Short communication

Effects of Euglena *(Euglena gracilis)* supplemented to diet (forage: concentrate ratios of 60:40) on the basic ruminal fermentation and methane emissions in *in vitro* condition

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ABSTRACT

An *in vitro* study was conducted to investigate the effect of different concentrations of Euglena (*Euglena gracilis*) on CH_4 production, dry matter (DM) digestibility, volatile fatty acid (VFA) and ammonia N(NH_3-N) concentration as well as on the protozoa population. The treatments considered were Euglena at concentrations of 0.0, 50, 100, 200, 400 and 1000 g/kg dry matter (DM) of the substrate (60:40 forage: concentrate ratio) incubated for 24 and 96 h using an *in vitro* continuous gas production and *in vitro* two-stage digestion procedure, respectively. The data were subjected to polynomial regression analysis. Methane emissions (ml/g DM) decreased at an increasing rate, generally with increasing concentration of Euglena but also exhibited quadratic ($P < 0.001$) and cubic ($P < 0.001$) effects while NH_3-N (mg/ml) concentration increased at an increasing rate (linear $P < 0.001$; quadratic $P = 0.001$; cubic $P = 0.024$). Total VFA concentration (mmol/l) decreased significantly ($P < 0.001$), when the substrate was totally replaced by Euglena. There was a linear ($P < 0.001$) and cubic ($P = 0.047$) reduction in protozoa population as the concentration of Euglena increased. *In vitro* DM digestibility was improved (linear $P = 0.003$; quadratic $P = 0.04$; cubic $P < 0.001$). These findings demonstrate that Euglena at concentration of 100 g/kg DM reduce CH_4 emissions by 9.1% and improve DM digestibility by 14.3%. However, when the concentration of Euglena increases from 100 g/kg DM, while further reducing CH_4 emissions, have negative effect on NH_3-N concentration, protozoa population and VFA concentration.

1. Introduction

The contribution of livestock production towards environmental pollution is becoming of great concern because of the emissions of greenhouse gases, such as CO_2, CH_4 and ammonia. In addition, the production of CH_4 during the enteric fermentation of feeds in the rumen is correlated with the loss of gross energy (GE) from the consumed feed (Szumacher-Stabel and Cieslak, 2012). Thus, identifying alternative solutions to this major constraint is a concern of both environmental protection and nutrient utilization. The efficiency of ruminal fermentation can be facilitated by modifying the feeding system using natural feed additives, thereby reducing the emission of greenhouse gases and enhancing the efficiency of nutrient utilization.

Microalgae are one of the most promising biological resources, as these organisms are rich sources of vitamins, minerals, proteins, polyunsaturated fatty acids, antioxidants, *etc.* (Pulz and Gross, 2004) and can be used to enhance the nutritional value of animal feed, reflecting the well-balanced chemical composition of these microphytes. The inclusion of microalgal biomass in small quantity positively affects the physiology of animals, as antibacterial action, improve gut function, feed conversion and reproductive performance have been reported (Harel and Clayton, 2004). A number of nutritional studies have demonstrated the suitability of microalgae biomass as a potential substitute for conventional protein supplements, such as soybean and fish meal (Dajana et al., 2013).

Carbon dioxide fixation through *Euglena gracilis* is effective and economical (Chae et al., 2006), thereby lowering the greenhouse effect and climate changes through the absorption of increasing CO_2 emissions in the atmosphere. Microalgae can be cultivated in areas unsuitable for other plants with several fold higher production and can effectively utilize and remove pollutants (*e.g.*, nitrogen and phosphorus) from water (Gouveia et al., 2008). Thus, Euglena due to its high content of fatty acid, protein and other biologically active compounds inclusion of this micro algae in the ration of ruminants may influence CH_4 emissions, rumen fermentation and nutrient utilization. As far as our knowledge is concerned, there is no information available on the effect of Euglena on CH_4 emissions.

Therefore, our objective was to investigate the effect of different concentrations of Euglena on *in vitro* CH_4 emissions DM digestibility, VFA concentration, protozoa population and NH_3-N concentration.

