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EFFECTS OF MICROALGAE SPECIES ON *IN VITRO* RUMEN FERMENTATION PATTERN AND METHANE PRODUCTION

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Abstract

This experiment was conducted to establish the effects of two types of microalgae [*Chlorella vulgaris* (AI), *C. variabilis* (AII) and their combination (AI+AII)] with two substrates (wheat and corn silages) on rumen fermentation, gas and methane production. To each substrate, one of 3 algae treatment was supplemented at 0% and 25% of the total incubated dry matter. A series of 5 measurement points (3, 6, 12, 24 and 48 h) were completed and the gas production was monitored. The proximate and mineral composition of microalgae and substrates were examined. At 48 h incubation rumen fermentation variables and CH_4 production were also assessed. When compared with wheat silage, corn silage caused an increase in gas production (P<0.05). Ruminal gas production decreased in the algae groups when compared to the controls (0% algae, wheat and corn silages, P<0.05). Among algae, *C. vulgaris* had the strongest effect, decreasing gas production by 34%. Among algae, the total volatile fatty acids (VFA) and CH_4 production were found to be lower in *C. variabilis* (P<0.001). Ammonia-N increased with the algae inclusion (P<0.05). But, the ruminal gas production, pH, acetate, the total VFA, CH_4 and rumen fermentation efficiency were not affected by the substrate and algae interaction (P>0.05). The propionate was the highest (P<0.05) for corn silage when incubated with *C. vulgaris*. Ruminal butyrate was the lowest for the wheat silage when incubated with the mixture of algae (P<0.05). The NH_3-N was the highest in corn silage when incubated with all algae types (P<0.05). Careful selection and combination of substrate and algae may positively manipulate rumen fermentation and may inhibit CH_4 production. Further research is needed to validate these results *in vivo*.

Key words: *Chlorella*, microalgae, *in vitro*, methane production, rumen fermentation pattern

Methane (CH_4) and nitrous oxide (N_2O) are two major greenhouse gases produced by the animal industry. In the last decade, the atmospheric concentration of these gases has risen notably (Monteny et al., 2006). Anaerobic microorganisms called methanogens form CH_4 through the process of their energy metabolism (de Macario and Macario, 2009). Among various factors, the digestion of cellulose by ruminants accounts for the majority of CH_4 production (Fievez et al., 2007; Maia et al., 2016). Dietary ingredients such as grains, legume forages, protein supplements,

fats and oils have been used to reduce enteric CH_4 emissions (Beauchemin et al., 2008). These ingredients however did not show consistent results, and some showed a decrease in feed digestibility (Cottle et al., 2011).

Algae which have been commonly used in biofuel production (Medipally et al., 2015), have gained more attention as a source of protein and polyunsaturated fatty acids (PUFA) or as a feed additive with health improving characteristics for livestock (Fievez et al., 2007; Han and McCormic, 2014). *Chlorella* (Chlorophycea), an important group of microalgae species are single-celled freshwater microalgae and contain the highest amount of chlorophyll of any common plant, with protein content of about 600 g/kg dry matter (DM) and 18 amino acids as well as vitamins and minerals (Kholif et al., 2017). It can be a complex feed supplement for possible use with different animals. Within the complexity of this supplement comes the provision of pigments, antioxidants, provitamins, vitamins and growth factors, as well as all basic nutrients. It can help enhance the quality of animal products and stimulate animal physiology and health (Fievez et al., 2007; Han and McCormick, 2014; Kotrbáček et al., 2015). Studies show that with even exceptionally low levels of *Chlorella* supplementation (that are economically acceptable) it still produces positive results (Kotrbáček et al., 2015). It is even considered to aid feed palatability, feed intake and feed efficiency (Yan et al., 2012). The use of *Chlorella vulgaris* has been reported to enhance ruminal bacterial growth (Anele et al., 2016; Tsiplakou et al., 2017), increase total rumen volatile fatty acids (VFA) and improve milk yield (Kholif et al., 2017). *Chlorella vulgaris* was previously identified as promising candidate for reduction of CH_4 emissions (Bohutskyi et al., 2014; Tsiplakou et al., 2017; Wild et al., 2019). Despite this interest, no one to the best of our knowledge has studied the effects of *C. variabilis* or the combination of *C. vulgaris* and *C. variabilis* on rumen fermentation and methane production. Therefore the aim of this study is to evaluate the effectiveness of *Chlorella vulgaris*, *C. variabilis* and the combination of both algae on rumen total gas production, rumen fermentation characteristics and methane production, *in vitro.* We also compared these effects with each microalga alone and both microalgae together with different feed substrates (corn and wheat silages).

