Animal (2020), **14:5,** pp 952–962 © The Animal Consortium 2019
doi:10.1017/S1751731119002799

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Effect of altering ruminal pH by dietary buffer supplementation on methane emissions from sheep fed forage rape

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(Received 17 February 2019; Accepted 14 October 2019; First published online 18 November 2019)

*Low methane (CH_4) emissions from sheep fed forage rape (*Brassica napus*) might be related to low ruminal pH value. In this study, sodium carbonate (Na_2CO_3: **SC**) was supplemented to the diet to alter ruminal pH for evaluation of its role in CH_4 emissions from sheep fed forage rape. Fourteen intact and eight fistulated Romney sheep were adapted to forage rape over 32 days and then randomly allocated to one of two groups: diets supplemented with SC or not (control). Methane emissions were measured from intact sheep in seven experimental periods. In parallel, ruminal pH and fermentation characteristics were assessed using the fistulated sheep. In the first (P01) and the second (P02) periods, none of the sheep received SC to examine the baseline CH_4 emissions. The P01 period was used as a covariate for analysis of gas emission measurements in subsequent measurement periods. Sodium carbonate was offered at 5% of the forage DM in P03 and P04, increased to 8% in P05 and P06 to assess the effect of pH increase on CH_4 emissions and stopped in P07 to assess if the CH_4 emissions reverted to values similar to those measured before the supplementation started. Methane yield (g/kg forage DM intake) was similar for the sheep in both groups during P02 and P03, but sheep supplemented with SC in the diet emitted 36%, 49% and 30% more CH_4 per unit of forage DM intake than those in the control group during P04, P05 and P06, respectively. Emissions returned to similar levels when SC supplementation was ceased in P07. Ruminal pH was 0.412 to 0.565 units higher in SC supplemented sheep than for the control group during the SC treatment periods. Based on the lack of an immediate response in CH_4 emissions to the supplementation of SC in P03, the positive responses in P04 to P06 and the rapid disappearance of the response after supplementation with SC stopped in P07, we propose a new hypothesis that ruminal pH effects on CH_4 emissions are possibly through medium-term changes in microbial and methanogenic communities in the rumen, rather than a direct, short-term impact on methanogens* per se*. In conclusion, SC supplemented to the forage rape diet of sheep increased rumen pH, leading to an increase in CH_4 emissions. Low ruminal pH in sheep fed forage rape explains, at least partially, the reported low CH_4 emissions from sheep fed with this forage crop.*

Keywords: sodium carbonate, greenhouse gas, ruminal fermentation, forage brassica, pH monitoring bolus

Implications

This study manipulated ruminal pH value by adding sodium bicarbonate to the diet and provided evidence that ruminal pH is a mechanism behind the low methane emissions from sheep fed forage rape. This finding would enhance our understanding in the mechanism of methane production in the rumen and provide a potential approach to mitigation of methane emissions from ruminants.

Introduction

Enteric methane (CH_4) emissions from farmed ruminants account for 35% of the total anthropogenic greenhouse gas emissions in New Zealand (Ministry for the Environment, 2018) and globally they contribute to approximately a fifth of the agricultural emissions (Hristov *et al.*, 2018). Nutritional manipulations are recognised as tools to mitigate CH_4 emissions (Beauchemin *et al.*, 2008; Hristov *et al.*, 2013), but the implementation of such tools can be of limited relevance to pastoral systems (Buddle *et al.*, 2011; Clark, 2013). In New Zealand, farmed ruminants mostly graze grass-dominated pastures, but grazing of forage crops such as brassicas also occurs. Our previous studies

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indicated that feeding forage brassicas to sheep (Sun *et al.*, 2012a, 2016), especially forage rape (*Brassica napus* L.) resulted in lower CH_4 emissions than feeding ryegrass, with the effect lasting over 3 months (Sun *et al.*, 2015a). Thus, this group of crops may be a viable option for CH_4 mitigation in pastoral animal production systems (Sun *et al.*, 2016).

The mechanism mediating the low emissions when feeding forage rape is still unclear. Forage rape is characterised by a low content of NDF and a high content of readily fermentable carbohydrates (Sun *et al.*, 2012a, 2015a) and we have observed that sheep fed forage rape had lower ruminal pH values, a greater proportion of propionate in the rumen than sheep fed ryegrass, and their microbial community profile being similar to that of grain-fed ruminants (Sun *et al.*, 2015a). Others observed that forage rape was utilised by sheep in a manner more typically resembling a concentrate than a forage diet (Lambert *et al.*, 1987). Therefore, we speculate that forage rape leads to a rumen environment similar to that observed when concentrates are fed, characterised by low ruminal pH and increased propionate proportion, both of which have been associated with less CH_4 formation (Lana *et al.*, 1998).

Buffers such as sodium bicarbonate ($NaHCO_3$) or sodium carbonate (Na_2CO_3: **SC**) have been used to increase ruminal pH values (Krause and Oetzel, 2006; Enemark, 2008). Buffer treatment increased the total short chain fatty acid (**SCFA**) concentration and altered the proportions of individual SCFAs (Cruywagen *et al.*, 2015). Our hypothesis was that the increase in ruminal pH would cause CH_4 yield to increase and the proportion of propionate in total SFCA formed would decrease. In this study, we used SC to increase the ruminal pH of sheep fed forage rape to values similar to those from sheep fed ryegrass. This would allow us to evaluate the role of ruminal pH as a mechanism underlying the low emissions with forage brassicas relative to ryegrass.

