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The effect of *Ulva lactuca* on *in vitro* ruminal gas production kinetics

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Abstract

The aim of this study was to determine whether the inclusion of a marine algae found in South African waters, *Ulva lactuca*, can reduce total gas and methane production *in vitro* when it replaces lucerne hay in a mixed sheep feed at incremental levels. Four treatments were prepared and incubated using bovine rumen fluid as inoculum: (1) 0 g *U. lactuca* kg^{-1} feed dry matter (DM) (0U), (2) 25 g *U. lactuca* kg^{-1} feed DM (25U), (3) 50 g *U. lactuca* kg^{-1} feed DM (50U), and (4) 100 g *U. lactuca* kg^{-1} feed DM (100 U). Total gas and CO_2 production was determined with the aid of an automated system and methane production was estimated by difference. Cumulative gas production data were fitted to a non-linear model ($Y = b(1-\exp^{-c(t-L)})$) to estimate values for total gas production (b, mL), rate of gas production (c, mL/h), and a discrete lag time (L, hours). The extent of total gas production was lower for 100U than for 25U, but neither differed from 0U or 50 U. The lag time observed was lower for 50U than 0U, but neither differed from 25U or 100U. No differences were found for the rate of gas production. No differences for any gas production values were observed between treatments. The ratio between methane and total gas production was highest for 100U, which differed from 25U, but not from 0U or 50U. The higher methane ratio observed in the 100U treatment may be attributed to the lower total gas production in this treatment due to the lower fermentability of *U. lactuca* compared to lucerne.

Keywords: climate change, global warming, greenhouse gases, *in vitro*, marine algae, ruminants, seaweed
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Introduction

Seaweed has been consumed by humans across the globe for centuries and has become increasingly popular in recent years due to its alleged nutritional and health benefits (Sugumaran *et al.*, 2022). The global seaweed industry is worth US$6 billion, with the majority of this being for use as human food (McHugh, 2003). In 2018, 32.4 million tonnes of seaweed were used globally (FAO, 2020; Chopin & Tacon, 2021), in contrast to 19.9 million tonnes in 2010 (FAO, 2012; Paul & Tseng, 2012). Of the former, 97.1% originated from commercial seaweed farms. Despite Africa's rich diversity of seaweed species and approximately 30 000km of shoreline, its contribution to the development of the seaweed industry is insignificant (FAO, 2002, 2010b; Kadinkiz & Uzun, 2023).

According to Amosu *et al.* (2013), the South African seaweed industry has existed for over 60 years. Wild harvesting of *Ecklonia maxima* (Osbeck) Papenfuss and *Laminaria pallida* Greville ex J. Agardh started in the 1940s when there was a scarcity of kelp (and consequently, alginate) in Japan during the Second World War (McHugh, 1987). Hand-picking of *Gelidium* species started in 1957 in the Eastern Cape, of which most were exported to Europe, North America, and Asia for the extraction of agar (Anderson *et al.*, 1989). Powdered kelp is exported to Japan for use in formulated fish feed, whereas wet kelp is harvested along the west coast of South Africa to produce plant-growth stimulants,

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soil conditioners, and abalone feed (Anderson *et al.*, 1989, 2003; Robertson-Andersson *et al.*, 2006, Troell *et al.*, 2006; Khan *et al.*, 2009). Seaweed and associated extracts are also used in food and phycocolloid production (Dhargalkar & Verlecar, 2009), in molecular and microbiology (Amosu *et al.*, 2013), in cosmetic creams and pharmaceuticals (Bhakuni & Rawat, 2005; Leonel, 2011; Lewis *et al.*, 2011), as well as in the medical field (Stein & Borden, 1984; Khan & Satam, 2003; Barsanti & Gualtieri, 2006).

