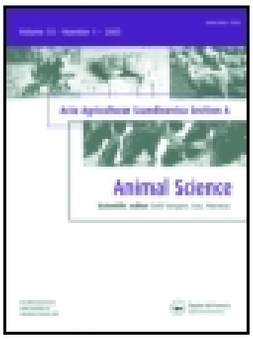

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BRIEF REPORT

Methane inhibition by *Asparagopsis taxiformis* with rumen fluid collected from ventral and central location – a pilot study

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ABSTRACT

We assessed the effect of rumen fluid (RF) collection site (central vs. ventral), seaweed (with or without *Asparagopsis taxiformis*) and their interaction on *in vitro* total gas and methane yield, and fermentation parameters. Rumen fluid was collected via esophageal tubing from six cows, controlling sampling locations manually through the rumen cannula. Linear mixed-effect model analysis revealed no interactions between rumen fluid collection site and seaweed. Seaweed reduced ($P < 0.01$) total gas and methane yield (mL/g DM) by 10% and 93%, respectively. Collection site had no effect on total gas yield, but methane yield was slightly lower ($P = 0.03$) with central than ventral RF. Our pilot study is the first report on gas parameters for incubated rumen fluid from different collection sites, and the observed effect of collection site on CH_4 yield provides further evidence for the importance of representative rumen sampling.

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KEYWORDS
Methane mitigation; seaweed; ruminant; rumen; Norwegian Red; esophageal tubing; fermentation

Introduction

Studies on methane-reducing feeds and feed additives are often carried out *in vitro* before they are tested *in vivo*, for cost reasons, but also for animal welfare reasons. For *in vitro* tests, rumen contents collected from animals fitted with a rumen cannula can follow 'a clearly defined and standardized sampling protocol' (Yáñez-Ruiz et al., 2016), for example as in Meale et al. (2012) and Ellis et al. (2016) but access to cannulated animals can be very limited. By contrast, rumen content collection by esophageal tubing is more applicable, but standardization of the sampling is challenging as the target location of the probe in the rumen cannot be identified readily. Despite a thorough and regular mixing of the rumen content by coordinated reticulo-rumen contractions, three layers of rumen contents are recognized. Intensive fermentation takes place in the intermediate layer and moderate fermentation in the ventral layer and upper fibre mat of the rumen content (Figure 1). Accordingly, sampling one or more layers of rumen contents could be expected to influence the degree of methane reduction by feeds and feed additives in the *in vitro* batch culture. Also studies comparing fermentation parameters between different layers of rumen content collected via cannula include a detailed description of the rumen content collection site for obvious reasons. However, results are inconsistent. Total short-chain fatty acid (SCFA) concentrations in rumen content collected from central rumen layers were greater than those from ventral layers in studies by Tafaj et al. (2004) and Bryant (1964), but did not differ in Shen et al. (2012) and Song et al. (2018). In studies testing the methane (CH_4) reduction potential of feeds and feed additives, information about the site of rumen fluid collection is often incomplete and no details are given about the insertion depth of the tube in intact animals or the collection sites within the rumen (central, ventral, cranial, caudal) in cannulated animals (Machado et al., 2014, 2016a, 2016b; Roque et al., 2019). Vucko et al. (2017) stated that the contents were collected 'from four quadrants of the rumen' in cannulated steers, with no indication of sampling height (central/ventral). It could be speculated that the rumen contents in the Vucko

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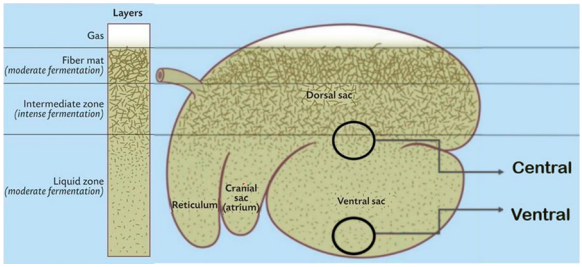

Figure 1. Illustration of the rumen fluid collection sites in the central and ventral rumen (modified from Physiology of Domestic Animals, Sjaastad et. al. 2016, reprinted with permission).

et al. (2017) study were sampled from the central layer, as almost complete inhibition of 99% of CH_4 yield (mL/g DM) was achieved *in vitro* by the addition of 2% of the seaweed *Asparagopsis taxiformis*. We hypothesized that the CH_4 reduction by *Asparagopsis taxiformis* will be more pronounced using rumen content from central than ventral location in *in vitro* batch culture incubations. The objective of this study was to assess the effect of the rumen content collection site (central, ventral) on the CH_4 reducing effect of *Asparagopsis taxiformis* and their interaction. We measured total gas and CH_4 production as well as other fermentation parameters in *in vitro* batch incubations.