Material and methods

Experimental design

Romney sheep, 14 intact wethers (5-year-old; liveweight 76.0 ± 2.6 kg, mean ± SD) and 8 fistulated wethers (3-year-old; 75.4 ± 8.9 kg), were gradually adapted from grazing 100% pasture to 100% forage rape over 7 days and then grazed on forage rape for 17 more days before being transferred to AgResearch Grasslands Research Centre (Palmerston North, New Zealand) on day 25 for the commencement of the indoor phase of the experiment (Table 1).

The sheep were randomly divided into two groups balanced with intact ($n = 7$ per group) and fistulated sheep ($n = 4$ per group). Sheep received Na_2CO_3 (sodium carbonate: SC group) or not (control group) as a supplement in their diet. The SC powder was mixed evenly with the forage immediately before each feeding. Methane emissions were measured from intact sheep on days 33 to 34 (measurement period 1: P01) and days 39 to 40 (P02) (Table 1) without SC supplementation. Then, the diet of sheep in the SC group was supplemented with SC at 5% of forage DM from days 41 to 50 and at 8% of forage DM from days 51 to 63. Supplementation with SC was removed from days 64 to 66. Methane emissions from sheep in both treatments were measured on days 41 to 42 (P03), days 48 to 49 (P04), days 55 to 56 (P05), days 61 to 62 (P06) and days 64 to 66 (P07).

Animals and forage

Forage rape (cv. Titan) was sown in autumn and harvested in winter (plant height from the ground to the top of plants *ca.* 68 cm) using a sickle bar mower, leaving a stubble height of *ca.* 8 cm. The forage was harvested daily in the morning between 1000 and 1200 h, transported to the animal experimental site before 1300 h, sampled between 1300 and 1330 h and then kept in a cold room (4°C) until the afternoon meal on the same day and for the meal on the next morning. Sheep were fed at 1.6 times their maintenance energy requirements (Australian Agricultural Council, 1990), offered as two meals of equal size at 0830 and 1600 h. The metabolisable energy of forage rape was assumed to be 12 MJ/kg forage DM (Sun *et al.*, 2012a) for the purpose of calculation of feed allowance.

Harvested forage was sampled for the determination of DM content (in triplicates, 105°C, 48 h) and chemical composition (one sample, 65°C, 48 h). Sub-samples for chemical composition were ground in a hammer mill fitted with a 1-mm sieve (Wiley Mill; Arthur H. Thomas, Philadelphia, PA, USA) and pooled over each CH_4 measurement period before analysis. Chemical composition of the crop is shown in Table 2.

Sheep were weighed weekly during the adaptation period in the paddock and before and after each chamber measurement period. At the end of the experiment, the mean (±SD) liveweight was 86.6 ± 4.4 kg, and 79.9 ± 9.8 kg for intact and fistulated wethers, respectively.

Methane measurement

A sheep respiration chamber facility containing three clusters with eight individual chambers each (detailed in Pinares-Patiño *et al.*, 2014) was used to determine CH_4, hydrogen (H_2) and carbon dioxide (CO_2) emissions. In brief, each chamber was 1.8 m long, 0.85 m wide and 1.2 m high, with a fresh air inlet in the front and an air outlet in the back. Each cluster of eight chambers was fitted with an air pump (UNI-JET 40; ESAM, Parma, Italy) to blow fresh air through the inlet at a constant flow rate which was measured by differential pressure using a custom-made Venturi flowmeter calibrated with a diaphragm gas meter (AL425; Elster American Meter Company, Essen, Germany). Each cluster was also equipped with a multi-gas analyser (4 900 Continuous Emission Analyser; Servomex Group Ltd, Crowborough, UK) and an electrochemical H_2 detector (7HYT Citicel; City Technology Ltd, Portsmouth, UK) to measure CH_4 and H_2 concentrations in the air samples. The sensitivity of the measurement was 0.5 and 5 µl/l, detection range 0 to 200 and 0 to 50 µl/l, recovery rate 98.2 ± 0.60 and 100.5 ± 4.01, for CH_4 and H_2, respectively. The recovery rates are determined once a

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Table 1 *Experimental design, showing the allocation of sheep to experimental groups, and their locations and the experiments being performed over the course of the trial*