The cultivation of seaweed in South Africa started and increased with the development of the abalone industry (Amosu *et al.*, 2013). The industry has grown from exploitation of only six genera of seaweed (*Ecklonia*, *Gracilaria*, *Gelidium*, *Gigartina*, *Laminaria,* and *Porphyra*) in the 1950's to nine genera to date (*Ecklonia*, *Gracilaria*, *Gracilariopsis*, *Gelidium*, *Gigartina*, *Laminaria*, *Plocamium*, *Porphyra,* and *Ulva*). Southern Africa has one of the richest marine algae specie diversities in the world, with 900 known species (Payne *et al.*, 1989; Bolton, 1999; Bolton *et al.*, 2003; Maneveldt *et al.*, 2008). The cool, temperate, rocky shore of the west coast of South Africa is dominated by kelp forests, mainly *Ecklonia maxima* and *Laminaria pallida* (Bolton & Anderson, 1997). The southern coast has a warm temperate biota with high seaweed endemism (Anderson *et al.*, 2009), whereas most species of the extreme east of South Africa are of tropical affinity (Bolton *et al.*, 2004). Currently, the most important seaweed in South Africa is fresh kelp (mainly *E. maxima* and *L. pallida*) for use as feed in abalone aquaculture and the production of agricultural growth stimulants via the collection of beach cast kelp for alginate and the small harvest of the red seaweed, *Gelidium pristoides,* for the production of agar (Anderson *et al.*, 2003; Rothman *et al.*, 2020). *Ulva* species, rich in bioactive compounds (Maray *et al.*, 2023), are by far the most cultivated marine algal species in southern Africa, flourishing in paddle-wheel raceways on abalone farms (Chopin *et al.*, 2008). Official figures of production volumes are not obtained from farms by the government as the *Ulva* is not sold but is instead used directly as abalone feed. Current wet production has, however, been estimated at approximately 2000 tonnes per annum (Rothman *et al.*, 2020).

Increased pressure to reduce methane emissions from ruminants in recent years to slow the global warming phenomenon has led to studies on the effect of seaweed as a dietary supplement to reduce methane emissions. Several species with a strong potential to reduce methane emissions have been identified. Specific species include *Ascophyllum nodosum*, *Alarium esculenta*, *Chondrus crispus* and, most notably, *Asparagopsis taxiformis* (Wang *et al.*, 2008; Machado *et al.*, 2014a; Kinley *et al.*, 2016; Ramin *et al.*, 2019). A study by Kinley *et al.* (2020) demonstrated that the inclusion of *A. taxiformis* in the diet of steers at a rate of 0.10% and 0.20% decreased methane emissions by 40% and 98%, while improving live weight gain by 53% and 42%, respectively. A previous study showed that pure *Ulva* spp. reduced total gas production by 21–29% and methane production by 45–50%, compared to decorticated cottonseed meal (Machado *et al.*, 2014a). Another study found no difference in *in vitro* total gas and methane production between diets containing 0%, 3%, and 5% *Ulva lactuca* (El-Waziry *et al.*, 2015). In the South African context, brown algal species are allocated to existing industries of great economic importance, making it unlikely to become available for use in the animal feed industry. Red algal species, which have been shown to reduce methane emissions in ruminants, are not currently cultivated in South Africa. This creates the opportunity for green alga species, such as *Ulva*, to be studied, both *in vivo* and *in vitro*.

According to Cone *et al.* (1996), the technique of *in vitro* fermentation was originally developed as a method to evaluate the nutritive value of forages and feeds by the determination of degradation of different feed components. Gas measurements have been used increasingly in the study of fermentation kinetics, and they provide important information on how both soluble and insoluble fractions are digested (Getachew *et al.*, 1998). The basic *in vitro* gas production technique (IVGPT) simulates ruminal fermentation of feed and feedstuffs under controlled laboratory conditions using natural rumen microbes and has been used for decades (Rymer *et al.*, 2005; Ayasan *et al.*, 2021). The original *in vitro* batch fermentation technique was first detailed by McBee (1953). Tilley and Terry (1963) detailed a two-stage *in vitro* method to evaluate forages and to determine the organic matter degradability of feedstuffs with microorganisms, which became the standard on which future methods for evaluation of ruminant feeds were based. However, its limitation was that it could not provide information on the kinetics of forage fermentation without applying long duration, labour-intensive studies (Tilley & Terry, 1963; Theodorou *et al.*, 1994).