Materials and methods

This study was conducted at the Faculty of Biosciences at the Norwegian University of Life Sciences (NMBU) as part of the course HFX407/307 'Emissions and Microbiota: A Path to Sustainable Animal Production' and the draft of this manuscript was written by the course participants, 3 Master students and 4 PhD students. The experimental protocol complied with the Norwegian legislation for animal welfare and was authorized by the Norwegian Animal Research Authority (FOTS-ID 18/256940).

Animals and feeding

Six dry, non-pregnant, rumen-cannulated Norwegian Red cows aged between 7 and 13 years and with an average (±SD) body weight of 776 ± 86 kg were used in the study. The cows were moved from pasture to tie-stalls 10 days prior to sampling. The cows were fed a standard diet in the morning and evening as used for the *in sacco* procedure according to the Nordic feed evaluation system (NorFor, 2011). In brief, 5.2 kg dry matter (DM) composed of 57% haylage, 35% concentrates and 8% wheat straw was fed in two equal portions per day. The contents (in g/kg DM) of organic matter, neutral detergent fibre (NDF), crude protein (CP) and crude fat (CF) in the composited haylage-straw mixture were 937, 695, 79.2 and 15.1, respectively, and in the concentrates 924, 168, 211 and 29.3, respectively. The tie-stall accommodation was bedded with rubber mats and fresh sawdust. The cows had *ad libitum* access to fresh drinking water.

In vitro incubation

The feed incubated *in vitro* was prepared as total mixed ration consisting of 50% grass silage and 50% concentrates on DM basis and was ground to pass through a 1 mm screen using a cutter mill (SM 200, Retsch GmbH, Germany). The contents (in g/kg DM) of organic matter, NDF, CP and CF in the total mixed ration were 938, 391, 167 and 22.8, respectively (Kidane et al., 2018, described as dietary treatment with CP 175). Freeze-dried red seaweed *Asparagopsis taxiformis* (called hereafter *Asparagopsis*) was obtained from seaExpert (Horta, Azores, Portugal), stored at −20°C and ground with a mortar.

Rumen fluid was collected from the cows at about 5 h after morning feeding via an esophageal tube (Selekt Collector Set, Virbac; Kolding, Denmark). The 6 cows were divided into two groups (cows 1–3, and cows 4–6), and 400 mL of rumen fluid was obtained from each cow per sampling site, resulting in a total of 1200 mL pooled rumen fluid per group and sampling site. Rumen fluid was collected first from the central rumen and then ventrally, as illustrated in Figure 1. The location of the collection tube was controlled manually via the rumen cannula. The first 500 mL of rumen fluid from the central location was discarded to avoid saliva contamination whereas the initial 500–1000 mL of rumen fluid from the ventral location was discarded to avoid rumen fluid from the central rumen location. The esophageal tube was cleaned after each cow. The rumen fluid was collected into four (2 sampling sites × 2 groups) pre-warmed thermos flasks and screened through 200 µm pore size filter cloth (SEFAR NITEX, Sefar AG, Heiden, Switzerland) into glass bottles maintained at 39°C in water-bath. Samples of the pre-incubation rumen fluid were taken for pH, ammonia nitrogen (N) and SCFA determination (as described in the section below).

