Journal of Agricultural Science (2017), **155**, 494–507. © Cambridge University Press 2016
doi:10.1017/S0021859616000812

ANIMAL RESEARCH PAPER

The effects of three total mixed rations with different concentrate to maize silage ratios and different levels of microalgae *Chlorella vulgaris* on *in vitro* total gas, methane and carbon dioxide production

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(*Received 10 March 2016; revised 8 August 2016; accepted 23 September 2016; first published online 2 November 2016*)

SUMMARY

The aim of the current study was to assess the effects of adding *Chlorella vulgaris* algae at different levels on *in vitro* gas production (GP) of three total mixed rations (TMR) with different concentrate (C): maize silage (S) ratios (25C : 75S, 50C : 50S, 75C : 25S). *Chlorella vulgaris* was added at 0, 20, 40 and 80 mg/g dry matter (DM) of the TMR and total gas, methane (CH_4) and carbon dioxide (CO_2) production were recorded after 2, 4, 6, 8, 10, 12, 24 and 48 h of incubation in three runs. Increasing concentrate portion in the TMR linearly increased the asymptotic GP and decreased the rate of GP without affecting the lag time. Addition of *C. vulgaris* at 20 mg/g DM to the 25C : 75S TMR increased the asymptotic GP, CH_4, CO_2 and GP at 48 h. Addition of *C. vulgaris* to the 50C : 50S TMR decreased the asymptotic GP and GP at 48 h. Higher CH_4 production was observed at 48 h of incubation when *C. vulgaris* was included at (per g DM): 20 mg for the 25C : 75S ration, 40 mg for the 50C : 50S ration and 80 mg for the 75C : 25S ration. Inclusion of *C. vulgaris* linearly increased CH_4 production for the 50C : 50S ration and increased CO_2 production at 10 and 12 h of incubation for the 50C : 50S ration, whereas 20 and 40 mg *C. vulgaris*/g DM of the 75C : 25S TMR decreased CO_2 production. The 25C : 75S TMR had the highest *in vitro* DM disappearance with *C. vulgaris* addition. *Chlorella vulgaris* addition was more effective with rations high in fibre content than those high in concentrates. It can be concluded that the optimal level of *C. vulgaris* addition was 20 mg/g DM for improved ruminal fermentation of the 25C : 75S TMR.

INTRODUCTION

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that have the ability to convert sunlight, carbon dioxide (CO_2) and inorganic elements into nutrient-rich biomass with good essential nutrients including lipids, proteins, carbohydrates, glycoproteins and calories (Hudek *et al*. 2014). Furthermore, some microalgae species, e.g. *Schizochytrium*, are considered rich sources of *n*–3 polyunsaturated fatty acids (PUFA) (Pereira *et al*. 2012).

One important microalgae species is *Chlorella vulgaris*. It is a fresh-water, single-celled microalgae which contains all the essential amino acids in proportions more suitable for humans and animal feed than soybean, canola, maize and wheat (Tibbetts *et al*. 2015), making it a nutrient-dense food. *Chlorella vulgaris* contains about 580 g protein/kg dry matter (DM) with about 18 amino acids, and various vitamins

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and minerals with a chlorophyll content as high as many common plants (Priyadarshani & Rath 2012). More than 20 vitamins and minerals including calcium, phosphorous, iron, magnesium, potassium, vitamins A, B complex, C, E and K, biotin, inositol and folic acid were reported by Priyadarshani & Rath (2012). In addition to its content of peptides and amino acids, which are stimulatory factors for ruminal microbial growth and digestion, the use of *C. vulgaris* as an animal feed additive has many advantages including increasing the concentration of some bacterial species, e.g. *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Clostridium sticklandii* with forage-based diet in *in vivo* studies, resulting in improved bacterial growth and promotion of ruminal trans C18 : 1, trans-11 C18 : 1 fatty acids and monounsaturated fatty acids formation in goats (Anele *et al*. 2016; Tsiplakou *et al*. 2016). Consequently, microalgae rich in fats could be considered a potential option to reduce methane (CH_4) emissions from ruminants because PUFA have antimicrobial effects on methanogens and protozoa due to their ability to disrupt microbial cell membranes (Martin *et al*. 2010). However, Tsiplakou *et al*. (2016) reported that microalgae rich in protein and low in fat content increased the populations of CH_4-producing bacteria and protozoa.

The density and activity of ruminal microflora depend on the chemical composition and the forage : concentrate ratio of diets fed to host animals (Elghandour *et al*. 2016*b*). Increasing the dietary portion of forage has been shown to increase CH_4 production (Elghandour *et al*. 2016*a*). The *in vitro* gas production (GP) technique is a useful tool for studying potential ruminal degradation of feeds (Rodriguez *et al*. 2015; Vallejo *et al*. 2016). This method allows for the estimation of how much substrate is used to produce volatile fatty acids and the energetic value of feed as well as to determine the amount of substrate truly fermented, which is converted into microbial protein (Elghandour *et al*. 2015*a*, *b*). During the first few hours of incubation (e.g. the first 24 h of incubation), the fermentation process is very active and more fermentation products are released. Therefore, it is important to measure the activity of fermentation processes at close intervals (i.e. every 2 h), and then extended to every 24 h. The aim of the present study was to assess the effects of adding *C. vulgaris* algae at different levels on *in vitro* rumen gas, CH_4 and CO_2 production of total mixed rations (TMR) with different maize silage to concentrate ratios.