2. Materials and methods

2.1. Euglena (Euglena gracilis)

Euglena *(E. gracilis)*, powder form with 100% purity, was obtained from Euglena Co., Ltd., Japan. The chemical composition of Euglena and the substrate (grass hay and concentrate mixture) are indicated in Table 1.

2.2. Rumen fluid sampling

Two ruminally fistulated non-lactating Holstein cows (average of 600 kg BW) were used as rumen fluid donors. The cows were maintained on a daily diet of 10 kg Orchardgrass hay (organic matter (OM), 980 g/kg; crude protein (CP), 132 g/kg; neutral detergent fiber (NDF), 701 g/kg; acid detergent fiber (ADF), 354 g/kg; lignin, 40 g/kg and GE, 18.02 MJ/kg; DM basis), with free access to clean drinking water and mineral block (Fe, 1836 mg; Cu, 377 mg; Co, 66 mg; Mg, 1046 mg; Zn, 1235 mg; I, 77 mg; Se, 33 mg; Vit E, 5000 mg; and NaCl, 962 g/3 kg). The rumen fluid from the two cows was sampled prior to morning feeding using a vacuum line and strained through a woven nylon cloth into a thermos flask, pre-heated to 39 °C with hot water. In the laboratory, the samples were pooled in equal proportions and continuously flushed for one hour with CO_2. The inoculum was immediately dispensed after preparation. Animal management and sampling procedures were approved through the animal care and use committee of Obihiro University of Agriculture and Veterinary Medicine.

Table 1
Chemical composition (g/kg DM) of experimental feeds.

	Euglena	Klein grass hay	Concentrate mixture	Euglena concentrations (g/kg DM)						SEM
				0	50	100	200	400	1000	
Dry matter (g/kg)	960	956	958	957	957	957	957	958	960	0.358
Organic matter	961	908	918	912[b]	914[b]	916[b]	921[b]	929 [ab]	961[a]	0.803
Ash	34.5	84.4	76.5	81.2[a]	79.1[a]	77.0[ab]	72.8[b]	64.4[c]	34.5[d]	0.147
Crude protein	240	147	164	154[d]	158[d]	162[cd]	169[c]	185[b]	240[a]	0.301
GE (MJ/kg DM)[1]	12.8	17.1	16.7	16.9[a]	16.7[a]	16.5[ab]	16.1[ab]	15.4[b]	12.8[c]	0.029
Ether extract	138	15.9	33.6	23.0[d]	28.2[d]	33.4[cd]	48.8[c]	64.7[b]	138[a]	0.353
NDF[2]	0.0	609	232	458[a]	447[a]	435[ab]	412[b]	366[c]	0.0[d]	1.979
ADF[3]	0.0	303	78.3	213[a]	209[a]	205[a]	198[ab]	182[b]	0.0[c]	1.102

[a–d] Means within a raw with different superscripts differ ($P < 0.05$).

[1] GE = gross energy.
[2] NDF = neutral detergent fiber.
[3] ADF = acid detergent fiber.

2.3. Experimental treatments and in vitro fermentation

The experimental samples were oven-dried at 60 °C for 48 h and stored under dry and cool conditions in sealed containers prior to use. Six treatments were prepared containing different concentrations of Euglena, Klein grass (*Panicum coloratum*) hay and concentrate mixture. The following treatment were evaluated: 6 g of Klein grass hay + 4 g concentrate (Control, T1); 6 g of Klein grass hay + 3.5 g of concentrate + 0.5 g Euglena (T2); 6 g of Klein grass hay + 3 g of concentrate + 1 g of Euglena (T3); 6 g of Klein grass hay + 2 g of concentrate + 2 g of Euglena (T4); 6 g of Klein grass hay + 4 g of Euglena (T5) and 10 g of Euglena (T6). The effects of each treatment (10 g of DM) on CH_4 production, VFA concentration, NH_3-N concentration, pH, oxidation reduction potential (ORP) and protozoan population were tested *in vitro* for 24 h at 39 °C using a continuous gas quantification system as previously described (Sar et al., 2005). The buffer was prepared according to McDougall (1948), sterilized by autoclaving and flushed with CO_2 for 1 h prior to dispensing into fermentation vessels. Fermentation was continued for 24 h at 39°C. Rumen fluid was added to buffer at a ratio of 1:4. The gas output from each fermentation vessel was measured for 10 min at 30-min intervals. Samples of the incubation medium were collected at the end of each incubation period (24 h) and stored at −20°C for NH_3-N and VFA analysis. Then, the contents were discharged, and the fermentation vessels were thoroughly washed and autoclaved. The experiment was repeated four times on separate days, with treatments randomly assigned to the four fermentation vessels for each incubation period.