Material and methods

Microalgae cultivation was performed with flat panel type photo-bioreactor under controlled conditions without any contamination. Harvested algae were naturally dried in a room in which climatic conditions were 25±1°C and 60±5% relative humidity. Algae samples and commercially available wheat and corn silages were ground through a 1 mm screen. Dry matter (DM), ash and total protein content of all samples were analyzed according to AOAC (981.10, 942.05 and 984.13 respectively; 1990). The fat content in dry microalgae was determined by extracting with hexane for 4 h in Soxhlet. Afterwards, a second extraction was performed with chloroform-methanol (2:1 v/v) for 4 h (Horwitz, 1975). The fat content of silages was determined in Soxhlet according to AOAC Official Method 920.39 (2012). Neutral

detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to Van Soest et al. (1991) by using an ANKOM fibre analyzer (ANKOM200, Macedon, NY, USA). The ground samples were digested using a mixture of 3 ml of HNO_3 and 3 ml of H_2O_2 in a microwave oven (Berghof MWS 2) (Wu et al., 1997). Phosphorus, magnesium, iron, manganese, copper, zinc, boron amounts were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, Perkin Elmer Optima 2100DV) (Isaac and Johnson, 1998). The cations (sodium, potassium and calcium) were determined by the Eppendorf Elex 6361 model flame photometer (Eppendorf, Hamburg, Germany).

The protocol used in this research was approved by the Institutional Animal Care and Use Committee (UÜHADYEK) (approval date: 20.03.2018; no: 2018-05/03). No health problems were detected in the experiment and sheep remained free of disorders during the overall experimental period. Three Merino male sheep, about 2 years of age, were used in this experiment. The donor sheep were acclimated to cages for 7 days prior to the experiment. They were kept in individual cages for rumen liquor collection. Animals were fed 50% alfalfa hay and 50% concentrate (consisting of wheat 74%, sunflower meal 24%, $CaCO_3$ 1.4%, NaCl 0.5% and mixture of vitamins and minerals 0.1%) to ensure balanced cellulolytic and amylolytic activity of the rumen fluid. They received 18% protein and 2750 kcal/kg DM energy. Water was offered *ad libitum*. Rumen fluid was taken from the sheep prior to the morning feeding by stainless steel stomach tube into a warm Thermos flask. Rumen fluid was filtered through four layers of cheesecloth before mixing with buffer and was maintained at 39°C. *In vitro* gas measurements were performed according to Menke and Steingass (1988). Experimental treatments consisted of 250 mg dry sample of totals of 100% wheat silage (0% algae, as control) and 100% corn silage (0% algae, as control). We also used 6 different combinations (3 with wheat silage, 3 with corn silage) as a 75% substrate 25% algae mixture (Maia et al., 2016) using our 3 different algae mixtures [Algae groups: *Chlorella vulgaris* (AI), *C. variabilis* (AII) and AI+AII]. Experimental treatments were incubated in triplicate (n=3) and treatments were randomly assigned to the experimental units. All samples were added to 150 ml gas tight syringe, together with a buffered incubation medium (20 mL) and rumen fluid inoculum (10 mL) bubbled with CO_2 (2:1 vol/vol). Three syringes containing 30 mL of inoculum with no sample (blanks) were also incubated alongside each of the dietary samples to correct for gas production. Syringes were incubated in a horizontal rack at 39°C to determine the cumulative gas production at various time intervals (3, 6, 12, 24 and 48 h). At 48 h the incubation was stopped and the rumen fluid was analyzed for pH, VFA, CH_4 and ammonium-N. The VFA analysis was performed with liquid chromatography (ICS 3000, Dionex Corporation, San Francisco, CA) equipped with an Acclaim 4x250 mm organic acid column. *In vitro* CH_4 concentration in headspace gas was measured according to description of Kinley et al. (2016). Ammonia-N (NH_3-N) was determined by using Kjeltech auto analyzer (Gerhardt, Bonn, Germany) without a digestion step according to AOAC (1990). Fermentation efficiency was calculated using the equation of Chalupa (1977): FE (%) = (0.62 acetate + 1.09 propionate + 0.78 butyrate)/(acetate + propionate + butyrate)×100. Metabolizable energy (ME) and net energy of lactation (NEL) values in all samples