Table 1. *Amino and fatty acid profiles of* Chlorella vulgaris *algae* (as provided by the manufacturer)

Items	Content
Essential amino acid content (g/kg algae protein)	
Arginine	38·8
Histidine	11·5
Isoleucine	19·3
Leucine	59·5
Lysine	42·4
Methionine	10·1
Phenylalanine	30·7
Threonine	29·4
Tryptophan	0·019
Valine	38·6
Non-essential amino acid content (g/kg algae protein)	
Alanine	52·1
Aspartic	59·0
Cysteine	0·069
Glutamic	77·6
Glycine	32·0
Proline	30·7
Serine	25·3
Tyrosine	23·8
Fatty acid profile (g/kg total fatty acids)	
Myristic acid (C14 : 0)	0·287
Palmitic acid (C16 : 0)	23·8
Palmitoleic acid (C16 : 1n7)	1·30
Stearic acid (C18 : 0)	2·71
Oleic acid (C18 : 1n9c)	4·13
Linoleic acid (C18 : 2n6c)	37·3
Alpha linolenic acid (C18 : 3n3)	40·9
Eicosadienoic acid (C20 : 2)	0·110
Docosanoic acid (C22 : 0)	0·202
Lignoceric acid (C24 : 0)	0·213
Nervonic acid (C24 : 1n9)	0·112

MATERIAL AND METHODS

Chlorella vulgaris, substrate and treatments

Chlorella vulgaris microalgae (Xuhuang Bio-Tech Co., Ltd., Shaanxi, China) containing 949 g DM/kg, with 944 g organic matter (OM)/kg DM was used in the current study. The crude protein (CP) content was 591 g/kg DM and the total carbohydrate content was 173 g/kg DM with 18·8 kJ energy/kg DM. The neutral detergent fibre (NDF) content was 121 g/kg DM, while the fat content was 134·2 g/kg DM. The amino and fatty acid profiles of *C. vulgaris* are shown in Table 1. *Chlorella vulgaris* was tested at 0, 20, 40 and 80 mg/g DM of TMR.

https://doi.org/10.1017/S0021859616000812

Table 2. *Chemical composition (g/kg DM) of the three total mixed rations with different concentrate* (C) to maize silage (S) ratios (adapted from Elghandour* et al. *2015a, b)*

	Rations			
	25C : 75S	50C : 50S	75C : 25S	S.E.M.
Organic matter	944	940	933	14·8
Crude protein	92	139	133	9·3
Neutral detergent fibre	372	302	218	12·6
Acid detergent fibre	149	127	88	11·0
Acid detergent lignin	15·0	12·6	10·3	1·42

* Contained (g/kg): 200 maize grain flaked, 260 maize grain cracked, 154 sorghum grain, 100 molasses sugarcane, 100 distilled dry grain, 96 soybean meal, 70 wheat bran, 10 $NaCOOH_3$, 10 mineral premix (vitamin A [12 000 000 IU], vitamin D_3 [2 500 000 IU], vitamin E [15 000 IU], vitamin K [2·0 g], vitamin B_1 [2·25 g], vitamin B_2 [7·5 g], vitamin B_6 [3·5 g], vitamin B_{12} [20 mg], Pantotenic acid [12·5 g], Folic acid [1·5 g], Biotin [125 mg], Niacin [45 g], Fe [50 g], Zn [50 g], Mn [110 g], Cu [12 g], I [0·30 g], Se [200 mg], Co [0·20 g]).

Three TMR of different concentrate (C): maize silage (S) ratios (25C : 75S, 50C : 50S and 75C : 25S) were prepared and used as fermentation substrates. Samples of TMR were dried at 65 °C for 48 h in a forced air oven until constant weight, ground in a Wiley mill to pass through a 1 mm sieve and stored in plastic bags for subsequent determination of chemical composition and *in vitro* incubation. Chemical composition of the TMR is shown in Table 2.

In vitro gas production determination

Rumen inoculum was collected from a ruminally cannulated Brown Swiss cow of 450 ± 20 kg body weight, fitted with a permanent rumen cannula. The cow was fed *ad libitum* with a TMR made of a commercial concentrate (PURINA®, Toluca, Mexico) and alfalfa hay in the ratio of 1 : 1 and formulated to meet all nutrient requirements according to NRC (2001). Fresh water was available at all times.

Rumen contents were collected before the morning feeding, flushed with CO_2, mixed and strained through four layers of cheesecloth into a flask with O_2-free headspace. Samples (0·5 g) of each TMR were weighed into 120 ml serum bottles with appropriate addition of *C. vulgaris* level/g DM. Consequently, 10 ml of rumen fluid was added to each bottle followed by 40 ml of the buffer solution recommended by Goering & Van Soest (1970), with no trypticase added.

Three incubation runs were performed in 3 weeks. Three hundred and twenty-four bottles with O_2-free headspace (three bottles for each TMR × four levels of *C. vulgaris* × three replication × three different runs) plus three bottles as blanks for each run (rumen fluid only) were incubated for 48 h. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in an incubator at 39 °C. The volume of total gas, CH_4 and CO_2 productions were recorded at 2, 4, 6, 8, 10, 12, 24 and 48 h of incubation. Total GP was recorded using the Pressure Transducer Technique (Extech Instruments, Waltham) of Theodorou *et al.* (1994) while CH_4 and CO_2 production was recorded using a Gas-Pro detector (Gas Analyser CROWCON Model Tetra3, Abingdon, UK).

At the end of incubation at 48 h, the fermentation process was stopped by swirling the bottles in ice, then the bottles were uncapped and the pH was measured using a pH meter (Conductronic pH15, Puebla, Mexico) and the contents of each bottle filtered to obtain the non-fermented residue for determination of degraded substrate.

Degradability and sample analysis

Degradability and sample analysis were determined as described in Elghandour *et al.* (2014). Briefly, after 48 h of incubation, the fermentation process was stopped and the contents of each serum bottle filtered under a vacuum through glass crucibles (coarse porosity no. 1, pore size 100–160 µm; Pyrex, Stone, UK) with a sintered filter. The fermentation residues were dried at 65 °C for 72 h to estimate DM disappearance.

Chemical analyses and calculations

Samples of the TMR and *C. vulgaris* were analysed for DM (method 934·01), ash (method 942·05), and

https://doi.org/10.1017/S0021859616000812

nitrogen (method 954·01) according to AOAC (1997), while TMR contents for NDF (Van Soest *et al*. 1991), acid detergent fibre (ADF) and lignin (AOAC 1997; method 973·18) analyses were carried out using an ANKOM^{200} Fibre Analyser Unit (ANKOM Technology Corp., Macedon, NY). Neutral detergent fibre was assayed with the use of an alpha amylase and sodium sulphite. Both NDF and ADF are expressed without residual ash. The fatty acid composition of *C. vulgaris* was determined on a Perkin-Elmer chromatograph (model 8420, Beacons-field, Perkin Elmer, Beaconsfield, UK) equipped with a flame ionization detector (analysis method ID: GB 5413.27–2010) according to the Chinese national standard methods (National Standards of People's Republic of China 2010) as provided by the manufacturer. Fatty acids were esterified using 5% methanolic hydrogen chloride with pentacosanoic acid as the internal standard (Sigma, Chemical Co., St. Louis, MO). Fatty acids were identified by comparing the retention times of the peaks with those of known standards. *Chlorella vulgaris* amino acid content was determined using a Hitachi High-Speed Amino Acid Analyser (HITACHI L-8900, Chome Nishishinbash, Minato-ku, Tokyo, Japan) according to Chinese national standard methods (analysis method ID: GB/T 5009·124-2003) as provided by the manufacturer. The analysis was based on the separation of amino acids using strong cation exchange chromatography followed by the ninhydrin colour reaction and photometric detection at 570 nm.