The increase in the interest in greenhouse gas emissions from agriculture in recent years has led to the inclusion of methane production measurements in traditional IVGPTs (Navarro-Villa et al., 2011; Pellikaan et al., 2011). The current basis of modern IVGPT for *in vitro* ruminant methane emission studies is based on Goering and van Soest's further development in 1970 of the Tilley & Terry (1963) method. The basic IVGPT provides an opportunity to determine the potential of feed substrates and

additives to reduce methane productions before using *in vivo* gas measurement techniques, which are costly, time-consuming, and laborious (Getachew *et al.*, 1998; Ayasan *et al.*, 2020).

Considering the current economic value of brown and red seaweeds and their previous allocation to such industries, research into the populous green marine algae in South Africa and its potential to reduce methane emissions is required.

Methods and materials

Ethical approval was granted by the Animal Care and Use Committee of the Stellenbosch University (ACU-2022-24681). Seaweed was harvested from four paddle-wheel raceways at Buffeljags Abalone Farm (Viking Aquaculture) in Gansbaai, South Africa. The collected seaweed was from the species *U. lactuca*, a green algae in the taxon, *Chlorophyta*. Representative samples were taken from 600 kg of hydrated *U. lactuca*. The *U. lactuca* samples were rinsed in fresh water to remove sand, debris, and epiphytes. It was then sun-dried in tunnels for 7 d, followed by oven-drying at 60 °C for 60 h. All samples were milled through a standard laboratory mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec) to pass through a 1-mm screen and subsequently stored in airtight honey jars. After preparation, samples were stored at 4 °C to prevent pest infestation.

Four lamb feedlot diets with increasing amounts of *U. lactuca* were used as substrate treatments to determine the effect on *in vitro* ruminal gas production kinetics: (1) 0U: 0 g *U. lactuca* kg^{-1} DM; (2) 25U: 25 g *U. lactuca* kg^{-1} DM; (3) 50U: 50 g *U. lactuca* kg^{-1} DM; and (4) 100U: 100 g *U. lactuca* kg^{-1} DM. A total mixed ration (TMR) was formulated for each treatment to ensure that the diet was nutritionally balanced (Table 1). Lucerne hay was incrementally replaced by *U. lactuca* (97% DM, 68% organic matter (OM), 260.5 g crude protein (CP) kg^{-1} DM, 219.4 g neutral detergent fibre (NDF) kg^{-1} DM).

Table 1 Physical and chemical composition (g kg^{-1} DM) of the experimental diets

	Treatments[5]			
	0U	**25U**	**50U**	**100U**
Physical composition				
Lucerne hay	300	275	250	200
Barley	420	420	420	420
Molasses syrup	40	40	40	40
Commercial Bypass Protein Concentrate[1]	40	40	40	40
Commercial Sheep Fattening Concentrate[2]	200	200	200	200
Ulva lactuca	0	25	50	100
Artificial Sweetener[3]	0.2	0.2	0.2	0.2
Chemical composition[4]				
Dry Matter (as-is)	901.0	922.9	909.1	904.8
Organic Matter (as-is)	919.8	907.3	911.2	909.5
Ash (as-is)	80.2	92.7	88.8	90.5
Crude Protein	178.5	186.6	203.3	236.7
Ether Extract	21.6	20.5	19.5	21.1
Starch	256.5	261.2	266.5	294.9
NDF*	277.9	316.6	281.8	272.1
ADF*	152.5	175.8	172.6	165.0
Bromoform	Not detected			