Days	Period	Location	Activity
1 to 24		Paddock	Adaptation to forage rape while grazing. pH boluses inserted into the rumen of fistulated sheep on day 21
25 to 28	Adaptation	Pens	Moved indoors in group pens
29 to 32		Crates	
33 to 34	P01	Chambers	Methane measurement
35		Crates	
36 to 37		Pens	
38	P02	Crates	
39 to 40		Chambers	Methane measurement
41 to 42		Chambers	Methane measurement. Treatment group received 5% Na_2CO_3 from days 41 to 50
43	P03	Crates	Rumen samples taken from fistulated sheep at 0, 1, 2, 4, 6, 8, 12, 16 and 24 h after morning feeding. pH boluses taken out
44		Pens	
45		Pens	
46 to 47		Crates	
48	P04	Chambers	pH boluses inserted into the rumen of fistulated sheep. Methane measurement
49		Chambers	Methane measurement
50		Crates/pens	Rumen samples taken from fistulated sheep at 0, 1, 2, 4, 6, 8, 12, 12, 16 and 24 h after morning feeding. Intact sheep to pens, fistulated sheep in crates
51 to 52		Pens	Na_2CO_3 increased to 8% from days 51 to 63
53 to 54		Crates	
55 to 56	P05	Chambers	Methane measurement
57		Crates	Rumen samples taken from fistulated sheep at 0, 1, 2, 4, 6, 8, 12, 12, 16 and 24 h after morning feeding
58 to 59		Pens	
60		Crates	
61 to 62		Chambers	Methane measurement
63	P06	Crates	Animals stayed in chambers, but with doors opened and the treatment group animals still received 8% Na_2CO_3. Rumen samples taken from fistulated sheep at 0, 1, 2, 4, 6, 8, 12, 16 and 24 h afternoon morning feeding
64 to 66	P07	Chambers	Methane measurement. Supplementation of Na_2CO_3 stopped
67		Pens	pH boluses taken out

year by an independent contractor (National Institute of Water and Atmospheric Research, Wellington, NZ, USA). The CH_4, H_2 and CO_2 concentrations in the outflow air from each chamber within a cluster were measured sequentially in a cycle so that a measurement point was obtained from each chamber every 6.8 to 7.2 min. The concentrations of these gases in the inflow air (i.e. 'background' concentrations) were measured every hour. Immediately before the chamber doors were closed, each cluster was calibrated using a standard gas mix containing 200 ± 4 ppm CH_4, 2000 ± 20 ppm CO_2, $21.1 \pm 0.1\%$ O_2 and 50 ± 1 ppm H_2 with N_2 as a carrier (BOC Limited, Auckland, New Zealand). Temperature, relative humidity and CO_2 concentrations inside the chambers were monitored to comply with animal welfare regulations. Total CH_4, H_2 and CO_2 emissions were calculated from the differences between the concentrations in the outflow and inflow air and the rate of airflow through the chambers corrected for humidity, temperature and air pressure.

Feed refusals were collected once daily before the morning feeding and dried at 65°C for 48 h. Twice daily, during feeding, doors were opened and chambers cleaned, faeces and urine trays replaced and drinking water replenished, all of which took about 10 to 15 min. The data missed while the doors were open were estimated using the mean of the previous 10 readings immediately before door opening. The measurement of gas emissions lasted for 48 (from P01 to P06) or 72 h (P07) in each measurement period.

Methane emissions were expressed as daily emissions (g CH_4/day) and CH_4 yield (g CH_4/kg forage DM intake). Sodium carbonate ingested was not included in the daily DM intakes for CH_4 yield calculations because it does not contain fermentable material that produces CH_4.

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Table 2 *The chemical composition of forage rape-fed sheep in the study*

	Period		
Composition	1 to 2	3 to 5	6 to 7
DM (g/kg)[1]	95	102	115
Ash	129	119	109
Organic matter	871	881	891
CP	261	202	171
Lipid	33	29	27
Hot water-soluble carbohydrate	167	228	241
Pectin	98	87	83
Starch	3	4	6
aNDF[2]	227	225	240
ADF	175	170	170
RFC : SC	1.84	2.39	1.99
Lignin (sa)	82	92	74
Gross energy (MJ/kg)	17.6	17.6	17.5

[1] $n = 6$ for periods 1 to 2, 9 for periods 3 to 5 and 7 for periods 6 to 7; g/kg fresh weight.
[2] aNDF = neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; ADF = acid detergent fibre expressed inclusive of residual ash; RFC = ratio of readily fermentable carbohydrate (hot water – soluble carbohydrate + pectin); SC = structural carbohydrate (aNDF – lignin(sa)).

Rumen pH measurement

The pH monitoring boluses (Sentinel™ Bolus; Kahne Limited, Auckland, New Zealand) were placed in the rumen of fistulated sheep on day 21 and taken out on day 43 for data retrieval and placed back in the rumen again on day 48 and removed on day 67. The bolus was tethered with a double string of fishing line (*ca.* 30 cm in length) and an animal identification tag (Allflex New Zealand Limited, Palmerston North, New Zealand) for easy recovery. The bolus was set to record ruminal pH values every 15 min. Prior to insertion into the rumen via fistula, the bolus was placed in RO water overnight and then in standard buffers (pH 4.0 and 7.0) for 3 h in each solution. After removed from the rumen, the bolus was washed with RO water and then placed in buffer solutions again for the same duration. The pH values recorded when the bolus was in the buffer solutions were used to evaluate the accuracy of pH values. These readings were in agreement with the pH values of the buffers before and after being in the rumen. Therefore, the data recorded during the measurement periods were used directly without any adjustments. From the pH values recorded during each period, the daily maximum, minimum and average pH values were obtained and the length of time at specific ranges (i.e. 5.0 to 5.49, 5.5 to 5.79, 5.8 to 5.99, 6.0 to 6.19 and ≥6.2) and the area (pH × hour) below certain pH values (i.e. 5.5, 5.8, 6.0 and 6.2) were calculated.