To estimate kinetic parameters of GP, gas volumes recorded (ml/g DM) were fitted using the NLIN procedure of SAS (2002) according to the France *et al*. (2000) model:

$$y = A \times [1 - e^{-c(t-L)}]$$

where y is the volume of GP at time t (h); A is the asymptotic GP (ml/g DM); c is the fractional rate of fermentation (/h) and L (h) is the discrete lag time prior to any gas being released.

Metabolizable energy (ME, MJ/kg DM) was estimated according to the method of Menke & Steingass (1988) as follows:

$$\text{ME} = 2{\cdot}20 + 0{\cdot}136\ \text{GP(ml/0·5 g DM)} + 0{\cdot}057\ \text{CP (g/kgDM)}$$

where GP is net GP in ml from 200 mg of dry sample after 24 h of incubation.

The partitioning factor at 24 h of incubation (PF_{24}; a measure of fermentation efficiency) was calculated as the ratio of DM degradability *in vitro* (mg) to the volume (ml) of GP at 24 h (i.e., *in vitro* DM disappearance (DMD)/total GP (GP_{24})) according to Blümmel *et al*. (1997).

Statistical analyses

For each end-point studied, and for each TMR, values recorded from the three repetitions within each incubation run were averaged. Thus, within each TMR there were three replicates per treatment (each corresponding to the average value recorded at each incubation run) and each replicate was considered as an experimental unit. Results of *in vitro* GP and rumen fermentation parameters were analysed as a factorial experiment using the PROC GLM option of SAS (2002) as:

$$Y_{ijk} = \mu + R_i + A_j + (R \times A)_{ij} + E_{ijk}$$

where Y_{ijk} is every observation of the ith ration type (R_i) with jth *C. vulgaris* level (A_j), μ is the general mean, $(R \times A)_{ij}$ is the interaction between ration type and *C. vulgaris* level and E_{ijk} is the experimental error. Linear (i.e. additive = values are average) and quadratic (i.e. synergistic = values are higher than the average) polynomial contrasts were used to examine responses of different silage to concentrate ratios to increasing addition levels of *C. vulgaris*. Statistical significance was declared at $P < 0{\cdot}05$.

RESULTS

In vitro gas production

In vitro GP (ml/g DM) of the three TMR with different C : S ratios at different levels of *C. vulgaris* addition is shown in Fig. 1. There were significant interactions ($P < 0{\cdot}05$) between ration type × *C. vulgaris* level for the asymptotic GP and CH_4 production at 48 h of incubation (Table 3). Increasing the concentrate portion of the TMR linearly increased ($P < 0{\cdot}001$) the asymptotic GP and decreased ($P < 0{\cdot}001$) the rate of GP without affecting lag time. Addition of *C. vulgaris* to the 25C : 75S TMR increased the asymptotic GP (quadratic effect, $P = 0{\cdot}047$). On the other hand, addition of *C. vulgaris* to the 50C : 50S TMR decreased the asymptotic GP (quadratic effect, $P = 0{\cdot}021$). There were no effects of *C. vulgaris* addition to the 75C : 25S TMR on GP (Table 3).

Increasing the concentrate portion in the TMR linearly increased ($P < 0{\cdot}001$) GP, CH_4, CO_2 and GP