A buffer solution was prepared according to Goering and Van Soest (1970) and gas production was measured using the ANKOM^{RF} Gas Production System (ANKOM Technology; Macedon NY, USA). For this, 1.0 g DM of feed was weighed into 250 mL glass bottles incubated at 39°C with 33.3 mL rumen fluid and 66.7 mL preheated buffer solution. The incubation was performed with rumen fluid from 2 groups of cows (cows 1–3, cows 4–6), sampled from 2 rumen locations (central, ventral),

incubated with no or 9 mg DM seaweed (Control and Inhibitor, respectively). Each treatment combination was incubated in triplicates with additional triplicates of blanks (mixture of rumen fluid and buffer only). The incubation was performed for 24 h in a 39°C heated cabinet with continuous gentle shaking on Stuart SSL3 3D gyro-rocker (Cole-Palmer Ltd, Staffordshire, UK). The incubation time of 24 h was chosen following Yanez-Ruiz et al. (2016) and based on the good agreement of the 24-h CH_4 yield in vitro and in vivo (Terranova et al. 2021). The incubation bottles, except for the blanks, were equipped with septa for gas sampling.

To determine the CH_4 concentration in the incubation bottles at completion of the incubation, duplicate gas samples of 2 mL (from each incubated medium with the exception of blanks) were withdrawn through the septa and transferred into clean, helium-filled 10 mL glass vials (Cleam Pack, Matriks As, Oslo, Norway). Thereafter, 9.5 mL of the incubation fluid was transferred to centrifuge glass tubes enriched with 0.5 mL concentrated (98–100%) formic acid and stored at 4°C until analysis. The pH of the remaining incubation fluid was measured as described below.

Sample analyses

The pH, SCFA concentrations and ammonia-N were determined both in the fresh mixed pre-incubation rumen fluid ($n = 4$; 2 sampling sites × 2 groups) and in the incubated fluids ($n = 24$). The pH was measured using a portable pH-meter (WTW, pH 3310, Germany with a Hamilton Bonaduz AG, Polyplast Pro, Switzerland, pH sensor). The SCFA were analysed by gas chromatography (TRACE 1300 Gas Chromatograph with Stabliwax-DA column 30 m, 0.25 mm i.d., 0.25 µm; Thermo Fischer Scientific S.p.A., Milan, Italy) and NH_3-N was determined using AOAC Official Method 2001.11 (AOAC International, 1995) as described by Thiex et al. (2002) with the Kjeltec 8400 (Foss Analytical, Hilleroed, Denmark).

Methane concentrations in the collected gas samples were analysed by gas chromatography (GC, Model 7890A Agilent, Santa Clara, CA) after separation by a Poraplot U capillary column operated isothermally at 50°C with helium as the carrier gas. The GC was equipped with a flame ionization detector, calibrated to certified standards (Linde Gas AS, Oslo, Norway). Samples were pumped by an autosampler through a peristaltic pump (Minipuls, Gilson) and injected automatically via a 250 µL sample loop.

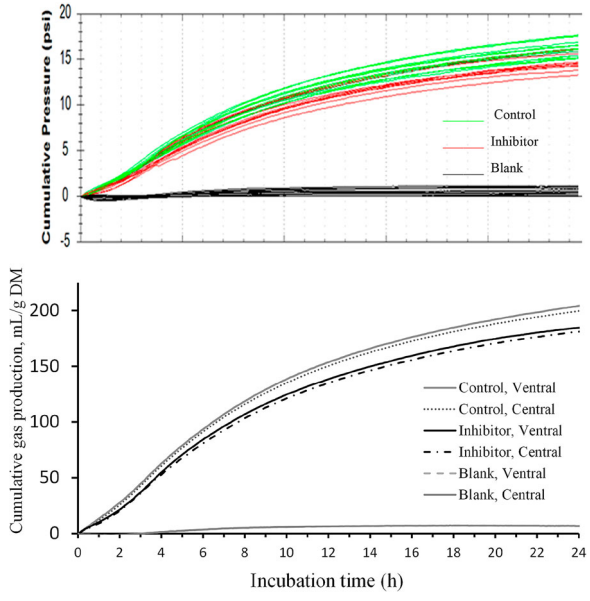

Figure 2. Cumulative gas pressure (pounds of force per square inch of area, psi) (top) and cumulative gas production (mL/g dry matter, DM) (bottom) over time of incubation for control (feed without inhibitor), inhibitor (feed with inhibitor seaweed, *Asparagopsis taxiformis*) and blanks (buffered rumen fluid without feed, without inhibitor).