2.4. Analysis of methane and volatile fatty acids

The CH_4 production from each fermentation vessel was continuously measured using auto infrared CH_4 (EXA IR, Yokogawa Electric Corporation, Tokyo, Japan) analysers, installed in an *in vitro* continuous gas quantification system (Takasugi Seisakusho Co., Ltd., Tokyo, Japan). The components and total VFA were determined through gas chromatography (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm ID × 30 m, 3.0 μm) using 2-ethyl-*n*-butyric acid as an internal standard. The samples were prepared for analysis according to Sar et al. (2005). The pH and ORP of the fermentation media were monitored in each vessel at 1-min intervals (TS mk-250, Takasugi-ss Co., Ltd., Japan). All data were pooled and stored on a computer through an interface using the analysers.

2.5. In-vitro DM and OM digestibility

In vitro nutrient digestibility was estimated using the two-stage digestion technique according to Tilley and Terry (1963). Duplicate samples of 0.3 g of Klein grass hay + 0.2 g of concentrate (control, T1), 0.3 g of Klein grass hay + 0.175 g of concentrate + 0.025 g Euglena (T2), 0.3 g of Klein grass hay + 0.15 g of concentrate + 0.05 g of Euglena (T3), 0.3 g of Klein grass hay + 0.1 g of concentrate + 0.1 g of Euglena (T4), 0.3 g of Klein grass hay + 0.2 g of Euglena (T5) and 0.5 g of Euglena (T6) were weighed and placed into a 100-ml plastic bottle, and 40 ml of McDougall's buffer (McDougall, 1948) was added to each bottle and pre-warmed to 39 °C. Subsequently, 10 ml of strained rumen fluid was dispensed into each bottle and sealed under continuous supply of CO_2 gas. The mixture was incubated at 39°C for 48 h, with occasional careful shaking. The acid-pepsin solution was subsequently added, and the contents were incubated for another 48 h at 39 °C. Then the contents were filtered through pre-weighed Gooch crucibles, and the residual DM was determined. The loss in weight was determined as *in vitro* dry matter digestibility (IVDMD), followed by ashing the residues to estimate *in vitro* organic matter digestibility (IVOMD).

2.6. Amino acid and fatty acid composition of Euglena

Amino acid and fatty acid composition of Euglena samples were analyzed by Japan Food Research Laboratories, Japan. The amino acid composition except for tryptophan was carried out by an automated amino acid analyzer (JLC-500/V, JEOL Ltd., Japan; Column, LCR-6 with 4 mm × 120 mm ID, JEOL, Co. Ltd., Japan). Tryptophan was analyzed by high performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Co., Ltd., Japan; Column, CAPCELL PAK C18 AQ, 4.6 mm ID × 250 mm, Shiseido Co., Ltd., Japan; detector, Flourospectro photometer (RF-20Axs, Shimadzu, Co. Ltd., Japan). Mobile phase consisted of perchloric acid and methanol (80:20). The flow rate was 0.7 ml/min and the fluorescence excitation was at 285 nm and 40°C.

Fatty acid composition of Euglena was determined by Gas chromatography, GC-1700, Shimadzu Co., Ltd., Japan equipped with FID. The fatty acids were separated on 30 m × 0.25 mm ID, DB-23 capillary column. Helium was used as a carrier gas at a flow-rate of 1.5 ml/min with split less injection at 250 °C and the detector temperature was 250 °C.

2.7. Chemical analysis

Samples of Euglena, Klein grass hay and concentrate were analysed for DM after drying at 135°C for 2 h (930.15), OM and total ash (942.05), and ether extract (EE) (920.39) according to the procedures of the Association of Official Analytical Chemists (AOAC, 1995). Nitrogen was determined through the Kjeldahl method (984.13) (AOAC, 1995) using an electrical heating digester (FOSS Tecator™ Digestor, Tokyo, Japan) and an automatic distillation apparatus (FOSS Kjeltec™ 2100, Tokyo, Japan), and CP was determined as N × 6.25. The NDF and ADF content were determined according to the method of Van Soest et al. (1991). Both NDF and ADF were estimated without amylase and expressed inclusive of residual ash. The

Table 2
Fatty acid and amino acid profile of Euglena.