https://doi.org/10.1017/S0021859616000812

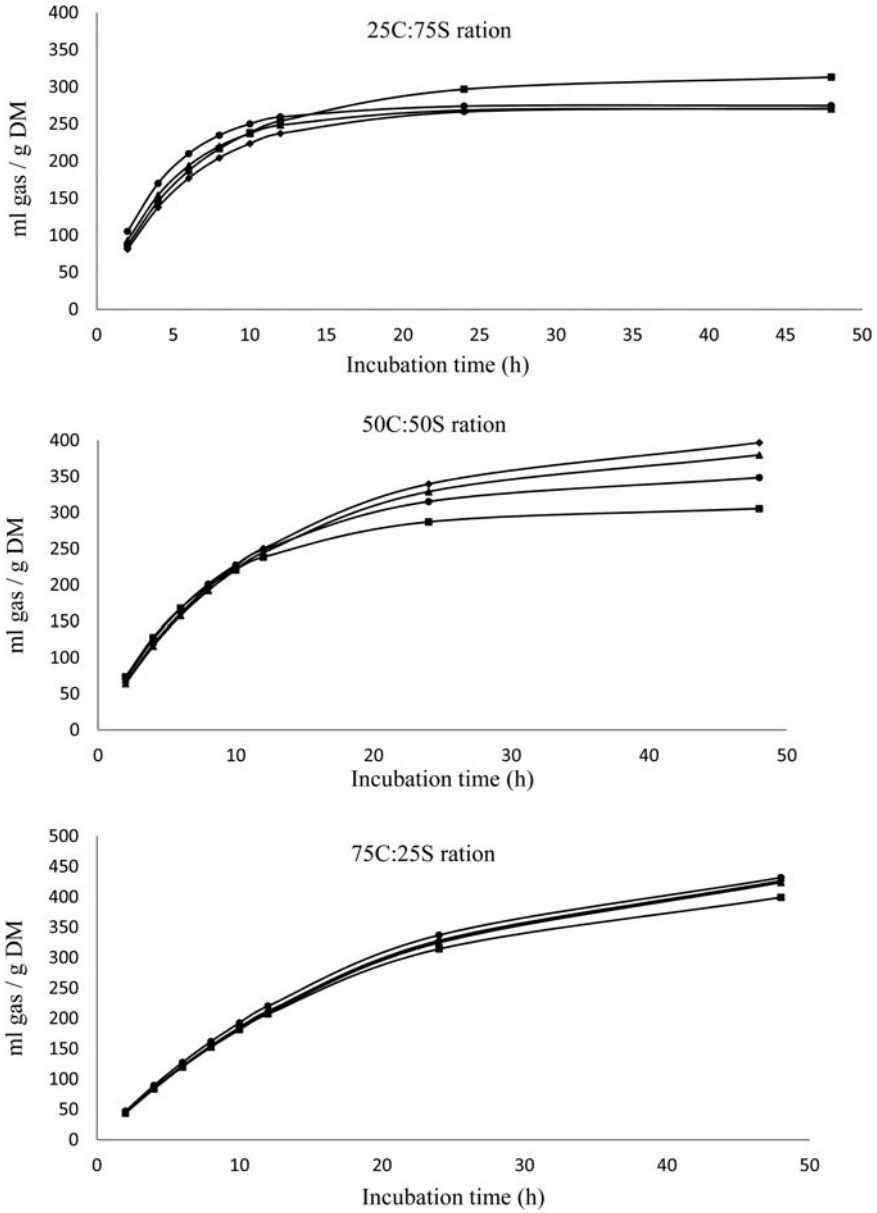

Fig. 1. The effects of three total mixed rations with different concentrate (C) to maize silage (S) ratios and *C. vulgaris* algae addition at 0 (-◆-), 20 (-■-), 40 (-▲-) and 80 (-●-) mg/g DM of the diet on *in vitro* gas production (ml/g DM).

at 48 h of incubation. The highest (quadratic effect, $P = 0{\cdot}035$) CH_4 production at 48 h of incubation was observed with 20 mg/g DM for the 25C : 75S TMR, 40 mg/g DM for the 50C : 50S ration and 80 mg/g DM for the 75C : 25S ration. There were no effects of *C. vulgaris* addition on CO_2 production at 48 h of incubation (Table 3).

In vitro methane and carbon dioxide production

In vitro CH_4 production (ml/g DM) of the three TMR with different C : S ratios at different levels of *C. vulgaris* addition is shown in Fig. 2. There were significant interactions ($P < 0{\cdot}05$) between ration type × *C. vulgaris* level for CH_4 production at 12 and 24 h of incubation (Table 3). Ration type quadratically affected CH_4 production at 24 h ($P = 0{\cdot}020$) and 48 h ($P = 0{\cdot}042$) of incubation. Algae addition had no effect on CH_4 production. For the 25C : 75S TMR, addition of *C. vulgaris* at 80 mg/g DM had the lowest (quadratic effect, $P = 0{\cdot}022$) CH_4 production at 10 h of incubation. On the other hand, addition of *C. vulgaris* at all levels to the 50C : 50S ration linearly increased ($P = 0{\cdot}009$) CH_4 production. Linear

https://doi.org/10.1017/S0021859616000812

Table 3. *The effects of three total mixed rations with different concentrate (C) to maize silage (S) ratios and different levels of microalgae* Chlorella vulgaris *on* in vitro *gas production (GP) kinetics, total GP, methane (CH_4) and carbon dioxide (CO_2) production at 48 h of incubation*

		GP parameters			Gas, CH_4 and CO_2 production (ml/g DM) at 48 h of incubation		
Ration	Algae (mg/g DM)	*A* (ml/g DM)	*c* (ml/h)	*L* (h)	GP	CH_4	CO_2
25C : 75S	0	271	0·18	1·7	270	40	158
	20	316	0·18	1·7	315	46	203
	40	270	0·21	1·5	271	40	172
	80	275	0·24	1·5	279	35	153
	S.E.M.	12·8	0·027	0·33	11·6	5·3	17·3
	Linear	0·981	0·452	0·671	0·028	0·936	0·601
	Quadratic	0·047	0·587	0·844	0·148	0·399	0·109
50C : 50S	0	414	0·10	1·5	397	56	226
	20	309	0·14	1·5	306	48	179
	40	393	0·10	1·5	380	69	249
	80	357	0·12	1·1	349	62	245
	S.E.M.	26·2	0·035	0·38	19·1	11·7	33·0
	Linear	0·802	0·969	0·966	0·011	0·449	0·645
	Quadratic	0·021	0·364	0·969	0·207	0·339	0·184
75C : 25S	0	469	0·050	1·9	427	64	274
	20	432	0·055	1·8	399	49	225
	40	468	0·049	1·0	424	52	244
	80	469	0·053	1·4	432	68	311
	S.E.M.	25·5	0·0025	0·53	20·4	4·6	20·5
	Linear	0·976	0·788	0·282	0·930	0·108	0·330
	Quadratic	0·277	0·115	0·639	0·329	0·164	0·212
P value							
Ration							
Linear		<0·001	<0·001	NS	<0·001	<0·001	<0·001
Quadratic		NS	NS	NS	NS	NS	NS
Algae							
Linear		NS	NS	NS	NS	NS	NS
Quadratic		0·029	NS	NS	NS	0·035	NS
Ration × Algae		0·007	NS	NS	NS	0·038	NS

NS, not significant; *A*, asymptotic gas production; *c*, rate of GP; *L*, the initial delay before GP begins.

reductions in CH_4 production were observed at 12 h ($P = 0·005$), 24 h ($P = 0·013$) and 48 h ($P = 0·029$) of incubation with the 75C : 25S ration (Table 4).

In vitro CO_2 production (ml/g DM) of the three TMR with different C : S ratios at different levels of *C. vulgaris* addition is shown in Fig. 3. There were significant interactions ($P < 0·05$) between ration type × *C. vulgaris* level for CO_2 production at 2, 6, 10, 12, 24 and 48 h of incubation (Table 4). Addition of *C. vulgaris* linearly increased CO_2 production at 4 h ($P = 0·028$) and 6 h ($P = 0·039$) of incubation. Ration type linearly increased ($P = 0·013$) CO_2 production at 2 h of incubation. For the 25C : 75S ration, *C. vulgaris* addition had no effect on CO_2 production at all incubation times. For the 50C : 50S ration, addition of *C. vulgaris* linearly increased CO_2 production at 10 h ($P = 0·035$) and 12 h ($P = 0·048$) of incubation. Addition of *C. vulgaris* to the 75C : 25S ration linearly increased CO_2 production at 10 h ($P = 0·035$) and 12 h ($P = 0·045$) of incubation (Table 4).