Rumen sampling

Rumen samples were collected at 0, 1, 2, 4, 6, 8, 12, 16 and 24 h after morning feeding on days 43, 50, 57 and 63 from fistulated sheep. The pH value of the rumen samples was immediately measured using an electronic pH meter (Model EZDO 7011; GOnDO Electronic Co., Ltd, Taipei, China), and then stored in ice until processing in the laboratory.

Laboratory analysis

Rumen samples were brought to the laboratory within 30 min after collection and a sub-sample centrifuged (4°C, 10 min, 21 000 ***g***). The supernatant was acidified and analysed for SCFA using gas chromatography (Tavendale *et al.*, 2005) and ammonia using the phenol hypochlorite reaction method (Weatherburn, 1967).

Feed samples were analysed for DM, organic matter, CP, lipids, hot water-soluble carbohydrates, pectins, starch, NDF, ADF, ADL and gross energy by the Nutrition Laboratory at Massey University (Palmerston North, New Zealand) with procedures detailed by Sun *et al.* (2012b).

Statistical analysis

In vivo CH_4 emission data were analysed for each experimental period using a mixed model with SC treatment as a fixed effect and chamber cluster as a random effect. The differences between periods were compared using the paired *t* test. *In vivo* CH_4 emissions were different for the two groups in P01, as a result of individual animal variations in the absence of SC supplementation. To account for these background differences, CH_4 emission data from P01 were used as a covariate for the remaining measurement periods. Rumen fermentation parameters of fistulated sheep were analysed using a REML model for repeated measurements with treatment (SC supplementation or control), sampling time and the interaction of treatment and sampling time as fixed effects, and sampling time treated as a repeated measurement. pH values measured using boluses were analysed using one-way ANOVA for each period. All data analyses were performed in GenStat for Windows (16th edition, VSN International, Hemel Hempstead, UK, www.genstat.co.uk).

Results

Forage intake and methane

During each of CH_4 measurement periods, forage DM intakes were similar (*P* values between 0.15 and 0.92) for the SC and the control groups. Although the forage DM intakes changed across periods, the difference between the two groups was less than 6% (Table 3).

There were no differences in CH_4 yield (i.e. CH_4 emitted per unit of forage DM intake) between the two groups over the first 40 days ($P = 0.72$ for P02; Figure 1) when SC was not supplemented. During the periods with 5% SC supplementation (P03 and P04), CH_4 yield was similar for the two groups during P03 ($P = 0.81$) immediately after SC supplementation began, but a week later during P04, sheep in the SC group emitted 36% more ($P = 0.02$) CH_4 per unit of forage DM intake than those in the control group. During the periods with 8% SC supplementation (P05 and P06), sheep in the SC group continued to emit 49% ($P < 0.01$) and 30% ($P = 0.02$) more CH_4 per unit of forage DM intake when

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Table 3 *Forage intake and methane emissions in intact sheep (n = 7 per treatment for each period) fed winter forage rape supplemented with sodium carbonate (Na_2CO_3: SC) or unsupplemented control diet (C)*

	Treatment			
	C	SC	SEM	*P* value
Forage intake (kg/day)				
P01	1.56	1.56	0.045	0.51
P02	1.94	1.89	0.029	0.35
P03	1.82	1.92	0.052	0.08
P04	1.73	1.83	0.082	0.35
P05	1.71	1.68	0.100	0.82
P06	1.98	1.92	0.117	0.63
P07	1.96	1.89	0.089	0.33
CH_4 (g/day)				
P01	22.0	22.5	2.05	0.87
P02	24.1	22.8	1.64	0.57
P03	22.6	23.2	2.17	0.85
P04	20.0	29.1	2.84	0.16
P05	19.5	29.0	2.20	0.01
P06	24.8	31.6	1.74	0.03
P07	27.8	27.8	2.16	0.96
CH_4 (g/kg forage intake)				
P01	14.1	14.3	1.48	0.56
P02	12.4	12.0	0.75	0.72
P03	12.5	12.1	1.08	0.81
P04	11.5	15.9	1.06	0.02
P05	11.5	17.3	1.29	<0.01
P06	12.5	16.5	1.07	0.02
P07	14.2	14.8	1.13	0.73
H_2 (g/kg forage intake)				
P01	0.37	1.39	0.441	0.13
P02	2.13	0.82	0.526	0.10
P03	2.52	0.70	0.590	0.05
P04	1.24	1.34	0.250	0.78
P05	1.10	2.16	0.715	0.31
P06	0.61	1.51	0.262	0.03
P07	0.34	0.63	0.125	0.13
CH_4 energy/intake energy				
P01	0.047	0.043	0.0037	0.45
P02	0.042	0.036	0.0024	0.10
P03	0.042	0.036	0.0034	0.24
P04	0.039	0.048	0.0033	0.09
P05	0.038	0.052	0.0041	0.04
P06	0.042	0.050	0.0034	0.13
P07	0.047	0.044	0.0036	0.56

The SC diet contained 0%, 0%, 5%, 5%, 8%, 8%, 0% Na_2CO_3 of forage DM in P01 to P07, respectively.

compared to the control group in P05 and P06, respectively. During P07, SC was removed from the diet of the SC group and the CH_4 yield returned to similar values for both treatment groups ($P = 0.73$).