Calculations and statistical analyses

Cumulative gas pressure (psi, pounds of force per square inch of area; Figure 2 top) from the gas production system was converted to total gas production (mL, Figure 2 bottom) following the ANKOM^{RF} manufacturer's protocol. Total gas produced was corrected for the blank measurements. The concentration of CH_4 in samples from the headspace was corrected for dilution by the carrier gas helium. The CH_4 production (mL) was calculated according to Cattani et al. (2016) using the following equation: total CH_4 production (mL) = $-0.0064 \times$ [CH_4 concentration in the headspace × (headspace volume + total gas production volume)]^2 + $0.9835 \times$ [CH_4 concentration in the headspace × (headspace volume + total gas production volume)]. This equation was recommended by Alvarez Hess et al. (2019) for estimating CH_4 production in vented *in vitro* systems in cases where collection of vented gas in bags is not possible.

Statistical analysis was performed using R statistical software (R Core Team 2016; version 4.0.2) and included 24 observations, i.e. 2 groups (cows 1–3, cows 4–6) × 2 rumen locations (central, ventral) × 2 treatments (with/without seaweed) × 3 replicates for the parameters pH, SCFA, ammonia-N and total gas production. However, for CH_4 analysis, one incubation glass was not equipped with a septum, and a total of 46 gas samples were analysed from 23 incubation glasses sampled in duplicates. The linear mixed-effects models (lmer) procedure was used with location (central, ventral) and treatment

(with/without seaweed) as fixed effects and cow group (1-3,4-6) as random effect. The interaction between location and treatment was also included in the model. The anova function in base R was used to obtain the overall effect of location, treatment, and their interaction. Effects were considered as statistically significant at $P < 0.05$ and as trends at $0.05 \leq P < 0.10$.

Results

Pre-incubation rumen fluid

In the fresh mixed pre-incubation rumen fluid, the average ($n = 2$ cow groups) pH, concentration of ammonia-N (mmol/L) and total SCFA (mmol/L) in the central and ventral rumen location were 6.65 vs. 6.73, 3.26 vs. 3.17 and 69.2 vs. 64.4, respectively. The molar proportions of acetate (C_2), propionate (C_3), butyrate, valerate, iso-butyrate and iso-valerate to total SCFA in the central vs. ventral rumen location were 68.5 vs. 66.2, 17.2 vs. 17.4, 11.7 vs. 13.1, 0.95 vs. 1.06, 0.87 vs. 1.14, and 0.82 vs 1.14, with C_2/C_3 ratio being 3.99 vs. 3.81, in respective order.

Incubated fluid

No interactions ($P > 0.10$) between rumen content collection site (central/ventral) and treatment (with/without inhibitor) were observed for any of the fermentation and gas parameters tested in this study (Table 1, Figure 3). Whether rumen fluid was collected centrally or ventrally had no effect ($P > 0.10$) on total gas yield, pH, concentrations of ammonia-N and total SCFA, molar proportions of acetate, propionate, butyrate, valerate and the ratio of acetate to propionate in the incubated fluid (Table 1, Figure 3a). However, central rumen fluid was characterized by a lower CH_4 yield expressed per unit dry matter ($P = 0.034$) and per unit total gas production ($P = 0.078$) (Figure 3b,c), and lower molar proportions of *iso* butyrate and *iso* valerate ($P < 0.05$) compared to the ventral rumen fluid (Table 1).

The addition of the inhibitor *Asparagopsis* increased ($P < 0.05$) the molar proportion of propionate (+32%), butyrate (+29%), and valerate (+9%), in the incubated fluid from both locations to a similar extent (Table 1). By contrast, *Asparagopsis* addition decreased ($P < 0.05$) ammonia-N (−13%) and total SCFA (−12%) concentrations, and the molar proportions of acetate (−15%), *iso* butyrate (−14%), *iso* valerate (−25%), and thus the C2:C3 ratio (from 3.3–2.1) across both rumen locations (Table 1).

The treatment *Asparagopsis* affected total gas and CH_4 yield to a different extent. The addition of the inhibitor *Asparagopsis* only slightly reduced ($P < 0.05$) total gas yield by 10%, with a similar degree of inhibition for central (9%) and ventral (11%) rumen fluid (Figure 3a), whereas inhibition of CH_4 yield (93%) was more substantial, both when expressed as mL of CH_4 production per g dry matter and per L of total gas production (both $P < 0.05$). Because there was no statistically significant interaction between collection site and *Asparagopsis* treatment, the reduction of CH_4 yield (mL/g DM and mL/L total gas production) was only numerically greater with central rumen fluid (both 99%) than ventral rumen fluid (87% and 85%, respectively) (Figure 3b,c).