Fermentation kinetics

No interactions were observed between ration type and *C. vulgaris* addition for measured fermentation parameters, except for pH ($P = 0·001$) which increased for the 25C : 75S and 75C : 25S rations but remained the same for 50C : 50S ration (Table 5). Addition of

https://doi.org/10.1017/S0021859616000812

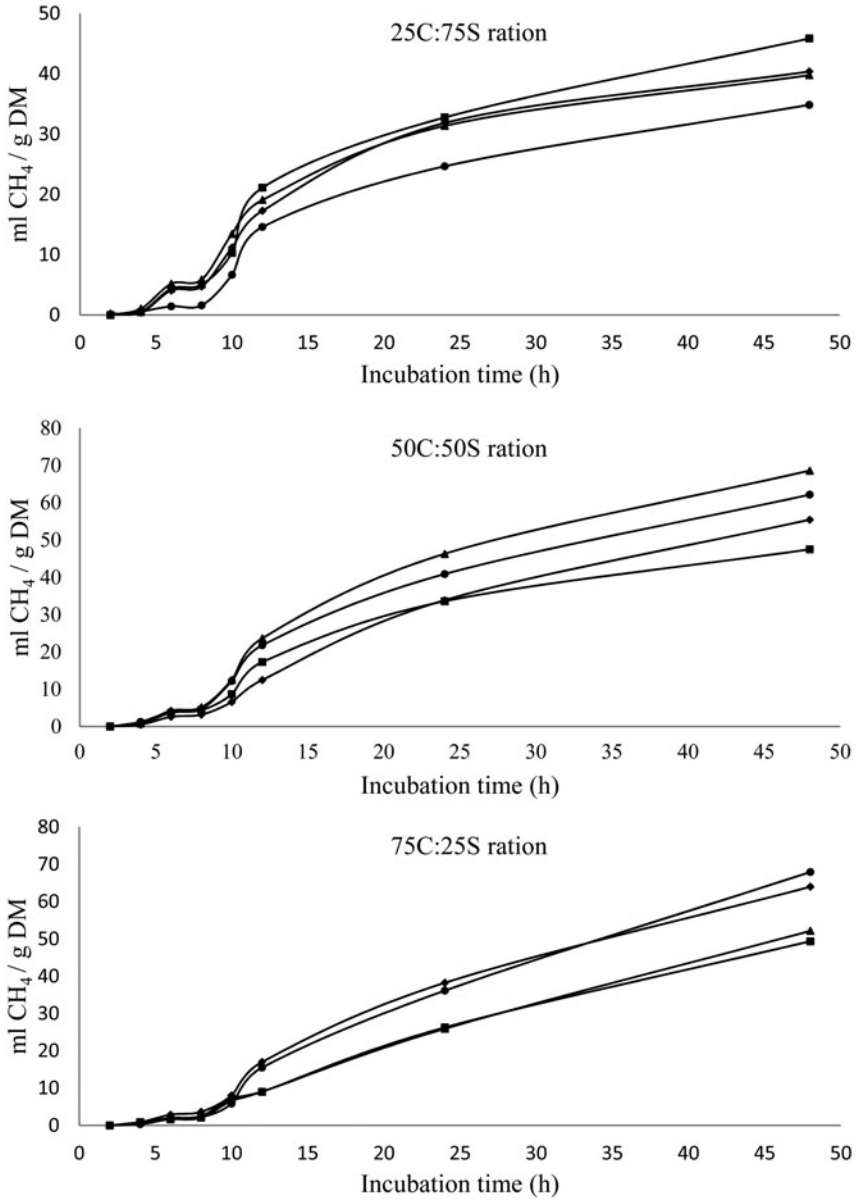

Fig. 2. The effects of three total mixed rations with different concentrate (C) to maize silage (S) ratios and *C. vulgaris* algae addition at 0 (-◆-), 20 (-■-), 40 (-▲-) and 80 (-●-) mg/g DM of the diet on *in vitro* methane (CH_4) production (ml/g DM).

C. vulgaris had no effect on DMD for the 75C : 25S ration but decreased DMD for the 25C : 75S ration (quadratic effect, $P = 0{\cdot}009$) and 50C : 50S (linear effect, $P = 0{\cdot}026$) ration. Addition of *C. vulgaris* had no effect on ME or PF_{24} for all rations (Table 5).

DISCUSSION

Chemical composition of *Chlorella vulgaris* algae

The chemical composition of *C. vulgaris* algae in the present study was consistent with some studies but not with others. Fiogbe *et al.* (2004) reported that *C. vulgaris* algae contained (/kg DM): 200–255 g CP, 31 g fat, 349 g carbohydrates and 85–117 g cellulose, with a good profile of essential amino acids and rich content of some vitamins. Janczyk *et al.* (2006) reported that *C. vulgaris* algae contained (/kg DM) 528 g CP, 81 g fat, 56 g carbohydrates, 208 g fibre, 251 g saturated fatty acids (SFA), 157 g mono-SFA and 585 g poly-SFA. Becker (2007) reported that *C. vulgaris* had a high protein content with a balanced amino acids profile compared with other referenced food proteins, e.g. soybean and egg. In their review,

https://doi.org/10.1017/S0021859616000812

Table 4. *The effects of three total mixed rations with different concentrate (C) to maize silage (S) ratios and different levels of microalgae* Chlorella vulgaris *on proportional methane (CH_4) and carbon dioxide (CO_2) production (ml/100 ml gas) after 48 h of incubation*