were calculated using the equations of Menke and Steingass (1987): ME (MJ/kg DM) = 0.136 GP + 0.0057 CP + 0.000286 EE^2 + 2.20, NEL (MJ/kg DM) = 0.096 GP + 0.0038 CP + 0.000173 EE^2 + 0.54, where GP is 24-h net gas production (mL 200 mg-1 DM), and CP, EE, are crude protein and ether extract (% DM), respectively.

In vitro gas production value was analyzed by GLM procedure for repeated measurements of SAS, considering the effects algae, substrate and the interaction between algae and substrate and the residual error. The results from post ruminal fermentation were analyzed as a complete randomized design (2 substrates, 4 algae treatments: no algae, AI, AII or AI+II, with 3 replicates each) with all possible interactions using SAS Proc GLM. Tukey's Pos Hoc tests were carried out in order to know the extent of interactions between substrates and algae treatments.

The statistical model was:

For the gas production parameter: $Y_{ijkl} = \mu + F_i + A_j + T_k + (FA)_j + E_{ijkl}$

For the rumen fermentation parameters: $Y_{ijk} = \mu + F_i + A_j + (FA)_{ij} + E_{ijkl}$

where:

$Y_{ijkl} - Y_{ijk}$ = observed values,

F_i = the offects of ith forage (wheat and corn silage),

A = the effects of jth algae (*C. vulgaris* (AI), *C. variabilis* (AII) and AI + AII),

T_k = the effects of kth time of incubation for gas production (3, 6, 24, 24 and 48 h),

$(FA)_{ij}$ = the effects of interaction between forage and algae type,

$E_{ijkl} - E_{ijk}$ = the effects of random error.

The significant differences among means were declared at $P<0.001$ and a tendency was declared at $P<0.10$.

Results

The proximate and the mineral composition of the substrates (wheat and corn silage) and two microalgae species (*Chlorella vulgaris* and *C. variabilis*) are shown in Table 1. Wheat silage and corn silage presented 331 and 334 dry matter (DM, g/kg), 406 and 439 NDF (g/kg DM), respectively. The chemical composition of the studied *C. vulgaris* and *C. variabilis* showed a variation with respect to the protein content which was found to be 136 and 108 (g/kg DM), respectively. The algae species were found to be rich in phosphorous, potassium, magnesium, sodium and iron.

No interaction was observed between substrate and algae supplementation for gas production, *in vitro* ($P=0.125$, Figure 1). The ruminal gas production was influenced by the types of substrate and algae supplementation in *in vitro* incubations ($P<0.001$, Table 2). After 3, 6, 24 and 48 hours of incubation, the gas production value of corn silage was higher than the corresponding value for wheat silage ($P<0.05$). On the other hand, the types of substrate did not affect the volume of total rumen gas production at 12 h of incubation. Overall, the algae supplementations led to less gas production than the control (0% algae, wheat and corn silages, $P<0.001$, Table 2). Among algae, *C. vulgaris* (AI) had the lowest gas production producing 51 mL after

48 h incubation (P<0.001) with 34% less gas than the control (0% algae, wheat and corn silages, 77 mL, P<0.001).