* NDF (Neutral detergent fibre); ADF (Acid detergent fibre)
[1] Epol: DM = 880 g kg^{-1}, CP = 300 g kg^{-1}, CF = 120 g kg^{-1}, Ca = 4.5–8 g kg^{-1}, P = 6 g kg^{-1}
[2] Molatek: DM = 870 g kg^{-1}, CP = 320 g kg^{-1}, CF = 100 g kg^{-1}, Ca = 14–45 g kg^{-1}, P = 6 g kg^{-1}, Mg = 2.5 g kg^{-1}, S = 9.9 g kg^{-1}, K = 19 g kg^{-1}, Mn = 150 mg kg^{-1}, Zn = 130 mg kg^{-1}, Co = 0.5 mg kg^{-1}, I = 2 mg kg^{-1}, Se = 1 mg kg^{-1}, Vitamin A = 20 000 IU kg^{-1}, Vitamin D = 5 000 IU kg^{-1}, Vitamin E = 50 IU kg^{-1}, Urea = 55 g kg^{-1}
[3] Pancosma: Flavouring compounds = 243g kg^{-1}, Sodium saccharin E954 = 173g kg^{-1}
[4] Values derived from proximate analysis of feed (AOAC International, 2005)
[5] Treatments: 0U (0g *Ulva* kg^{-1} DM), 25U (25g *Ulva* kg^{-1} DM), 50U (50g *Ulva* kg^{-1} DM), 100U (100g *Ulva* kg^{-1} DM)

Fresh rumen fluid was collected prior to each *in vitro* run from two ruminally-cannulated Holstein dairy cows at 10h00, exactly three hours after the morning feeding. A three-hour feed retention period was selected to ensure rumen fill with fresh TMR and adequate activity of rumen microbes (Weimer, 2017). Cows were from the Welgevallen Experimental Farm's herd of the Stellenbosch University, South Africa. The cows received the same total mixed ration (TMR) and were fed twice daily at 07h00 and 16h00. According to Weimer (2017), the specific diet of the rumen fluid donor is not important, given that all donors receive the same diet for the duration of an *in vitro* trial. Rumen fluid was collected from multiple areas within the rumen and filtered through two layers of cheesecloth prior to being poured into pre-warmed thermos flasks. Flasks were filled to the brim to ensure that the contents remained anaerobic. Upon arrival at the laboratory, the rumen fluid was strained through another two layers of cheesecloth before being pooled in equal amounts to create a single combined rumen fluid inoculant. The pH of the final combined rumen fluid was taken, and it was then continuously gassed with a gentle stream of pure CO_2 gas to maintain an anaerobic environment. Samples from each treatment were fermented with pooled rumen fluid in six different runs over the course of six weeks, resulting in a total of six repetitions per treatment. A blank vial, containing only rumen fluid, was included in each run to determine a correction factor for gas production from the rumen fluid alone without substrate. Pooled rumen fluid pH varied between 6.69 and 6.84 between runs. This small variation can be attributed to various factors, such as a difference in feed and water intake, dominance, and the stage of reproductive cycle on the day of rumen fluid collection (Weimer, 2017).

The laboratory-scale AMPTS II and Gas Endeavour (GE) system (Bioprocess Control, Lund, Sweden) were used to assess the *in vitro* fermentation of the treatment diets and consequently, the effect of *U. lactuca* within the diets. Pre-warmed glass bottle anaerobic digesters (500 mL) containing 4.0 g of substrate were filled with 80 mL rumen fluid and 320 mL *in vitro* solution. Pure CO_2 gas was used to displace O_2 from the reactors to ensure an anaerobic environment by flushing the bottles for 60 s. The thermostatic water bath was used to maintain the temperature at 40 °C and the stirring motors were used to agitate the contents of the 500 mL glass bottles containing the substrate and *in vitro* solution.

The biogas volume measuring device uses the principle of liquid displacement and buoyancy to measure the volumetric biogas flowrate before the biogas is scrubbed in the CO_2-absorption unit. This unit uses a concentrated solution of sodium hydroxide (3M) with a thymolphthalein pH-indicator to remove acid gas fractions including CO_2 and H_2S (Bioprocess Control, 2016). The remaining gas, which would be mainly biomethane with less than 5% hydrogen and nitrogen (Clarke & Reid, 1972), exits the CO_2-absorption unit to be measured at the biomethane volume measuring device, which also uses the principle of water liquid displacement and buoyancy to measure the volumetric biomethane flowrate. The volumetric biogas and biomethane flowrates are automatically normalised to one atmosphere (atm), 0 °C, and zero moisture content (Bioprocess Control, 2016). *In vitro* fermentation of the substrate was allowed to proceed for 96 h. Gas measurements were recorded every 15 min resulting in 384 data points per sample.