		Proportional CH_4 production at								Proportional CO_2 production at							
Ration	Algae (mg/g DM)	2 h	4 h	6 h	8 h	10 h	12 h	24 h	48 h	2 h	4 h	6 h	8 h	10 h	12 h	24 h	48 h
25C : 75S	0	0·3	0·3	2·3	2·3	5·0	7	12	15	9	11	21	30	35	45	50	59
	20	0·0	0·7	2·3	2·3	4·3	8	11	15	7	15	25	32	42	49	55	65
	40	0·0	0·3	2·7	2·7	5·7	8	12	15	9	16	26	35	40	49	55	63
	80	0·0	0·3	0·7	0·7	2·7	6	9	13	5	11	18	25	32	39	46	0·6
	S.E.M.	0·17	0·33	0·33	0·33	0·29	1·1	1·2	2·0	1·1	1·8	1·9	2·2	3·9	3·6	4·7	4·3
	Linear	0·195	0·500	0·500	0·500	0·141	0·840	0·851	0·911	0·866	0·077	0·095	0·108	0·401	0·459	0·530	0·456
	Quadratic	0·438	0·694	0·694	0·694	0·022	0·564	0·745	0·948	0·169	0·560	0·521	0·886	0·383	0·752	0·609	0·478
50C : 50S	0	0·0	0·3	1·7	1·7	3·0	5·0	10	14	7·4	12	18	26	31	41	48	58
	20	0·0	0·7	2·3	2·3	4·0	7·3	11	15	6·8	12	21	27	34	43	50	58
	40	0·0	0·7	2·7	2·7	5·7	9·7	14	18	6·3	13	22	32	41	51	58	66
	80	0·0	1·0	2·3	2·3	5·3	8·7	13	17	8·2	17	25	34	42	52	60	70
	S.E.M.	0·00	0·29	0·53	0·53	0·90	0·96	1·3	1·7	0·78	2·1	2·2	2·9	2·9	3·1	3·6	3·4
	Linear	1·000	0·438	0·217	0·217	0·069	0·009	0·068	0·135	0·332	0·648	0·182	0·144	0·035	0·048	0·083	0·156
	Quadratic	1·000	0·650	0·803	0·803	0·769	1·000	0·845	0·757	0·903	0·880	0·649	0·647	0·560	0·475	0·432	0·433
75C : 25S	0	0·0	1·0	2·3	2·3	4·3	8·0	11·7	15·0	4	12	21	30	40	49	55	64
	20	0·0	1·0	1·3	1·3	3·7	4·3	8·3	12·3	4	13	18	27	30	40	39	56
	40	0·0	1·0	1·7	1·7	4·0	4·3	8·0	12·3	7	16	22	29	30	40	40	58
	80	0·0	0·3	1·3	1·3	3·0	7·0	10·7	15·7	8	16	25	34	44	54	54	72
	S.E.M.	0·00	0·17	0·33	0·33	0·80	0·69	0·82	0·71	1·1	1·5	1·8	2·6	2·7	2·6	2·6	2·8
	Linear	1·000	1·000	0·195	0·195	0·776	0·005	0·013	0·029	0·110	0·109	0·721	0·932	0·035	0·045	0·159	0·145
	Quadratic	1·000	1·000	0·141	0·141	0·623	0·061	0·172	0·162	0·226	0·459	0·165	0·417	0·155	0·167	0·226	0·195
P value																	
Ration																	
Linear		NS	0·041	NS	NS	NS	NS	NS	NS	0·013	NS	NS	NS	NS	NS	NS	NS
Quadratic		NS	NS	NS	NS	NS	NS	0·020	0·042	NS	NS	NS	NS	NS	NS	NS	NS
Algae																	
Linear		NS	NS	NS	NS	NS	NS	NS	NS	NS	0·028	0·039	NS	NS	NS	NS	NS
Quadratic		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ration × Algae		NS	NS	NS	NS	NS	0·002	0·031	NS	0·013	NS	0·031	NS	0·003	0·003	0·028	0·021

DM, dry matter; NS, not significant.

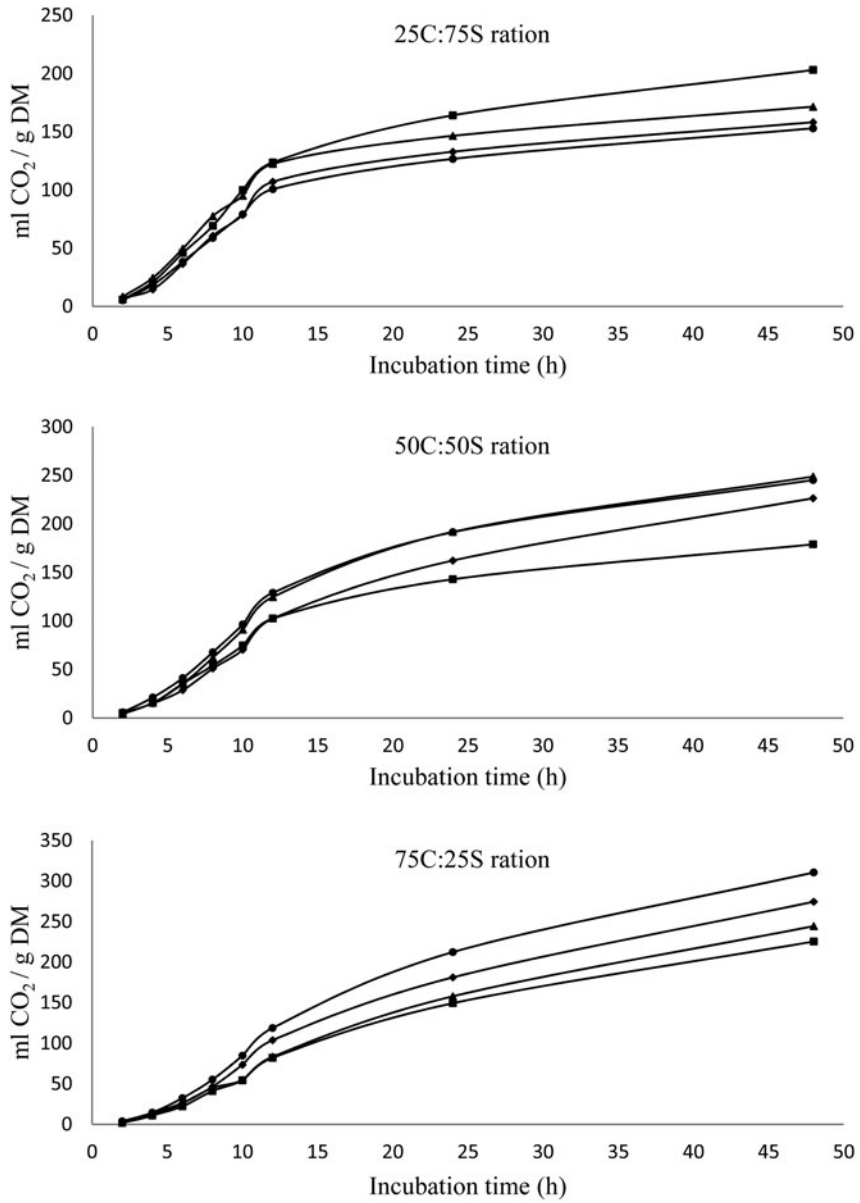

Fig. 3. The effects of three total mixed rations with different concentrate (C) to maize silage (S) ratios and *C. vulgaris* algae addition at 0 (-◆-), 20 (-■-), 40 (-▲-) and 80 (-●-) mg/g DM of the diet on *in vitro* carbon dioxide production (ml/g DM).