Table 1. The proximate composition of the two types of substrate (wheat and corn silages) and two types of algae species

Items	Wheat silage (WS)	Corn silage (CS)
DM (g/kg)	331.00	334.00
OM (g/kg DM)	266.00	245.00
Protein (g/kg DM)	111.50	61.20
NDF (g/kg DM)	406.80	439.00
ADF (g/kg DM)	342.70	278.50
Fat (%)	3.24	2.24
ME (MJ/kg DM)	10.74	10.46
NEL (MJ/kg DM)	6.57	6.37
P (g/kg)	2.65	3.90
Ca (g/kg)	2.27	1.41
K (g/kg)	14.84	8.84
Mg (g/kg)	2.25	1.29
Na (g/kg)	1.66	0.49
Fe (g/kg)	0.83	0.18
Cu (g/kg)	9.29	8.45
Zn (g/kg)	16.43	8.70
Mn (g/kg)	0.06	0.04
B (g/kg)	59.36	52.88
	***C. vulgaris* (AI)**	***C. variabilis* (AII)**
DM (g/kg)	72.20	71.30
OM (g/kg DM)	67.40	61.00
Protein (g/kg DM)	136.30	107.70
NDF (g/kg DM)	1.80	4.77
ADF (g/kg DM)	38.41	44.80
Fat (%)	15.30	14.10
ME (MJ/kg DM)	12.02	11.57
NEL (MJ/kg DM)	6.62	6.42
P (g/kg)	27.08	13.05
Ca (g/kg)	0.94	1.05
K (g/kg)	132.95	134.67
Mg (g/kg)	12.36	12.81
Na (g/kg)	16.45	16.40
Fe (g/kg)	5.40	5.64
Cu (g/kg)	0.00	0.00
Zn (g/kg)	0.53	0.46
Mn (g/kg)	1.27	1.40
B (g/kg)	1.64	1.79

SEM – standard error of the mean; DM – dry matter; OM – organic matter; NDF – neutral detergent fiber; ADF – acid detergent fiber; ME – metabolizable energy, NEL – net energy of lactation.

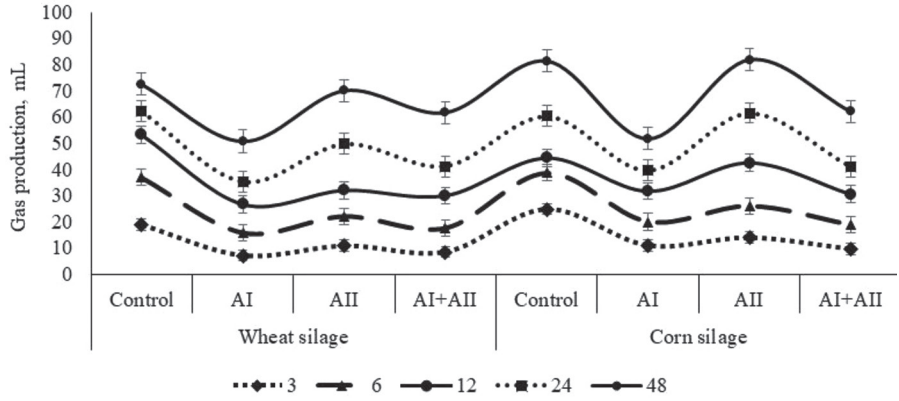

Figure 1. Effects of the interaction of substrate and algae supplementation on gas production, *in vitro* (mL, P=0.125). *Chlorella vulgaris* (AI), *C. variabilitis* (AII) and their combination (AI + AII)

Table 2. Effects of substrate and algae supplementation on *in vitro* total gas production (mL)

Hours	Wheat silage (WS)	Corn silage (CS)	SEM	Control	*C. vulgaris* (AI)	*C. variabilis* (AII)	AI+AII	SEM
3	12 b	15 a	0.367	22 a	9 c	13 b	9 c	0.519
6	23 b	26 a	0.729	38 a	18 c	24 b	18 a	1.030
12	36 a	37 a	0.902	49 a	29 c	37 b	30 c	1.274
24	47 b	51 a	1.901	61 a	38 b	56 a	41 b	1.543
48	64 b	69 a	1.523	77 a	51 c	76 a	62 b	2.154
P value		0.009				P<0.001		

SEM – standard error of the mean.
Means in the same column with different letters differ significantly (P<0.05).