Discussion

This pilot study included rumen fluid not only from 3 cows, as recommended by Yáñez-Ruiz et al. (2016), but from 2×3 cows run in technical triplicates, to partly compensate for the fact that we performed only one experiment in the frame of the student course instead of three independent experiments as recommended. The conclusions from our pilot study must be therefore drawn with caution. To the best of our knowledge, *in vitro* total gas and CH_4 production using inoculum obtained from different layers of rumen content have not been reported previously.

Pre-incubation rumen fluid

Based on descriptive statistics, greater SCFA concentration in pre-incubation rumen fluid from the central than ventral layer of the rumen content indicated more intensive fermentation centrally than ventrally in the rumen, which is in accordance with Sjaastad et al. (2016, Figure 1), Tafaj et al. (2004) and Bryant (1964).

Incubated rumen fluid

Unexpectedly, no interactions between rumen content collection site (central/ventral) and inhibitor treatment (with/without *Asparagopis*) were observed, indicating a similar direction and degree of effect by *Asparagopsis* in incubations with rumen fluid from central and ventral rumen layers.

Effect of rumen content collection site

Fermentation parameters like pH, ammonia-N, total SCFA, the molar proportions of acetate, propionate, butyrate, valerate and the ratio of acetate to propionate are commonly reported in fresh, but not incubated rumen fluid from different rumen collection sites. In

Table 1. Effect of rumen fluid collection site (central, ventral) and treatment (with/without inhibitor *Asparagopsis taxiformis*) on rumen fermentation parameters after 24 h *in vitro* incubation.

Rumen location	Central		Ventral			*P*-values		
Treatment	Control	Inhibitor	Control	Inhibitor	SEM	Loca tion	Treat ment	L × T
Trait								
pH	6.50	6.53	6.51	6.51	0.017	0.82	0.74	0.54
NH_3-N, mmol/L	12.2	10.5	12.2	10.7	0.292	0.55	<0.001	0.45
Total SCFA, mmol/L	76.2	65.5	71.5	65.0	0.757	0.29	0.002	0.38
Molar proportion, mmol/mol								
Acetate (C_2)	64.8	55.3	65.1	55.5	0.671	0.65	<0.001	0.75
Propionate (C_3)	20.0	26.3	19.7	26.1	0.667	0.20	<0.001	0.87
Butyrate	12.4	15.9	12.2	15.7	0.505	0.67	<0.001	0.79
iso butyrate	0.622	0.521	0.689	0.610	0.096	< 0.001	<0.001	0.37
Valerate	1.13	1.21	1.15	1.26	0.063	0.12	<0.001	0.54
iso valerate	1.07	0.771	1.13	0.892	0.196	0.003	<0.001	0.30
C_2/C_3	3.24	2.11	3.31	2.12	0.400	0.37	<0.001	0.67

Data are least square means, unless otherwise stated.
NH_3-N: ammonia nitrogen, SCFA: short-chain fatty acids

fresh rumen fluid collected centrally and ventrally, Shen et al. (2012) and Song et al. (2018) found no difference in any of these parameters, which is in line with our results for incubated fluids.

The lower molar proportions of iso-butyrate and iso-valerate in incubated rumen fluid from the central compared to the ventral part of the rumen are interesting. Iso-butyrate and iso-valerate are synthesized by rumen microbes in the oxidative deamination and the oxidative decarboxylation of the branched amino acids valine and leucine (Andries et al., 1987). However, these SCFA are of minor relevance as an energy source for the animal compared to acetate, propionate and butyrate.