Priyadarshani & Rath (2012) reported that *C. vulgaris* contains (/kg DM) about 410–580 g CP, 120–170 g carbohydrate and 100–220 g fat. The differences observed between these reports and the results in the present study may be due to different cultivation conditions and nutrition (Priyadarshani & Rath 2012). Protein is the most expensive nutrient in animal feed, thus developing natural alternatives to conventional protein meals may be cost-effective. Among all dietary amino acids in ruminant nutrition, lysine and methionine are the first and second limiting amino acids, respectively. The profile of amino acids in *C. vulgaris* shows relatively high amounts of lysine and methionine (Lum *et al*. 2013; Kholif *et al*. in press).

Influence of ration type on *in vitro* gas production

The interaction between ration type × *C. vulgaris* level suggests that the fermentation kinetics are ration- and algae-level-dependent, thus underpinning the importance of identifying optimal supplemental levels of *C. vulgaris* for each ration type.

Rations with higher concentrate portions had higher asymptotic GP with lower rates of GP compared with

https://doi.org/10.1017/S0021859616000812

Table 5. *The effects of three total mixed rations with different concentrate (C) to maize silage (S) ratios and different levels of microalgae* Chlorella vulgaris *on* in vitro *rumen fermentation profile after 48 h of incubation*

Ration	Algae (mg/g DM of diet)	pH	DMD (mg/g DM)	ME (MJ/kg DM)	PF_{24} (mg DMD/ml gas)
25C : 75S	0	6·8	560	10·2	5·11
	20	6·7	667	11·0	5·02
	40	6·7	655	10·3	5·10
	80	6·7	665	10·4	5·08
	S.E.M.	0·22	14·0	0·39	0·046
	Linear	0·087	0·001	0·921	0·892
	Quadratic	0·333	0·009	0·138	0·154
50C : 50S	0	6·65	580	12·2	4·89
	20	6·65	642	10·8	5·03
	40	6·68	635	11·9	4·92
	80	6·65	638	11·6	4·96
	S.E.M.	0·021	22·0	0·72	0·070
	Linear	0·212	0·026	0·785	0·771
	Quadratic	0·456	0·404	0·184	0·192
75C : 25S	0	6·7	588	11·6	4·91
	20	6·7	598	11·3	4·96
	40	6·8	679	11·6	4·92
	80	6·8	602	11·9	4·89
	S.E.M.	0·33	39·1	0·35	0·036
	Linear	0·431	0·142	0·853	0·885
	Quadratic	0·481	0·483	0·462	0·398
P value					
Ration					
Linear		NS	NS	0·005	0·001
Quadratic		0·001	NS	NS	NS
Algae					
Linear		NS	0·004	NS	NS
Quadratic		NS	NS	0·046	NS
Ration × Algae		0·001	NS	NS	NS

DMD, *in vitro* dry matter disappearance; ME, metabolizable energy; PF_{24}, partitioning factor at 24 h of incubation; NS, not significant.

high silage rations, implying an effect of the chemical composition of the feeds, in particular its fibre and protein contents, on GP and fermentation kinetics (Elghandour *et al*. 2014, 2015*a*). Gas production is generally a good indicator of digestibility, fermentability and microbial protein production (Rodriguez *et al*. 2015). Higher proportions of concentrates in the rations indicate a better nutrient availability for rumen microorganisms (Elghandour *et al*. 2014), which will stimulate the degradability of different nutrients (Hamid *et al*. 2007). Increasing fibre content as a result of increased maize silage portion may have negative effects on microbial growth and fermentation due to the decreased readily available energy and protein content and increased structural carbohydrates content of those rations (Elghandour *et al*. 2015*b*), causing a decrease in ration digestibility and fermentability (Kumar *et al*. 2013). Elghandour *et al*. (2015*a*, *b*) observed that increasing the maize silage portion in TMRs, instead of concentrate, lowered GP and negatively affected fermentation.

Influence of *Chlorella vulgaris* level on *in vitro* gas production

Addition of *C. vulgaris* at 20 mg/g DM to the 25C : 75S ration increased GP, which suggests increased ruminal microbial activity. Dubois *et al*. (2013) observed that algae rich in protein content increased GP in the rumen. *Chlorella vulgaris* contain a unique

https://doi.org/10.1017/S0021859616000812

phytonutrient called *Chlorella* growth factor (CGF), which is concentrated in the nucleus of the algae cells. It comprises nucleic acid associated with peptides, proteins, amino acids, vitamins and sugars, and it is an agent for improved growth in bacteria (Kotrbáček *et al*. 2015). Addition of *C. vulgaris* at 20 mg/g DM (low level of *C. vulgaris* in the present study) had a positive impact on ruminal fermentation compared to the higher levels. Moreover, *C. vulgaris* contains β-glucan, which has a role in scavenging free radicals (Iwamoto 2004), thus improving fermentation.

Chlorella vulgaris at high levels has been recognized as an antimicrobial agent that acts against bacteria, protozoa and fungi, thus resulting in reduced fermentation activity. In the present study, increasing *C. vulgaris* levels negatively influenced GP. Microalgae contain toxic metabolites (phycotoxins), which have antibiotic and antifungal activities (Garcia-Camacho *et al*. 2007). Janczyk *et al*. (2009) showed that *C. vulgaris* had a high antimicrobial activity due to the presence of cyclic peptides, alkaloids and lipopolysaccharides, in addition to the presence of polysaccharides, phenolic substances and aromatic compound. This supports the hypothesis that an optimal *C. vulgaris* level could improve fermentation efficiency. The high nucleic acid content in algal cells may be another reason for the negative effect on fermentation with increasing algae levels.