Ruminal pH was influenced by the types of substrate and algae supplementation (P<0.001, Table 3) whereas no interaction was found between substrate and algae inclusion (P=0.413). Corn silage had lower ruminal pH than wheat silage (P<0.001). Overall, algae affected ruminal pH in the range of 5.98–6.18. Among algae, *C. vulgaris* (AI) had the highest ruminal pH at 48 h of fermentation (P<0.001, Table 3), *in vitro*. Volatile fatty acids (VFA) composition was influenced by the types of substrate and algae inclusion (P<0.001, Table 3). Algae inclusion decreased the acetate (P<0.001), butyrate (P<0.001) and the total VFA concentrations (mmol/L, P<0.001) but increased the propionate (mmol/L, P<0.001, Table 3). An interaction between the types of substrate and algae inclusion was found for propionate (P<0.001, Table 3, Figure 2) and butyrate (P<0.001 Table 3, Figure 2) and a trend was observed for total VFA concentrations (mmol/L, P=0.07) whereas no interaction was found for the acetate concentration (mmol/L, P=0.582). When corn silage was used as a substrate *C. vulgaris* had the highest concentration of propionate (mmol/L, P<0.001).

A significant interaction was found for NH_3-N production between substrate and algae inclusion (P<0.001, Table 3, Figure 2). When corn silage was used as a substrate, NH_3-N concentration was increased by more than 0.5 fold with each algae inclusion when compared with wheat silage (P<0.001). Methane production was strongly affected by the types of substrate and algae supplementation (P<0.001, Table 3) while it tended (P=0.10) to be affected by the substrate and algae mixture (P=0.10). After 48 h incubation, fermentation efficiency (FE, %) was affected by algae supplementation (P<0.001, Table 3) while the type of the substrate (P=0.31) or the substrate and algae interaction (P=0.228) did not affect FE. Fermentation efficiency was decreased by nearly 2% with algae inclusion.