	Fatty acid content																					
	C10:0	C12:0	C13:0	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2n-6	C18:3n-3	C20:2n-6	C20:3n-3	C20:3n-6	C20:4n-3	C20:4n-6	C20:5n-3	C22:4n-6	C22:5n-3	C22:5n-6
g/100 g Fatty acid	0.3	5	8.3	35.4	2.9	10.2	2.9	0.6	1.5	1.8	5.4	2	1.1	2.3	0.2	3.8	1	4.5	0.8	2.6	0.2	1.2
g/100 g Euglena	0.04	0.69	1.14	4.88	0.40	1.41	0.40	0.08	0.21	0.25	0.74	0.28	0.15	0.32	0.03	0.52	0.14	0.62	0.11	0.36	0.03	0.17

	Amino acid content[a]																		
	Arg	Lys	His	Phe	Tyr	Leu	Ile	Met	Val	Ala	Gly	Pro	Glu	Ser	Thr	Asp	Trp	Cys
g/100 g Euglena	1.53	1.59	0.63	1.06	0.8	1.92	0.93	0.49	1.51	1.79	1.18	1.43	2.66	0.98	1.14	1.95	0.4	0.36
g/100 g Amino acid	6.85	7.11	2.82	4.74	3.58	8.59	4.16	2.19	6.76	8.01	5.28	6.40	11.9	4.38	5.10	8.72	1.79	1.61

[a] Arg = Arginine; Lys = Lysine; His = Histidine; Phe = Phenylalanine; Tyr = Tyrosine; Leu = Leucine; Ile = Isoleucine; Met = Methionie; Val = Valine; Ala = Alanin; Gly = Glycine; Pro = Proline; Glu = Glutamic acid; Ser = Serine; Thr = Threonine; Asp = Aspartic acid; Trp = Triptophan; Cys = Cysteine.

GE content of the samples was analysed using a Shimadzu auto-calculating bomb calorimeter (CA-4AJ, Shimadzu Co. Ltd., Japan). The NH_3-N concentrations were analysed according to Conway and O'Malley (1942).

2.8. Statistical analysis

The data were analyzed using REG procedure of SAS (2010). The treatments with different concentration of Euglena were included as a fixed effect and the fermentation vessels/bottles as random effects in the model. *In vitro* digestibility was completed in four runs, with each sample replicated four times in a single run. The replication average within a run was considered as a statistical unit. In cases of *in vitro* gas production, each treatment was incubated four times in different runs (statistical replicates). The total effects included in the model for each variable were four replications and six treatments. Linear, quadratic and cubic contrasts of the treatment means were assessed. Differences among the means were identified using Tukey's multiple comparisons. The effects were considered significant at $P < 0.05$ and trends were discussed at $0.05 < P < 0.10$

3. Results

3.1. Chemical composition of Euglena

The chemical composition of the experimental feeds used in the present study indicated that Euglena has higher OM, CP and EE compared to grass hay and concentrate mixture (Table 1). Euglena contains all the essential amino acids (Table 2). Saturated, mono unsaturated and poly unsaturated fatty acid contents of Euglena were 64.5, 9.8 and 19.7 g/100 g of the total fatty acid respectively (Table 2). The GE content of Euglena is lower than that of the substrate but with higher digestibility.

3.2. The effects of Euglena inclusion on in vitro NH_3-N and VFA concentration and the protozoa population

Ammonia N concentration generally increased with increasing concentrations of Euglena, but also exhibited quadratic ($P = 0.001$) and cubic ($P = 0.024$) effects. Total VFA concentration decreased significantly (linear $P < 0.001$) when the substrate was totally replaced by Euglena (Table 3). Molar proportion of acetate increased (quadratic $P = 0.007$; cubic $P < 0.001$) and tended to increase linearly ($P = 0.057$), whereas the proportion of propionate decreased linearly ($P < 0.001$) and tended to decrease quadratically ($P = 0.068$), while butyrate increased (linearly $P = 0.022$; quadratic $P = 0.012$; cubic $P < 0.001$). The

Table 3
Effects of Euglena inclusion on VFA concentration, NH_3-N concentration and protozoa count after 24 h of incubation.