To the best of our knowledge, CH_4 production from different layers of rumen content has not been reported previously in fresh or incubated rumen fluid. We expected a higher *in vitro* CH_4 yield in rumen fluid collected from the site of more intensive fermentation, i.e. the central rumen layer. Increased fermentation is related to increased metabolic hydrogen ([H]) which is removed by CH_4 formation to maintain 'the normal functioning of microbial enzymes involved in electron transfer reactions, particularly NADH dehydrogenase', resulting in NAD^+ restoring, and ultimately efficient fermentation (Morgavi et al., 2010; Ungerfeld 2015). However, the rumen fluid from the central layer had a slightly lower CH_4 yield than the rumen fluid from the ventral layer in the present study. Since the collection site had no effect on the total amount and the molar proportions of SCFA, it is difficult to explain this observation. One explanation might be the provision of the same nutrient-rich feed as substrate in the *in vitro* batch cultures, which differs from *in vivo* conditions where more-degraded, more-dense feed particles accumulate in the ventral layer. In our pilot study the observed difference in CH_4 yield between collection sites was small, still our finding supports the recommendation by Yáñez-Ruiz et al. (2016) to use a mixture of rumen contents collected from several rather than one location 'to be as representative for the rumen environment as possible'.

Effect of the seaweed Asparagopsis taxiformis

The observed moderate reduction in total gas yield and relatively greater reduction in methane yield by *Asparagopsis* have been reported repeatedly (Machado et al., 2014; Machado et al., 2016a, 2016b). Decreased total gas yields indicate reduced fermentation which matches the observed moderate reduction of SCFA and ammonia-N concentrations in the *Asparagopsis* treatment in our study. The latter is likely due to a reduced degradation of protein by microorganisms as discussed for *Asparagopsis* and other seaweeds by Machado et al. (2016b) and Wang et al. (2008), respectively. In the present study, CO_2 and H_2 concentrations were not measured, yet the decrease in CH_4 accounted for most of the reduction in total gas.

In accordance with previous *in vitro* reports on *Asparagopsis* (Machado et al., 2014, 2016a, 2016b; Kinley et al., 2016; Roque et al., 2019), a substantial reduction in methane yield goes along with a shift in fermentation end products from acetate to propionate and butyrate. Methane is a hydrogen sink in the rumen, and inhibition of methanogenesis by *Asparagopsis* shifts the production towards hydrogen consuming products, such as propionate, rather than hydrogen producing products, such as acetate, which is believed to be due to reductive propionate production being more favourable than acetogenesis in the presence of excess hydrogen (Mitsumori et al., 2012).

As a protection against herbivory, *Asparagopsis* contains halogenated, bioactive secondary metabolites which are naturally produced and stored in gland