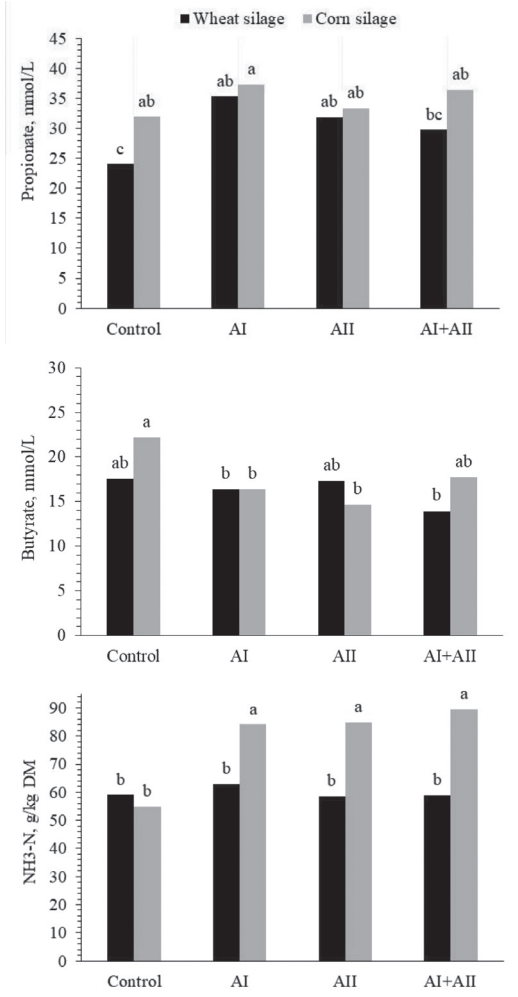

Figure 2. Effects of interaction of substrate and algae supplementation on propionate (mmol/L), butyrate (mmol/L) and NH3-N (g/kg DM) concentrations after 48 h of *in vitro* incubation. Mean values with different letters differ significantly (P<0.05). *Chlorella vulgaris* (AI), *C. variabilis* (AII) and their combination (AI+AII)

Table 3. Effects of substrate and algae supplementation on *in vitro* rumen fermentation parameters and methane production

	Substrate		SEM	Algae				SEM	P Value		
	Wheat silage (WS)	Corn silage (CS)		Control	*C. vulgaris* (AI)	*C. variabilis* (AII)	AI+AII		Substrate	Algae	Substrate* Algae
pH	6.18 a	5.88 b	0.032	5.93 b	6.18 a	5.98 b	6.00 b	0.045	P<0.001	P<0.001	P=0.413
Acetate (mmol/L)	42.09 b	50.25 a	1.201	55.22 a	43.55 b	41,18 b	44.73 b	1.705	P<0.001	P<0.001	P=0.582
Propionate (mmol/L)	30.23 b	34.96 a	0.681	27.98 c	36.32 a	32.62 b	33.10 b	0.923	P<0.001	P<0.001	P<0.001
Butyrate (mmol/L)	16.30 b	17.75 a	0.550	19.83 a	16.38 b	16.0 b	15.83 b	0.778	P=0.080	P<0.001	P<0.001
Total VFA (mmol/L)	88.63 b	102.78 a	1.949	103.08	96.25 ab	89.90 b	93.67 b	2.756	P<0.001	P=0.020	P=0.070
NH_3-N (g/kg DM)	59.90 b	78.43 a	1.580	57.0 b	73.60 a	71.78 a	74.27 a	0.254	P<0.001	P<0.001	P<0.001
Methane (mmol/L)	33.78 b	39.28 a	0.767	40.50 a	36.14 b	33.90 b	35.57 b	2.234	P<0.001	P<0.001	P=0.10
FE (%)	76.58 a	76.84 a	0.179	77.79 a	76.10 b	76.71 b	76.27 b	1.084	P=0.310	P<0.001	P=0.228

DM – dry matter; FE – fermentation efficiency; SEM – standard error of the mean; FE (%) = (0.62 acetate + 1.09 propionate + 0.78 butyrate)/(acetate + propionate + butyrate) ×100 (Chalupa, 1977). Means in the same column with different letters differ significantly (P<0.05).

Discussion

In this study, the effects of microalgae species on rumen total gas production, rumen fermentation characteristics and CH_4 production were examined in *in-vitro* conditions. Two types of algae have been assessed qualitatively and explored for the possibility of their use as a natural alternative to manipulating rumen efficiency and reducing greenhouse gas emissions produced by ruminants. This research also determined rumen total gas production, rumen fermentation characteristics and CH_4 production of two major substrates (wheat and corn silages) used in diets, which were incubated with two types of microalgae species alone or in combination.

In this study, the content of protein and fat found in both algae are comparable with or even higher than some feed ingredients (alfalfa hay, corn and wheat grains) that are commonly used in ruminant diets (Table 1, Lum et al., 2013). As in this study, Han and McCormic (2014) as well as Piorreck et al. (1984) suggested most algae that have a high protein content may be considered as a high protein supplement. It has been reported that algae residue have high fat content, soluble carbohydrates, macro and micro minerals and low fiber (Han and McCormic, 2014; Maia et al., 2016).