	Euglena concentrations (g/kg DM)						SEM	Effect		
	0	50	100	200	400	1000		Linear	Quadratic	Cubic
Volatile fatty acids (mol/100 mol)										
Acetic	62.7[bc]	63.6[b]	63.3[bc]	61.6[c]	62.8[bc]	65.8[a]	0.049	0.057	0.007	<0.001
Propionic	26.5[a]	25.2[ab]	25.3[ab]	23.6[bc]	21.9[cd]	20.2[d]	0.082	<0.001	0.068	0.893
Butyric	9.03[b]	9.36[b]	9.57[b]	11.9[a]	11.9[a]	9.61[b]	0.050	0.022	0.012	<0.001
Valeric	1.8[c]	1.87[c]	1.85[c]	2.95[b]	3.46[b]	4.4[a]	0.027	<0.001	<0.001	0.245
A:P[1]	2.36[c]	2.52[c]	2.50[c]	2.64[cb]	2.87[b]	3.25[a]	0.011	<0.001	0.002	0.113
TVFA (mmol/l)[2]	41.5[a]	35.2[a]	40.1[a]	30.4[ab]	30.6[ab]	19.4[b]	0.814	<0.001	0.171	0.392
Protozoa(cell/ml × 10^6)	2.50[a]	2.13[ab]	1.88[b]	1.88[b]	1.75[bc]	1.38[c]	0.013	<0.001	0.637	0.047
NH_3-N (mg/ml)[3]	15.9[c]	17.0[c]	17.5[c]	33.3[b]	60.3[a]	66.6[a]	0.428	<0.001	0.001	0.024
pH	7.09[b]	7.04[b]	7.18[b]	7.13[b]	7.39[ab]	7.81[a]	0.017	0.001	0.009	0.361
ORP (mV)[4]	−421[a]	−427[ab]	−430[ab]	−442[bc]	−456[c]	−480[d]	0.658	<0.001	0.003	0.430

[a–d] means within a raw with different superscripts differ($P < 0.05$).

[1] A: P = acetate to propionate ratio.
[2] TVFA = total volatile fatty acid.
[3] NH_3-N = ammonia N.
[4] ORP = oxidation reduction potential.

Table 4

Effects of Euglena inclusion on *in vitro* CH_4 emission, DM and OM digestibility.

	Euglena concentrations (g/kg DM)						SEM	Effect		
	0	50	100	200	400	1000		Linear	Quadratic	Cubic
Methane emission										
ml 24 h^{-1}	166[a]	150[b]	150[b]	150[b]	132[c]	85.5[d]	0.340	<0.001	<0.001	<0.001
ml/g DM	17.3[a]	15.7[b]	15.7[b]	15.7[b]	13.8[c]	8.91[d]	0.033	<0.001	<0.001	<0.001
ml/g Digestible DM	24.9[a]	20.9[bc]	19.6[cd]	21.7[b]	18.9[d]	9.02[e]	0.059	<0.001	0.02	<0.001
ml/g OM	18.2[a]	16.5[b]	16.4[b]	16.3[b]	14.2[c]	8.90[d]	0.035	<0.001	<0.001	<0.001
ml/g Digestible OM	29.9[a]	24.6[b]	22.8[bc]	25.2[b]	21.3[c]	9.39[d]	0.077	<0.001	0.024	<0.001
IVDMD[1]	0.70[d]	0.75[c]	0.80[b]	0.72[cd]	0.73[c]	0.99[a]	0.002	0.003	0.040	<0.001
IVOMD[2]	0.61[d]	0.67[c]	0.72[b]	0.65[cd]	0.67[c]	0.95[a]	0.002	0.001	0.022	<0.001

[a–e] Means within a raw with different superscripts differ ($P < 0.05$).

[1] IVDMD = *in vitro* dry matter digestibility.