All *in vitro* samples were incubated in a buffered incubation medium containing a rumen fluid inoculum, as described by Goering & van Soest (1970) and Van Soest & Robertson (1991). A 0.1% solution of resazurin, acting as a reduction indicator, was prepared by dissolving 0.1 g of resazurin into 100 mL of distilled water (Goering & van Soest, 1970). The solution was stored in a glass container at 4 °C. The reducing solution was prepared in two separate flasks, A and B. The content of each was stirred and left until fully dissolved, followed by the careful addition and mixing of the solution in flask B to that of flask A prior to mixing the buffer solution. The reducing solution was subsequently immediately mixed with the incubation medium prior to the onset of incubation. The final *in vitro* solution had a pH of 7.4. The various solutions that were used are presented in Table 2.

Table 2 Composition and mixing of *in vitro* solutions (Goering & van Soest, 1970)

Reagent	Quantity
1 L Buffer solution:	
Distilled water (dH_2O)	1 L
Ammonium bicarbonate (NH_4HCO_3)	4 g
Sodium bicarbonate ($NaHCO_3$)	35 g
1 L Macromineral solution:	
Distilled water (dH_2O)	1000 mL
Di-sodium hydrogen orthophosphate (Na_2HPO_4) (anhydrous)	5.7 g
Potassium dihydrogen orthophosphate (KH_2PO_4) (anhydrous)	6.2 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.6 g
100 mL Micromineral solution:	
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	13.2 g
Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)	10 g
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	1 g
Ferric chloride hexahydrate ($FeCl_3.6H_2O$)	8 g
12 L Incubation medium (30 samples):	
Distilled water (dH_2O)	6 L
Tryptose	30 g
Micromineral solution	1500 µL
Macromineral solution	3 L
Buffer	3 L
Resazurin	37.5 mL
600 mL Reducing solution (30 samples):	
Flask A:	
Distilled water (dH_2O)	300 mL
Cysteine hydrochloride ($C_3H_7NO_2$ HCl)	3.75 g
Potassium hydroxide pellets (KOH)	150 g
Flask B:	
Distilled water (dH_2O)	300 mL
Sodium sulphide nonahydrate ($NaS.9H_2O$)	3.75 g

A non-linear model that included a lag phase, along with the *Solver* option in Microsoft Office 365 Excel (Version 2304) was used to calculate the kinetic coefficients from *in vitro* gas production data. The model used for the estimations was derived from the one described by Ørskov and McDonald (1979). The model used is shown in Equation 1.

$$\boldsymbol{Y = b(1 - e^{-c(t-L)})} \quad (1)$$

Y = gas volume at time t
b = total gas production (mL g^{-1} DM)
c = rate of gas production (h^{-1})
t = incubation time (hours)
L = lag time (hours)

Potential gas production values (b), gas production rates (c), and lag times (L) were estimated from the non-linear model in Equation 1. Statistica 14 (TIBCO Software Inc. 2020) was used to analyse the model-derived data by means of a one-way ANOVA. Appropriate multiple comparisons (Fisher's LSD) were done *post hoc* to determine where significant differences occurred among the levels of the factors involved. Significance was declared at $P \leq 0.05$, whereas tendencies were declared at $P > 0.05 \leq 0.10$.

Results and Discussion

Gases, such as CH_4 and CO_2, are produced because of carbohydrates that are fermented in the rumen to form volatile fatty acids, of which propionate, acetate, and butyrate are predominant (Getachew et al., 1998). Whereas IVGPT can be used to evaluate the potential digestibility of a feedstuff and the potential gas production from its degradation, IVGPT does not simulate the emissions and digestibility of the entire animal (Storm et al., 2012). Enteric ruminal gases are mostly carbon dioxide

(on average, 65%) and methane (on average, 30%), according to Czerkawski, (1986). The basic IVGPT should thus be used as a first approach to evaluate potential feedstuffs for potential degradation and gas production, but the results should then be used to optimise whole-animal experiments.