In the current study, accumulated gas production from corn silage was higher than from wheat silage but the ruminal pH was lower (Tables 2 and 3). This could be explained by a difference in the total VFA, which is related to a more extensive fermentation of corn silage (Van Kessel and Russell, 1996). Similar to our results, Maia et al. (2016) also found out that the total rumen gas production increased and the rumen pH was reduced when corn silage was incubated for 24 h compared to meadow hay. The decline in gas production and the rise in ruminal pH by algae supplementation over the 48 h incubation were similar to previous *in vitro* study (Maia et al., 2016). In contrast to our results, in a recent *in vitro* study (Dubois et al., 2013) inclusion of algae with very high protein content increased the gas production in the rumen. It has been previously reported (Kinley et al., 2016) that red algae (*Asparagopsis taxiformis)* and lipid-extracted microalgae (Lodge-Ivey et al., 2014) added to forage diets significantly increased propionate and butyrate levels in the rumen. This is considered beneficial as the energy from propionate was utilized more efficiently than the energy from acetate in earlier studies (Chalupa, 1977; Lodge-Ivey et al., 2014). In our study high fat content of *Chlorella* used may inhibit cellulotic bacteria in the rumen and lead to a reduction of total VFA, much like the study of Fievez et al. (2007). The observations found in this study are consistent with earlier *in vivo* study (Boeckaert et al., 2008), in which the addition of algae to corn silage based diet increased the ruminal pH and decreased the total VFA by up to 18% after 19 d treatment. On the other hand, unlike our results, Lodge-Ivey et al. (2014) observed that the total rumen VFA concentration was greater when lipid extracted algae (*Chlorella* or *Nannochloropsis*) were added to the concentrate diet. This might be related to differences in supplementation level and oil extraction. The results from this study indicated that the corn silage when incubated with both algae produced the highest NH_3-N concentration in the rumen (Table 3, Figure 2). The increased NH_3-N concentration of rumen in the algae supplemented group also supports the findings of Durmic et al. (2014) and Tsiplakou et al. (2017). The result of high dietary protein will be

increased ruminal NH_3-N concentration. The amino acids and peptides in the presence of fermentable carbohydrates will be factors for microbial growth (Bach et al., 2005). Algae contain high protein so the amount of NH_3-N produced may contribute to the microbial protein synthesis, however more research is needed for partitioning algae proteins based on ruminal digestion. Algae supplementation will be beneficial in livestock feeding where corn silage is the major forage source. Since corn silage is low in protein, it can be increased by the addition of algae. Previous studies (Fievez et al., 2007; Machado et al., 2014; Durmic et al. 2014; Tsiplakou et al., 2017) noted a significant decrease of CH_4 production with the addition of algae species, but this could not be directly confirmed in our study. However there was a slight trend noted of a decrease in CH_4 production especially in wheat silage when incubated with combination of two algae. Dubois et al. (2013) and Moate et al. (2013) also found an unclear effect of algae on CH_4 production. The findings of other *in vitro* studies (Fievez et al., 2007; Machado et al., 2014; Durmic et al. 2014; Tsiplakou et al., 2017) showed DHA-rich microalgae or marine algae supplementation caused an increase in ruminal propionate and a reduction in total VFA and CH_4 production. There is a balance between propionate and acetate and butyrate formation having a significant role in H_2 availability for methanogenic archae. Redirection of metabolic hydrogen towards propionate was considered a CH_4 inhibiting strategy (Baker, 1999; Fieves et al., 2007). Reasons for the discrepancies in CH_4 production between *in vivo* and *in vitro* studies are not clear. But it should be considered that some ruminal fermentation experiments could be altered based on the microbial diversity of inoculum from the donor animal used (Boguhn et al., 2013).

Conclusions

We found low concentration of butyrate, and a high concentration of propionate and a trend of lower concentration of VFA in wheat and corn silages incubated with *Chlorella vulgaris*, *C. variabilis* and their combination. Although we cannot conclude from these findings the absolute for CH_4 reduction. Further research would be needed to evaluate the reduction of methane by algae *in vivo*. It is likely that microalgae supplemented feed could add protein especially in low protein forage diets.

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