There were large variations in H_2 emissions (per unit of forage DM intake) among animals and the differences were inconsistent over periods and not statistically different between SC and control treatments ($P > 0.05$). Carbon dioxide emissions (per unit of forage DM intake) were similar ($P > 0.05$) for the two groups with the difference being less

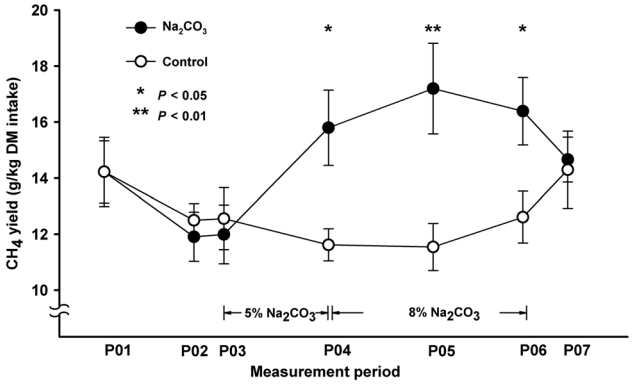

Figure 1 Methane (CH_4) emissions from intact sheep ($n = 7$ per treatment for each period) fed winter forage rape, with or without Na_2CO_3 supplemented, measured using open circuit respiration chambers for 48 or 72 h. Methane yield measurements in P01 were used as a covariate for the rest of the periods.

than 4% for any measurement period (data not shown). The ratio of CO_2 to CH_4 was smaller (data not shown) and CH_4 energy loss as a proportion of gross energy intake greater for the SC group than the control group during P05 ($P = 0.03$ and $P = 0.04$, respectively), but not significantly different during other periods ($P > 0.10$ and $P > 0.10$, respectively).

Rumen fermentation

Rumen samples were taken from fistulated sheep over a 24 h period, commencing immediately before morning feeding, during the periods when SC was provided. Most fermentation parameters in fistulated sheep differed markedly between the two groups (Figure 2). The total SCFA concentration was lower in the rumen of sheep supplemented with SC in the diet compared to the control group at most sampling times. The molar proportion of acetate was greater for the SC group (58.8 *v.* 55.6 mmol/100 mmol SCFA, $P < 0.01$) within 6 h after morning feeding, although the daily means were similar (averaging 59.9 mmol/100 mmol SCFA, $P = 0.38$) for the two groups. The molar proportion of propionate was lower in the SC group at most times of the day, although the difference between the two groups was not large in P04 ($P = 0.21$ for the overall difference). As a result, the ratio of acetate to propionate was greater for the SC group at most time points in the day ($P < 0.05$). The molar proportion of butyrate was similar (averaging 19.0 mmol/100 mmol SCFA, $P = 0.61$) for the two groups 4 h after morning feeding, with the exception of the first 6 h in P03, but 4 h after feeding the SC group had a greater butyrate molar proportion than the control group (21.2 *v.* 17.0 mmol/100 mmol SCFA, $P = 0.04$) and the difference lasted for the rest of the day. The ratio of acetate and butyrate to propionate and valerate was greater for the SC group than the control group during all periods with SC supplementation ($P < 0.01$). The proportion of minor SCFAs including *iso*-butyrate, *iso*-valerate and 2-methylbutyrate was lower for the SC group ($P < 0.02$).

Daily mean ruminal pH values were greater ($P \geq 0.05$) when SC was supplemented with the largest difference