The increased asymptotic GP and rate of GP without affecting lag time could be due to the presence of oligosaccharides, sugar sources and non-protein nitrogen in the algae, which can improve the growth of bacteria to stimulate microbial activity.

Addition of *C. vulgaris* was also more effective for TMR with a high silage (roughage) portion than those with high concentrate content. It was expected that low concentrate diets would have better fermentation with algae addition due to better nutrient availability for rumen microorganisms to stimulate the degradability of nutrients. Low-quality TMR such as 25C : 75S lack the nutrients for ruminal microflora growth activity and it is postulated that the addition of algae provided more nutrients for microbial growth and activity. In case of high concentrate TMR, the incubation medium already contained adequate nutrients required for microbial activity, and the addition of more nutrients from the algae had no effect on microbial activity. This suggests that algae supplementation is more effective with poor quality TMR than high quality TMR.

Influence of ration type on *in vitro* carbon dioxide and methane production

Fermentation of dietary carbohydrates produces gases in the rumen, composed of hydrogen, CO_2 and CH_4. In the present study, ration type had no effect on CO_2 production. However, increasing silage portion in the TMR increased total gas and CH_4 production. This was expected, because the digestion of fibrous rations results in the preferential production of acetate, butyrate and CH_4 compared to a concentrate ration (Kumar *et al*. 2013). The methanogenic *Archaea* can utilize hydrogen gas (H_2) produced from the ruminal degradation of structural carbohydrates for CH_4 production (Stewart *et al*. 1997). Furthermore, hydrogen-consuming acetogenic bacteria are able to use H_2 as an energy source for growth using CO_2, as H_2 and CO_2 are the dominant substrates of methanogenesis (Morgavi *et al*. 2010).

Influence of *Chlorella vulgaris* on *in vitro* methane and carbon dioxide productions

The 40 mg algae/g DM level of *C. vulgaris* addition decreased CH_4 production of the 25C : 75S TMR. This may be related to the decreased DMD with this level of *C. vulgaris* addition (Anele *et al*. 2016). As previously mentioned, decreased DMD may have resulted in decreased GP for the 25C : 75S TMR. Goel & Makkar (2012) suggested that CH_4 production was associated with the increase in fermented and digested feed nutrients. Besides this, and due to its high eicosapentaenoic and docosahexaenoic (DHA) contents, *C. vulgaris* has been considered as a possible additive for reduction of CH_4 emissions (Tsiplakou *et al*. 2016). This may be related to its content of unsaturated fatty acids, resulting in reduced CH_4 production (Martin *et al*. 2010). Fievez *et al*. (2007) observed up to 80% reduction in CH_4 production with the addition of a DHA-rich supplement. Tsiplakou *et al*. (2016) observed an increased methanobacteria and protozoa population in the rumen liquid of goats fed a forage-based diet supplemented with *C. vulgaris*. Anele *et al*. (2016) observed a negative correlation between CH_4 production and microalgae content of carbohydrate, oleic acid (C18 : 1n–9) and α-linolenic acid (C18 : 3n–3).

Addition of *C. vulgaris* at 80 mg/g DM increased CO_2 production with both 50C : 50S and 75C : 25S TMR. However, the levels of 20 and 40 mg/g DM of *C. vulgaris* addition with the 75C : 25S TMR decreased CO_2 production, suggesting that the effect of

https://doi.org/10.1017/S0021859616000812

C. vulgaris microalgae is ration-type and -level dependent. Generally, microalgae lack lignin (Chen *et al*. 2013), which gives them the ability to sequester more CO_2 into digestible biomass, e.g. carbohydrate, protein and lipids (Walker 2009) and may be used to produce biogas including CH_4 and hydrogen via anaerobic processing (Hughes *et al*. 2012), suggesting their potential as a strategy for carbon capture from fossil fuel manufacturing facilities (Sayre 2010).

Influence of ration type on *in vitro* fermentation kinetics

Improved fermentation with the 50C : 50S TMR could be because of the balanced concentration of nutrients, especially structural and non-structural carbohydrates (Elghandour *et al*. 2015*a*, *b*).

Influence of *Chlorella vulgaris* on *in vitro* fermentation kinetics

Improved ruminal fermentation with *C. vulgaris* addition at 20 mg/g DM was associated with increased activity of ruminal microbes. It has been shown that *C. vulgaris* contains growth-promoting substance such as *S*-nucleotide adenosyl peptide complex, which may affect nutrient digestibility of the animals (Yan *et al*. 2012). The addition of *C. vulgaris* might have provided the necessary nutrients required by the microbes to effectively degrade the TMRs for better fermentation (Anele *et al*. 2016). Halama (1990) suggested that the algal content of polysaccharides, phenolic substances and aromatic compounds had a nutritional and ecological importance to the fed animals. Tsiplakou *et al*. (2016) observed changes in cellulolytic and proteolytic bacteria with modifications in the cellulase and protease activity in the rumen liquid of goats receiving *C. vulgaris*. In addition, Carro & Miller (1999) demonstrated that peptides and amino acids are stimulatory factors for ruminal microbial growth and digestion. Drewery *et al*. (2014) reported increased OM digestibility with increasing levels of microalgae residue supplementation in steers fed oat straw. Tibbetts *et al*. (in press) reported that the dietary effects of algal supplementation on feed digestibility in ruminants are related in part to its lipid content. Inclusion of *C. vulgaris* increased the concentration of some bacteria *in vitro* (Fievez *et al*. 2007) and *in vivo* (Tsiplakou *et al*. 2016).

It can be concluded that *C. vulgaris* could be used as a feed additive to improve fermentation and feed utilization. The optimal level of *C. vulgaris* addition was 20 mg/g DM. *Chlorella vulgaris* addition was more effective with rations higher in fibre content than with rations high in concentrates. However, animal feeding trials are required to validate *in vivo* the utilization of *C. vulgaris* microalgae on animal performance.

The first author (A.E. Kholif) would like to thank the Mexican National Council for Science and Technology (CONACyT, Mexico) and The World Academy of Sciences (TWAS, Italy) for supporting his postdoctoral fellowship at the Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México.

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