[2] IVOMD = *in vitro* organic matter digestibility.

acetate to propionate ratio increased linearly ($P < 0.001$) and quadratically ($P = 0.002$) with increasing concentrations of Euglena. The pH increased linearly ($P = 0.001$) and quadratically ($P = 0.009$) while the ORP decreased linearly ($P < 0.001$) and quadratically ($P = 0.003$). There was a linear ($P < 0.001$) and cubic ($P = 0.047$) reduction in protozoa population as the concentrations of Euglena increased.

3.3. The effects of Euglena on in vitro CH_4 emissions, DM and OM digestibility

Methane emissions (ml/g DM) decreased at an increasing rate, generally with increasing concentration of Euglena but also exhibited quadratic ($P < 0.001$) and cubic ($P < 0.001$) effects (Table 4). *In vitro* DM digestibility was improved (linear, $P = 0.003$; quadratic $P = 0.04$; cubic $P < 0.001$) by addition of Euglena. Similar trend was followed by IVOMD.

4. Discussion

4.1. The effects of Euglena on in vitro CH_4 emission and digestibility

Methane emissions were reduced by 9–48%, when Euglena was included in a dose dependent manner. It has been reported that for every 1% addition of fat in the ration of ruminants, methanogenesis was reduced by 2.2–5.6% (Eugene et al., 2008; Beauchemin et al., 2008; Martin et al., 2010). The finding of our study confirms that addition of Euglena (100 g/kg DM) increased the fat content of the ration by 1% and reduced methane emission by 9.1%. Medium chain fatty acids (MCFA) such as lauric acid and myristic acid, identified as substances strongly reducing microorganisms participating in methanogenesis (Dohme et al., 1999). The application of 2.8% coconut oil suppressed methane release of lambs by about 25% relative to the control (Machmuller et al., 2000). Similarly the previous work by Machmuller and Kreuzer (1999) also indicated that methane production inhibition due to the addition of 3.5% coconut oil was 28% compared to the unsupplemented diet. In our study, the lauric acid (C12:0), tridecylic aicd (C13:0), myristic acid (C14:0) and palmitic acid (C16:0) constitute 59.2 g/100 g of the total fatty acid in Euglena. The presence of these fatty acids in higher proportions might be responsible for reduction of methane emission by influencing microorganisms involved in the process of methanogenesis.

The findings of our *in vitro* study indicated that the overall DM and OM digestibility was positively influenced by supplementation of Euglena. Inclusion of Euglena at the dose of 100 g/kg DM improved DM and OM digestibility by 14.3% and 18.0% respectively. However, when the levels of Euglena increased from 100 g/kg of DM, increased in digestibility was due to higher digestible nutrient content of Euglena. Dry matter digestibility of grass and concentrate mixtures, a major part of the diet, is 69% and that of Euglena is 99%. When Euglena was included at the concentrations of 50, 100, 200, 400 and 1000 g/kg of the diet, digestibility should have been 70, 72, 73, 76 and 82%, but the actual digestibility is 70, 75, 80, 72 and 73% respectively, which indicated that addition of Euglena beyond 100 g/kg of the diet depressed digestibility of the feed. Fats negatively affect degradability of DM and NDF component, which was perhaps due to decreases in numbers of rumen protozoa, bacterial population and activities of fiber degrading enzymes (Patra and Yu, 2013). In this study higher digestibility was obtained at lower levels of Euglena inclusion, which may be associated with the presence of balanced amino acid profile that improved the efficiency of dietary protein utilization by facilitating the growth of microbial population and increased efficiency of digestibility of fiber and starch. Study by McCann et al. (2014) suggested that supplementation of algal residue to steers increased forage utilization by increasing members of Firmicutes of the rumen microbiome. Other evidence shows that both essential and non essential amino acids play important role in regulating the intestinal microbiota and anti-oxidant response (Wu, 2009).

4.2. The effects of Euglena inclusion on in vitro NH_3-N concentration, VFA concentration and protozoa population

Ammonia N concentration was not influenced when Euglena was included at the doses of 50–100 g/kg DM of the substrate but when the concentrations increased above 100 g/kg DM, NH_3-N concentration increased two to four fold compared to the control. This is associated with the increased concentration of CP in the ration as the proportion of Euglena increased. The result of a previous study indicated that the presence of excess dietary protein leads to ammonia formation (Place and Mitloehner, 2010), reflecting the loss of dietary nitrogen and causing environmental pollution.