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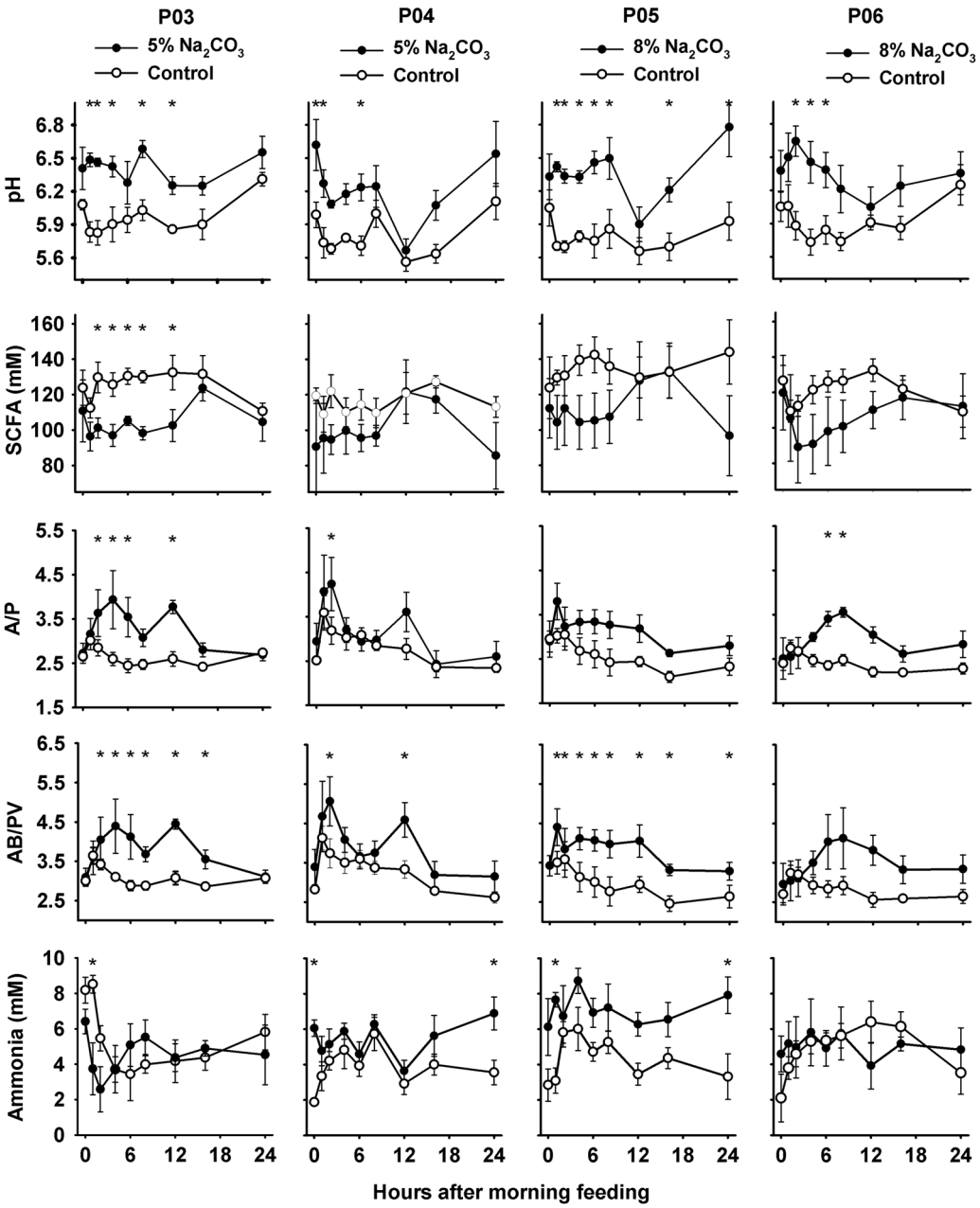

Figure 2 Rumen fermentation parameters of fistulated sheep ($n = 4$ per treatment for each period) fed forage rape at 0830 and 1600 h with or without Na_2CO_3 supplemented in the diet. Bars represent SEM; *, the difference between treatments was significant ($P < 0.05$). SCFA = short chain fatty acid; A/P = the ratio of acetate to propionate; AB/PB = the ratio of acetate and butyrate to propionate and valerate.

between groups in P05 (Figure 2). Supplementation with SC resulted in greater ruminal ammonia concentration in P04 ($P = 0.01$) and P05 ($P = 0.01$), although the difference was reversed at some time points of the day, which resulted in no significant difference in the mean daily concentration ($P = 0.67$ for P03 and $P = 0.81$ for P06, Figure 2).

Ruminal pH measured using boluses

Ruminal pH value in the fistulated sheep was continuously measured using pH boluses. There were large differences among animals in ruminal pH value. For example, the ruminal pH value of sheep #6394 was greater at all times than that in other sheep in the control group (Supplementary Material Figure S1). The diurnal pattern of ruminal pH was variable among animals as well. For example, the ruminal pH of sheep #6396 decreased from 0830 to 1430 h on day 48, whereas in sheep #6393 the pH value increased (Supplementary Material Figure S1). Sheep in the SC group seemed to have greater ruminal pH values after feeding, especially after the afternoon meal, during the periods when SC was supplemented, but sheep in the control group did not have obvious diurnal patterns in ruminal fluid pH value (Supplementary Material Figure S1).

The maximum, minimum and average pH values were similar for the two groups of animals during the measurement periods when SC was not supplemented (P01, P02

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Table 4 *Maximum, minimum and average pH values of fistulated sheep (n = 4 per treatment for each period) fed forage rape supplemented with sodium carbonate (Na_2CO_3: SC) or unsupplemented control diet (C), measured using pH monitoring boluses*

	Treatment			
	C	SC	SEM	*P* value
Maximum pH				
P01	6.13	6.42	0.150	0.23
P02	6.25	6.42	0.106	0.31
P03	6.22	6.50	0.067	0.02
P04	6.26	6.58	0.150	0.17
P05	6.31	6.49	0.153	0.44
P06	6.19	6.46	0.146	0.23
P07	6.34	6.30	0.143	0.88
Minimum pH				
P01	5.21	5.28	0.077	0.56
P02	5.34	5.27	0.072	0.51
P03	5.33	5.64	0.051	<0.01
P04	5.32	5.25	0.145	0.74
P05	5.48	5.50	0.185	0.96
P06	5.39	5.40	0.183	0.97
P07	5.38	5.29	0.117	0.61
Average pH				
P01	5.65	5.76	0.083	0.39
P02	5.77	5.84	0.106	0.65
P03	5.75	6.13	0.053	<0.01
P04	5.72	5.94	0.171	0.38
P05	5.88	6.03	0.172	0.56
P06	5.77	5.94	0.151	0.46
P07	5.81	5.74	0.113	0.68