The mean values of total gas production (TGP) parameters are presented in Table 3. The total gas volume produced in the 100U treatment was lower than that in 25U treatment. However, the gas volumes did not differ among any of the other treatments. No differences among treatment groups for rate of gas production were observed (Table 3; $P = 0.347$). Lag times for total gas production showed a tendency to differ (Table 3; $P = 0.063$), where the value for the 50U treatment was higher than that for the 0U treatment, but it did not differ from 25U or 100U (Figure 1). Gas production was completed for all treatments within 20–40 h and treatments containing *U. lactuca* produced similar amounts of gas as the standard feedlot lamb diet (Figure 1). The total volume of gas produced was lower for 50U and 100U compared to 0U, but not statistically different. This is in contrast with results obtained by Machado *et al.* (2014b), who reported that TGP from a sample that contained 170 g kg^{-1} of an *Ulva* spp was 22% lower than that of the control which contained no *Ulva*. El-Waziry *et al.* (2015) however, reported no statistical differences in total gas production between samples containing 0, 30, and 50 g kg^{-1} *U. lactuca*, which agrees with the results of the current study. It therefore appears that low inclusion rates of *Ulva* (up to 50 g kg^{-1}) will not impact total gas production, but that the apparently lower fermentability of Ulva vs. lucerne hay would have a negative effect on fermentability of a diet when included at 100 g kg^{-1} or more.

The effect of *U. lactuca* on *in vitro* methane production is shown in Figure 1 and Table 3. No differences were observed for either volume of methane produced, rate of methane production, or lag time (Table 3). This corresponds with the results obtained in a study by El-Waziry *et al.* (2015) where the maximum inclusion of *Ulva* was 50 g kg^{-1}. However, Machado *et al.* (2014b) found that the inclusion of *U. lactuca* at 170 g kg^{-1} reduced *in vitro* methane production by 50.3%. It is therefore speculated that the inclusion of *Ulva* at levels higher than 100 g kg^{-1} would be required to result in a meaningful reduction of *in vitro* methane production.

The ratio between methane production and total gas production indicates the potential ability of *U. lactuca* to reduce enteric methane production. A smaller ratio indicates a sample with a better ability to reduce enteric methane production. A difference for the ratio between methane and TGP was observed (Table 3; P = 0.022). The ratio was largest for 100U, differing from 25U, but not from 0U or 50U. Methane production was approximately half of that of total production for all treatments (Table 3). However, TGP was the lowest in the 100U treatment. This can be explained by the lower fermentability of *U. lactuca* compared to lucerne, which was replaced by *U. lactuca* in the samples. Thus, due to a larger reduction in total gas production for 100U, but no difference in methane production, the M:TG ratio was largest for 100U.

Table 3 The effect of incremental replacement of lucerne hay with *Ulva lactuca* as substrate on *in vitro* gas production parameters

Item[1]	Treatment[2]				*P*-value
	0U	25U	50U	100U	
Total gas production (NmL g^{-1} OM*)					
b	131.4^{ab} ± 4.052	138.7^{a} ± 4.052	129.6^{ab} ± 4.052	121.1^{b} ± 4.052	0.046
c	0.127 ± 0.002	0.125 ± 0.002	0.130 ± 0.002	0.125 ± 0.002	0.347
L	1.37^{x} ± 0.038	1.28^{xy} ± 0.038	1.21^{y} ± 0.038	1.29^{xy}± 0.038	0.063
Methane production (NmL g^{-1} OM*)					
b	63.6 ± 1.585	64.9 ± 1.585	62.4 ± 1.585	61.2 ± 1.585	0.426
c	0.193 ± 0.003	0.194 ± 0.003	0.193 ± 0.003	0.186 ± 0.003	0.395
L	1.09 ± 0.042	1.01 ± 0.042	0.99 ± 0.042	1.05 ± 0.042	0.451
M:TG*	0.49^{ab} ± 0.008	0.47^{a} ± 0.008	0.48^{ab} ± 0.008	0.51^{b} ± 0.008	0.022

Means within rows with different superscripts ^{a,b} differed significantly ($P \leq 0.05$), whereas means with different superscripts ^{x,y} tended to differ ($P \leq 0.10$)

* M – Methane; TG – Total gas; OM – Organic matter

[1] b – Gas volume produced (mL g^{-1} OM); c – Rate of gas produced (mL h^{-1}); L – Lag time (h)

