a valuable feedstuff for ruminant animals. The aME content of the control diet was 3.7 MJ kg^{-1} and was low compared with that of typical dairy industry forage [50] and was not significantly affected by WAB or LEB supplementation at any inclusion level at an average of 3.2 MJ kg^{-1}. However, there was a general downward trend with increasing dietary levels of algal material, with the exception of diets supplemented with *M. reisseri* that averaged 3.8 MJ kg^{-1} which is similar to the control diet. Although little similar work has been done in this area, similar trends have been observed for certain other algae species (e.g., *Selenarstrum* sp., *Scenedesmus* sp., *Thalassiosira* sp.) evaluated *in vitro* [49].

Based on our *in vitro* digestion techniques for monogastric and ruminant animals, it seems clear that *C. vulgaris* and *M. reisseri* have a generally similar and higher nutritional value than *N. bacillaris* and *Tetracystis* sp. This trend was consistent across all *in vitro* assays used and for both monogastric and ruminant animals and is likely related to a key compositional difference between these two groups of algae. While the results showed that the four microalgae species were virtually indistinguishable in terms of growth performance and daily productivity [9], moderate differences existed in their lipid characteristics, PUFA content, ratio of n-3 to n-6 fatty acids, Ca:P ratios and Fe content. A large difference in the composition of carbohydrate fractions between these two groups was also noted. Although total carbohydrate contents of the microalgae were similar at 27–30%, *C. vulgaris* and *M. reisseri* were composed of a relatively equal mix of starch and fiber, while those of *N. bacillaris* and *Tetracystis* sp. were almost entirely fiber which represented ~95% of the total carbohydrate with only a small proportion of ~5% composed of starch. Whereas starch can be a readily available and inexpensive source of DE in animal feeds, fiber generally has low digestibility in monogastric animals and the extent of fiber utilization in ruminant animals can be highly variable depending on species, fiber composition and feed processing [11–13]. Han and McCormick [51] suggested that the carbohydrate fraction of microalgae may be less rumen fermentable that those of soybean meal and alfalfa hay and low OMD observed in the present study is consistent with that observation. In animal nutrition, dietary crude fiber is generally used to describe the sum of cellulose, hemicellulose, pectin and lignin; all of which are present to varying degrees in most plant-based ingredients used in modern animal feeds [10]. On the other hand, microalgae fiber is composed almost entirely of cellulose without (or very low) levels of hemicellulose, pectin or lignin [52]. Whereas, this may seem to be an opportunity for biotechnological applications such as production of bioethanol, the lack of these fibrous components may impair the functional efficiency of rumen microbes. In addition, preliminary findings using near infrared spectroscopy technology has indicated a low similarity between microalgal cellulose and that of conventional terrestrial crop-based sources [53] which could affect its bioavailability in the rumen and digestive tract of animals, however this area requires further exploration. The *in vivo* digestibility of crude fiber in *C. vulgaris* biomass was found to be low at 37–41% in rats [19]. Not only does fiber reduce the overall algal biomass digestibility, high levels can also reduce protein digestibility by entrapping them in a cellular matrix which reduces their solubility, ultimately rendering them less available to proteolytic enzymatic hydrolysis [54]. This is consistent with our PS data, where *N. bacillaris* and *Tetracystis* sp., with high fiber levels, had consistently lower PS at an average of 65% than *C. vulgaris* and *M. reisseri* which averaged 77% with lower levels of fiber. The effect of lower PS in these two high-fiber microalgae yielded consistently lower results for all other measured variables for monogastric digestion including DPD (61 vs 73%), $GPD_{Protein}$ (52 vs 76%) and GPD_{Energy} (44 vs 60%). Not surprisingly, ruminant digestion was not influenced to the same degree, having similar values for OMD of 32 and 35%, respectively and aME contents of 3.1 and 3.2 MJ kg^{-1}, respectively since ruminant animals are more specialized fiber fermenters compared with monogastric animals [26].

4.3. Conclusions

Based on the results from *in vitro* digestion using porcine enzymes, *M. reisseri* and *C. vulgaris* demonstrated the greatest potential for partial replacement of conventional feed ingredients for monogastric animals. Estimated DP levels of WAB were: *M. reisseri* (11%) > *C. vulgaris* (10%) > *Tetracystis* sp. (8%) = *N. bacillaris* (7%), while levels for LEB were: *C. vulgaris* (15%) = *M. reisseri* (14%) > *Tetracystis* sp. (13%) > *N. bacillaris* (12%). Estimated DE levels of WAB were: *M. reisseri* (15 MJ kg^{-1}) > *C. vulgaris* (14 MJ kg^{-1}) > *Tetracystis* sp. (12 MJ kg^{-1}) = *N. bacillaris* (12 MJ kg^{-1}), while levels for LEB were: *C. vulgaris* (16 MJ kg^{-1}) > *M. reisseri* (15 MJ kg^{-1}) > *Tetracystis* sp. (10 MJ kg^{-1}) > *N. bacillaris* (9 MJ kg^{-1}). Further work is required to develop cost-effective processing technologies that effectively rupture microalgae cell walls to improve digestibility and/or for the production of algal protein concentrates (APCs) with lower levels of indigestible carbohydrates and higher protein content for monogastric animal feeds. Based on the results from batch-culture *in vitro* ruminal fermentation, *M. reisseri* showed the best potential for ruminant animals as a roughage equivalent; although the other algal species were generally well-utilized as well. The reduction in methane production caused by LEB of all algal species tested was not entirely related to algal lipid content and this finding is consistent with a recent report by Anele et al. [55]; suggesting that research is needed to identify additional anti-methanogenic substances in microalgae. Optimum dietary inclusion levels for target animal species must be determined based on palatability, cost, animal health and performance, environmental impact and product safety and quality. Lastly, production of algal biomass needs to become scaled-up to industrial levels in order to ensure a reliable supply, consistent nutrient profile and cost-competitiveness that animal feed sectors will demand.

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