The SC diet contained 0%, 0%, 5%, 5%, 8%, 8%, 0% Na_2CO_3 of forage DM in P01 to P07, respectively.

and P07) (Table 4). From P03 to P06, when Na_2CO_3 was provided, the average and maximum pH values were statistically or numerically greater for the SC group compared with the control. However, the minimum pH was similar for the two groups of sheep, except during P03 where the minimum pH was higher for the SC than for the control. When considering the proportion of time in which the pH remained within a specific range, the two groups of sheep did not differ when SC was not supplemented (Table 5). However, once SC was introduced into the diet, the length of time when pH remained above 6.0 (i.e. pH 6.0 to 6.19 and pH $\geq$ 6.2) increased from 3.5 h/day for the control sheep to 18.8 h/day for the SC supplemented sheep (P03), and at the same time the duration of time when rumen pH was below 5.8 (i.e. pH 5.0 to 5.49 and pH 5.5 to 5.79) reduced to 1.2 h/day in the SC treatment sheep compared with 13.8 h/day in the control treatment group. After continued exposure to 5% SC in the diet (i.e. P04), the duration of pH above 6.0 decreased to 10.3 h/day and increased again to 13.2 h/day (P05) and 11.1 h/day (P06) when SC supplementation was increased to 8%. Overall, the dietary SC supplementation resulted consistently in smaller areas under the curve (pH $\times$ hour) below levels of pH 5.5, 5.8, 6.0 and 6.2, although

Table 5 *Duration (h/day) in a specific range of daily ruminal pH value of fistulated sheep (n = 4 per treatment for each period) fed forage rape supplemented with sodium carbonate (Na_2CO_3: SC) or unsupplemented control diet (C), measured using pH monitoring boluses*

	Treatment			
	C	SC	SEM	*P* value
pH 5.0 to 5.49				
P01	5.19	4.53	1.668	0.79
P02	4.00	3.41	1.895	0.83
P03	3.91	0	1.298	0.08
P04	9.50	1.50	2.644	0.08
P05	3.28	0.50	1.562	0.26
P06	4.25	1.91	1.768	0.39
P07	2.81	2.84	1.041	0.98
pH 5.5 to 5.79				
P01	12.84	9.59	1.618	0.21
P02	10.47	7.16	2.618	0.41
P03	9.91	1.25	2.218	0.03
P04	6.66	5.53	2.049	0.71
P05	10.00	4.09	3.038	0.22
P06	11.78	4.94	3.291	0.19
P07	10.84	13.31	3.185	0.61
pH 5.8 to 5.99				
P01	4.53	4.72	1.378	0.93
P02	4.31	5.56	1.107	0.46
P03	6.72	4.00	1.326	0.20
P04	1.47	6.69	0.723	<0.01
P05	4.19	6.16	2.630	0.62
P06	1.81	6.03	1.535	0.10
P07	3.63	4.22	1.366	0.77
pH 6.0 to 6.19				
P01	1.34	2.66	0.794	0.29
P02	2.97	4.66	1.735	0.52
P03	2.78	9.59	1.767	0.03
P04	0.78	5.75	0.727	<0.01
P05	0.63	5.25	0.703	<0.01
P06	1.00	6.63	1.322	0.02
P07	2.31	2.22	1.234	0.96
pH $\geq$ 6.2				
P01	0.09	2.50	1.478	0.29
P02	2.25	3.22	2.027	0.75
P03	0.69	9.16	1.175	<0.01
P04	5.59	4.53	4.128	0.86
P05	5.91	8.00	5.033	0.78
P06	5.16	4.50	4.196	0.92
P07	4.41	1.41	2.929	0.50

The SC diet contained 0%, 0%, 5%, 5%, 8%, 8%, 0% Na_2CO_3 of forage DM in P01 to P07, respectively.

the differences were not statistically significant due to large variation among animals (Supplementary Material Table S1).

Discussion

The chemical composition of forage rape used in the study (Table 2) was similar to the previously reported data (Sun *et al.*, 2012a, 2015a), and typical of this crop in

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New Zealand farms (Barry, 2013). Thus, forage rape used in this study can be considered representative of the crop used in previous studies in which methane emissions have been evaluated, and more generally, of the crop grown in New Zealand conditions.

Ruminal pH has a wide range of influences on rumen physiology and fermentation, including methanogenesis (Van Kessel and Russell, 1996; Janssen, 2010). Ruminal pH is a result of interactions between the production of organic acids from microbial fermentation of feed, bicarbonate flow into the rumen from saliva and from secretion across the ruminal epithelium, absorption and passage of SCFA and possibly ammonia absorption (Aschenbach *et al.*, 2011; Dijkstra *et al.*, 2012). The optimal ruminal pH value for the growth of methanogens is in the range of pH 6.0 to 7.5 with the highest growth rate of this microorganism occurring at pH near neutral, and drop in ruminal pH results in a slower rate of methanogen growth and lower activity (Van Kessel and Russell, 1996; Janssen, 2010).