[2] Treatments: 0U (0 g *Ulva* kg^{-1} DM), 25U (25 g *Ulva* kg^{-1} DM), 50U (50 g *Ulva* kg^{-1} DM), 100U (100 g *Ulva* kg^{-1} DM)

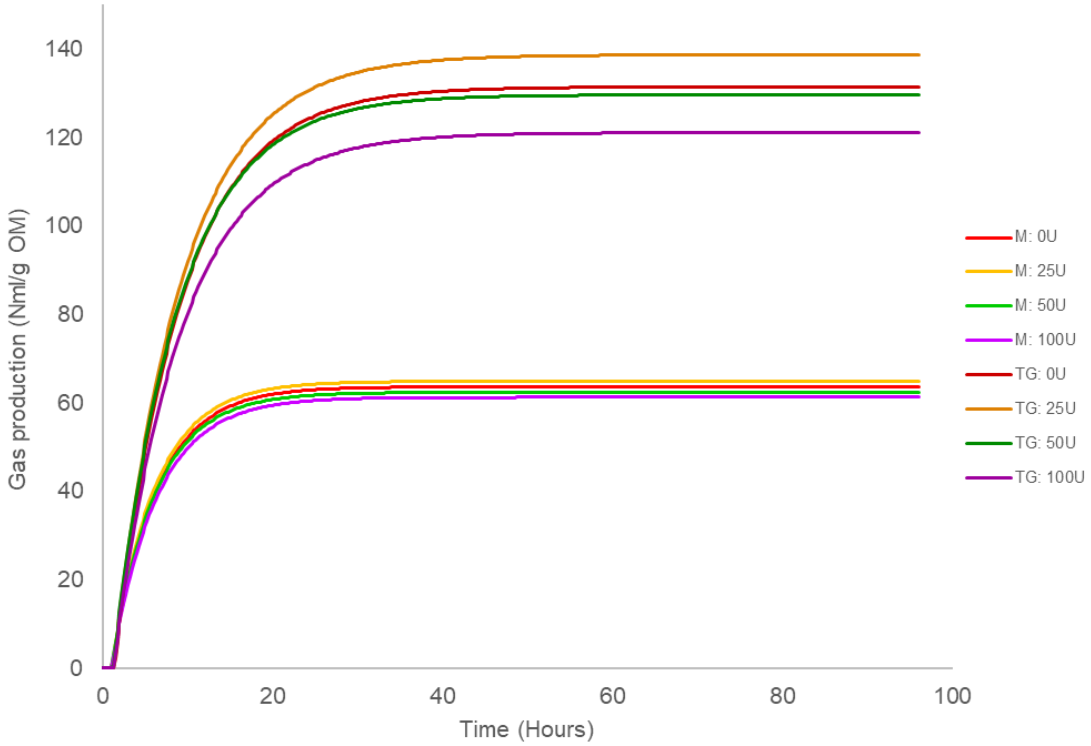

Figure 1 The effect of *U. lactuca* on *in vitro* total gas (TG) and methane (M) production

The reduction of methane emissions by marine algae reported in the literature was largely attributed to the haloalkane, bromoform (Machado *et al.*, 2015). Previous studies reported *U. lactuca* to release 150 ng bromoform g^{-1} of fresh weight per hour (Gschwend *et al.*, 1985; Manley *et al.*, 1992; Collén *et al.*, 1994; Carpenter & Liss, 2000). However, bromoform was not detected in the dried *U. lactuca* samples that had been used in the current study. It should be mentioned that the bromoform content appears to be substantially lower in *Ulva* spp. than in some other seaweeds. In a study by Min *et al.* (2021), it was found that the bromoform content was 1723 µg g^{-1} DM in *Asparagopsis taxiformis*, 1320 µg g^{-1} in *A. armata*, and only 150 µg g^{-1} DM in an *Ulva* spp. The disparity in results between the current study and those reported by the authors mentioned above could potentially be explained by the technique used to process the samples.