Total VFA concentration was not affected when Euglena was included up to 400 g/kg DM of the diet, but reduced significantly when the substrate was totally replaced by Euglena. This study indicated that the addition of Euglena at different levels (with fat content of 2.3–6.5% of the diet on a DM basis) did not affect total VFA concentration. Recent studies (Patra, 2013) showed that total VFA concentrations were not significantly changed with increasing concentration of fats in the diet. The proportion of propionate reduced by 11, 18 and 24% when the concentration of Euglena goes beyond 100 g/kg DM, while the proportion of acetate increased by 5% at the total substitution of the substrate with Euglena and butyric acid increased by 31–32%, when Euglena was included 200–400 g/kg DM of the substrate. In this study the proportion of propionate was not increased despite substantial inhibition of methanogenesis. Previous study by Dohme et al. (2001) indicated that there was a significant shift in the proportion of individual VFA, with significantly increased butyrate and decreased propionate. Fats are not fermented in the rumen and thus do not produce surplus hydrogen. Consequently methane production could be declined due to production of less hydrogen per unit of feed when higher levels of fats are included in the diet (Patra, 2013). The proportion of propionate was not increased, which may be due to less availability of hydrogen.

The protozoa population was also influenced by Euglena addition, showing a 14.8–44.8% reduction in a dose dependent manner. The decrease in CH_4 emission could be associated with the decrease in protozoa population influenced by the presence of higher proportion of saturated medium chain fatty acids (C12:0, C13:0, C14:0 and C16:0). Protozoans are the greatest producers of hydrogen in the rumen ecosystem (Szumacher-Strabel and Cieslak, 2012). Previous studies have shown that the addition of fatty acids in the ration of ruminants negatively affects not only the protozoa population (Szumacher-Strabel et al., 2004; Varadyova et al., 2007) but also affects methanogenic bacteria (Ipharraguerre and Clark, 2003; Szumacher-Strabel et al., 2004). Protozoa counts were reduced ($P < 0.05$) by 88% when diets contained 3.5% coconut oil (Machmuller and Kreuzer, 1999). However study by Kisidayova et al. (2006) indicated that rumen protozoa had no uniform response to fatty acids supplemented depending on the composition and resultant concentration of the main fatty acid compounds.

5. Conclusion

Euglena is rich source of amino acids and fatty acids. Addition of Euglena reduced methane emission and improved overall DM and OM digestibility. The reduction in methane emission is associated with saturated medium chain fatty acid contents of Euglena supplement used and its subsequent impact on rumen protozoa activity. From the results of this *in vitro* study it can be demonstrated that addition of Euglena up to 100 g/kg DM of the diet has the potential to mitigate methane emission and considerable improvement in DM and OM digestibility. More research is needed to identify and clearly explain the contribution of other biologically active compounds present in Euglena.

Conflict of interest

Acknowledgements

References

AOAC, 1995. Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Exp. Agric. 48, 21–27.

Chae, S.R., Hwang, E.J., Shin, H.S., 2006. Single cell protein production of *Euglena Gracilis* and carbondioxide fixation in an innovative photo-bioractor. Bioresour. Technol. 97, 322–329.

Conway, E.J., O'Malley, E., 1942. Micro diffusion methods: ammonia and urea using buffered absorbent(revised methods for ranges greater than 100 μg N). Biochem. J. 36, 655–661.

Dajana, J.K., Jelica, B.S., Olivera, B.B., Aleksandra, C.M., Ivan, L.M., 2013. Algae in food and feed. Food Feed Res. 40, 21–31.

Dohme, F., Machmuller, A., Wasserfallen, A., Kreuzer, M., 1999. The role of rumen ciliate protozoa for methane suppression caused by coconut oil. Lett. Appl. Microbiol. 29, 187–192.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M., 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Lett. Appl. Microbiol. 32, 47–51.

Eugene, M., Masses, D., Chiquette, J., Benchaar, C., 2008. Meta-analysis on the effects of lipid supplementation on methane production in lactating dairy cows. Can. J. Anim. Sci. 88, 331–337.