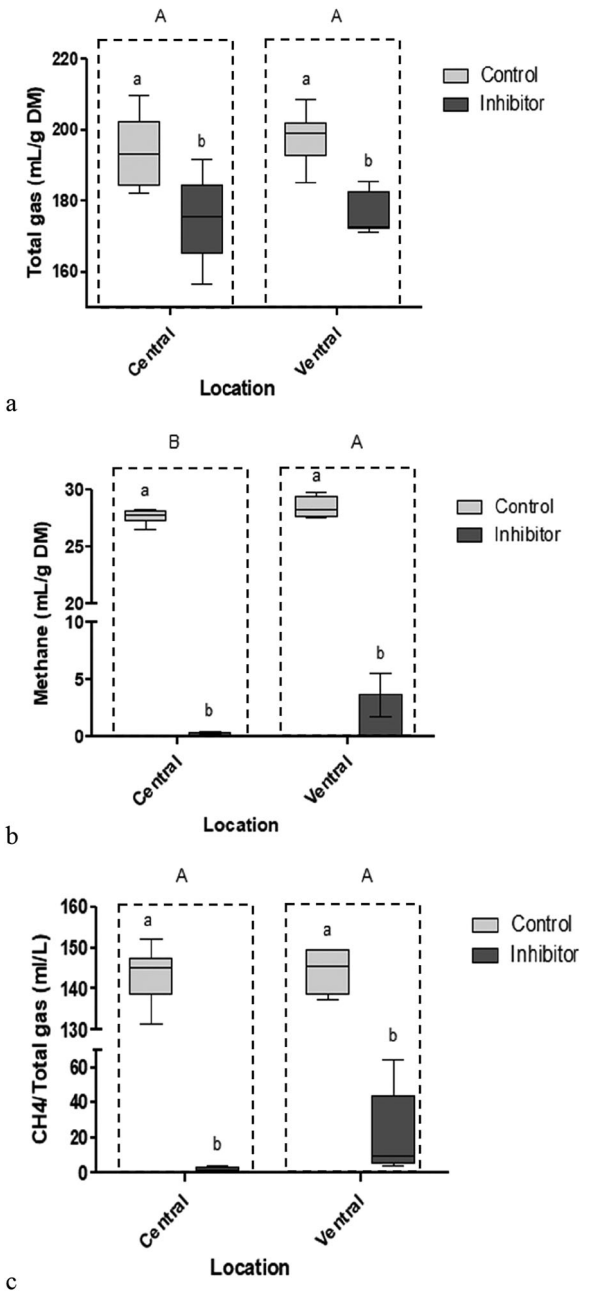

Figure 3. Effect of rumen sampling site (central, ventral) and treatment [without/with methane (CH_4)-inhibitor *Asparagopsis taxiformis*) on (a) total gas yield, (b) CH_4 yield, and (c) the proportion of CH_4 to total gas production. Least square means (LSM) carrying no common upper-case superscript are different at $P < 0.05$ between central and ventral rumen location. The LSM carrying no common lower-case superscript are different at $P < 0.05$ between with/without inhibitor treatment within location.

cells of the macroalgae (Vucko et al., 2017). Bromoform, which is the most abundant halogenated CH_4 analogue in *Asparagopsis*, reacts with reduced vitamin B_{12} which results in the enzymatic inhibition of cobamide-dependent methyl transferase needed in methanogenesis, hence inhibiting the formation of CH_4 in the rumen (Wood et al., 1968; Machado et al., 2016b).

Multiple studies with different inclusion levels of *Asparagopsis in vitro* (Machado et al., 2014, 2016a, 2016b, Roque et al., 2019) report a dose-dependent CH_4 inhibition. For example, inclusion of *Asparagopsis* at 1% on organic matter basis reduced CH_4 by 84.7% (Machado et al., 2016a) whereas at 2% inclusion *Asparagopsis* showed a nearly complete inhibition of CH_4 (i.e. a decrease of more than 99% compared to control) (Kinley et al., 2016; Machado et al., 2016a). Similarly, our inclusion rate of 1% on DM basis resulted in a reduction in CH_4 yield by 93%. Variation in the degree of CH_4 inhibition among studies using the same inclusion rates are likely explained by small differences in the bromoform concentrations between batches of *Asparagopsis*.

We hypothesized that the effect of *Asparagopsis* on CH_4 production would be more pronounced in rumen content taken from central than ventral location. Although a numerical difference was observed, the CH_4 reduction by *Asparagopsis* was not more effective in rumen content from central versus ventral location. A greater effect of the inhibitor in central rumen content could have been expected because several studies reported a higher abundance of total and fibrolytic bacteria and of *Methanobacteriales* in the central/dorsal rumen as compared to the ventral rumen (Bryant and Robinson, 1968; Martin and Michalet-Doreau, 1995; Zeitz et al., 2016). As discussed above, *Asparagopsis* is a strong inhibitor, resulting in almost complete CH_4 inhibition at relatively small inclusion rates (Kinley et al., 2016; Machado et al., 2016a). In future studies, the interaction of rumen content collection site with CH_4 mitigating feeds or feed additives rather than inhibitors could be worth testing. Studies using rumen fluid from one collection site (as with esophageal tubing) may under- or overestimate the CH_4 mitigation potential of a feed or feed additive and may conclude that a higher inclusion rate is necessary, or a lower inclusion rate might be sufficient for a proper CH_4 mitigation.

Conclusion

This is the first report on *in vitro* total gas and methane yields from rumen fluid collected at different locations in the rumen. We found a lower CH_4 yield in rumen fluid from central than ventral rumen layers, providing further evidence for the recommendation to use a mixture of rumen contents collected from several location in *in vitro* studies.

The CH_4 reduction by the inhibitor *Asparagopsis* was numerically but not significantly greater in incubations

using rumen fluid from central than ventral locations. Still, the interaction of rumen content collection site and CH_4-reducing feeds and feed additives could be worth exploring in future *in vitro* studies to ensure appropriate inclusion rates in *in vivo* studies.

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Disclosure statement

We confirm that there are no relevant financial or non-financial competing interests to report.

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