Bromoform is a natural product in seaweed, produced and stored in specialised gland cells (Paul *et al.*, 2006b). Functioning as a defence against herbivores (Paul *et al.*, 2006b; Vergés *et al.*, 2008) and microbes (Paul *et al.*, 2006a), bromoform is released onto the plant surface when required. Mendes *et al.* (2013) reported increased permeability of cell membranes and consequent loss of volatile compounds when fresh algae was dried at high temperature. In the current study, sun- and convection-drying methods were used to dry marine algae, as was also done by El-Waziry *et al.* (2015), whereas Machado *et al.* (2014b) used freeze-drying. Early work by Simnikov (1941) indicated that vapour pressure was the determining factor in the evaporation of bromoform from samples processed in hermetically-sealed vessels. If convection drying is done in such vessels at a temperature of 60 °C (334K), then, according to the Antoine equation, the vapour pressure would be ~0.05 bar (5 kPa). Since pure bromoform readily evaporates into air at 20 °C, one would assume that, at 60 °C and under vapour pressure of 5 kPa, a considerable portion of the bromoform in *Ulva* would be lost. Although the work by Simnikov (1941) was conducted in airtight vessels, vapour pressure is purely a function of temperature (Felder & Rousseau, 2004). Thus, it is safe to conclude that the drying methods used in this study were responsible for bromoform losses from the *Ulva* samples.

Uribe *et al.* (2018) investigated the effect of different drying methods (freeze-, solar-, vacuum- and convective drying) on the phytochemical content and amino acid and fatty acid profiles of *Ulva* spp. Convective drying at 70 °C for 120 min was the method that best retained proximate and other physiochemical parameters, including amino acids and antioxidants. They did not, however, measure bromoform. Five brown seaweed species were dried by Badmus et al. (2019) by means of oven-drying at 40 and 60 °C, freeze-drying, and microwave-drying to investigate the effect on a wide range of nutritionally-important chemicals, including antioxidant activity, but not bromoform content. Although

low-temperature methods, such as freeze-drying and oven-drying at 40 °C appeared to be the overall methods of choice, results were also affected by seaweed species. The authors suggested that, because there was no single procedure that was consistently superior for all parameters and all seaweed species investigated, a drying method should be selected that appears to be suitable for a specific species. No indications of bromoform losses from *Ulva* gland cells during oven-drying, or any other form of drying, could be found in the literature. It therefore remains a speculation that bromoform concentrations would be higher in freeze-dried samples than in oven-dried samples. It is suggested that a comparative study be done to test such a hypothesis.

Regarding in vitro methods to study treatment effects on methane production, it should be kept in mind that the adaptation period of the rumen liquid donor animals may play an important role. There are indications that the methane-producing population of ruminal microbes require an adaptation period of 30 d after switching to a new feed before any treatment effect on methane emissions can be expected to be observed (Williams *et al.*, 2009). The rumen fluid used in the current study was from cattle that had not been adapted to a diet that contained *Ulva*. It is suggested that the study should be repeated and that donor animals should first be adapted to *U. lactuca* in their diet for 30 d. An *in vivo* experiment of at least 30 d in length should also be considered to determine whether *U. lactuca* can reduce methane emissions from ruminants.

Conclusion

This study did not find any evidence of a dose-dependent reduction in in vitro total gas and methane production due to the inclusion of *U. lactuca* in samples. The lack of results can be attributed to the absence of bromoform in the dried *Ulva* samples used in the present study. To the knowledge of the author, there is no comparative study on the various drying techniques most suitable for the preservation of bromoform in *U. lactuca*. Due to economic factors and practicality, the decision was made to utilise sun- and oven-drying to preserve the algae. Bromoform analysis of the dried samples was only possible after the completion of the study, as the analytical facility had been awaiting standards for analysis.

Previous published results from Williams *et al.* (2009) indicated that the methane-producing microbiome within the rumen may need an adaptation period of 30 d for reductions in methane emissions to be observed. The unadapted rumen fluid used in this study yielded no differences between treatments for the amount of methane produced. Further research, using rumen fluid from adapted animals, is required to accurately determine whether the inclusion of *U. lactuca* can reduce total gas and methane production *in vitro*. Additionally, animal experiments are required to determine whether any *in vitro* reductions that might occur will be translated to *in vivo* methane production.

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