Gouveia, L., Batista, A.P., Sousa, I., Raymundo, A., Bandarra, N.M., 2008. Microalgae in novel food products. In: Papadoupoulos, K. (Ed.), Food Chemistry Research Developments. Nova Science Publishers, Inc., NY, USA, pp. 75–112, ISBN 978-1-60456-262-0.

Harel, M., Clayton, D., 2004. Feed formulation for terrestrial and aquatic animals. US Patent 20070082008 (WO/2004/080196).

Ipharraguerre, I.R., Clark, J.H., 2003. Usefulness of ionohpores for lactating dairy cows. A review. Anim. Feed Sci. Technol. 106, 39–57.

Kisidayova, S., Mihalikova, K., Varadyova, Z., Potkanski, A., Szumacher-Strabel, M., Cieslak, A., Certik, M., Jalc, D., 2006. Effect of microbial oil, evening primrose oil and borage oil on rumen ciliate population in artificial rumen (Rusitec). J. Anim. Feed Sci. 15, 153–156.

Machmuller, A., Kreuzer, M., 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65–72.

Machmuller, A., Ossowski, D.A., Kreuzer, M., 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci. Technol. 85, 41–60.

Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4, 351–365.

McCann, J.C., Drewery, M.L., Sawyer, J.E., Pinchak, W.E., Weckersham, T.A., 2014. Effects of postextraction algal residue supplementation on the ruminal microbiome of steers consuming low-quality forage. J. Anim. Sci. 92, 5063–5075, http://dx.doi.org/10.2527/jas.2014-7811.

McDougall, E.I., 1948. "Studies on ruminant saliva". I. The composition and output of sheep's saliva. Biochem. J. 43, 99–109.

Patra, A.K., 2013. The effect of dietary fats on methane emissions and its other effects on digestibility, rumen fermentation and lactation performance in cattle: a meta-analysis. Livest. Sci. 155, 244–254.

Patra, A.K., Yu, Z., 2013. Effects of coconut and fish oils on methane production, fermentation, abundance and diversity of rumen microbial populations in vitro. J. Dairy Sci. 96, 1782–1792.

Place, S.E., Mitloehner, F.M., 2010. Contemporary environmental issues: a review of the dairy industry's role in climate change and air quality and the potential of mitigation through improved production efficiency. J. Dairy Sci. 93, 3407–3416.

Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol. 65, 635–648.

Sar, C., Mweny, B., Santoso, B., Takaura, K., Morikawa, R., Isogai, N., Asakura, Y., Toride, Y., Takahashi, J., 2005. Effect of Escherichia coli W3110 on ruminal methanogenesis and nitrate/nitrite reduction in vitro. Anim. Feed Sci. Technol. 118, 295–306.

SAS Institute, 2010. SAS Version 9.3. SAS Inst. Inc., Cary, NC, USA.

Szumacher-Stabel, M., Cieslak, A., 2012. Dietary possibilities to mitigate rumen methane and ammonia production. In: Guoxiang Liu (Ed.), Greenhouse Gases, Capturing, Utilization and Reduction. InTech, http://dx.doi.org/10.5772/32105, ISBN: 978-953-51-0192-5 http://www.intechopen.com/books/greenhouse-gases-capturing-utilization-and-reduction/dietary-possibilities-to-mitigate-rumen-methane-and-ammonia-production.

Szumacher-Strabel, M., Martin, S.A., Potkanski, A., Cieslak, A., Kowalczyk, J., 2004. Changes in fermentation process as the effect of vegetable oil supplementation in in vitro studies. J. Anim. Feed Sci. 13, 215–218.

Tilley, J.M.A., Terry, R.A., 1963. A two stage technique for in vitro digestion of forage crops. J. Br. Grassland Soc. 18, 104–111.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fibre, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Varadyova, Z., Kisidayova, S., Siroka, P., Jalc, D., 2007. Fatty acid profiles of rumen fluid from sheep fed diets supplemented with various oils and effect on the rumen ciliate population. Czech J. Anim. Sci. 52, 399–406.

Wu, G., 2009. Amino acids: metabolism, function and nutrition. Amino Acids 37, 1–17.