There are several options to manipulate ruminal pH value including buffer agents and carbohydrate types. Most studies manipulating pH for testing its effects on methanogenesis are conducted *in vitro* (e.g. Deng *et al.*, 2018), but recently *in vivo* experiments were also reported in the literature (Hellwing *et al.*, 2012; Moate *et al.*, 2017; Bougouin *et al.*, 2018). Addition of 0.95% bicarbonate to a grass-clover silage-based diet rich in molasses (Hellwing *et al.*, 2012) or 1% bicarbonate to grass silage-based fibre- or starch-rich diets for dairy cows (Bougouin *et al.*, 2018) had no effects on CH_4 emissions, but the effect on rumen pH was small with an increase of mean pH value by 0.14 units and minimum pH value by 0.06 units (Bougouin *et al.*, 2018). When grain inclusion in the diet has been manipulated to assess the effects of pH on CH_4 emissions, the results have been variable. On the one hand, reduction in ruminal pH value as a consequence of feeding dairy cows with wheat-grain resulted in reductions of CH_4 emissions when compared to feeding of maize-grain-based diets (Moate *et al.*, 2017). On the other hand, increasing the proportion of barley in the diet of beef heifers did not decrease CH_4 emissions when compared to those from heifers fed a high-forage (55% of barley silage) diet (Hünerberg *et al.*, 2015). In contrast to these studies in which ruminal pH is either slightly changed by additional buffer or modified by altering the source of grain, here we reported an *in vivo* study with sheep in which pH value was modified substantially and without the confounding effect of changing the basal diet. Sodium bicarbonate is the most common buffering agent to manipulate ruminal pH value. However, when we tried it in a pilot experiment, a marked change in ruminal pH was not achieved and therefore we selected SC as the buffer for our experimental model.

Increased DM intake generally results in a reduced CH_4 yield in sheep (Blaxter and Clapperton, 1965; Sun *et al.*, 2012b; Hammond *et al.*, 2013) mediated by increases in the fractional outflow rate from the rumen (Hammond *et al.*, 2014). In this study, forage DM intakes were similar for the two groups in all periods of the experiment. Therefore, differences in CH_4 yield between the two groups were not confounded by feed intake level effect and thus differences in CH_4 yield between the groups can be considered a direct result of SC supplementation.

During P02, SC was not supplemented and CH_4 emissions were similar between the two groups as predicted, although the values were different from P01 which might be resulted from the changes in DM intake (Blaxter and Clapperton, 1965; Sun *et al.*, 2012b; Hammond *et al.*, 2013) and forage water content (Pacheco *et al.*, 2014). No difference detected between the two groups in P02 after inclusion of the covariate (P01 measurements) suggested that the background difference was consistent over periods and that the approach of using a background measurement period as a covariate was sound for the purposes of this study.

There were no immediate responses (i.e. within hours) in CH_4 yield to supplementation of the forage rape diet with SC (P03), despite the short-term response in rumen pH and the acetate to propionate ratio, which were greater in SC sheep compared with controls. This finding is contrary to an *in vitro* report by Lana *et al.* (1998) who suggested an immediate and direct effect of low ruminal fluid pH to inhibit CH_4 production, but supports the finding by Moate *et al.* (2019) who reported that ruminal fluid pH did not affect the instantaneous rate of ruminal methanogenesis in an *in vivo* study on dairy cows with wheat replacing corn in the diet. Methane measurements in P03 were performed on the first 2 days of SC supplementation and it is known that methanogens are slow-growing organisms compared with other inhabitants of the rumen (Janssen, 2010). We, therefore, speculate that the rumen methanogen community did not dramatically change within the first 2 days of SC supplementation. In some of our previous work, long-term feeding of forage rape to sheep resulted in a rumen microbial community similar to that of grain-fed animals (Sun *et al.*, 2015a). In the study by Van Kessel and Russell (1996), CH_4 production *in vitro* was inhibited when the inoculum was rumen fluid from concentrate-fed cows with a pH value of 5.45. In that study, CH_4 production was detected only after the pH value of the rumen fluid was adjusted to 7.0 for incubation.

After continuous supplementation of the diet with SC for a week, the mean ruminal pH was 5.96 for the control group and 6.41 for the SC group and a concomitant increase in CH_4 yield was observed for as long as SC was fed. The long-term higher rumen pH with SC supplementation of forage rape might cause rumen microbial community shifts that could emulate what is seen in animals eating ryegrass. During P04 to P06, the average difference in ruminal pH of sheep supplemented with SC in the diet was 0.47 units greater and stayed above pH 6.0 for longer than in the control group. In the study by Van Kessel and Russell (1996), the rumen fluid collected from cows fed timothy hay emitted a larger amount of CH_4 from the *in vitro* system than that collected from concentrate-fed cows when pH value was over 6.0. We speculate that the lack of immediate responses in CH_4 yield immediately after the addition of SC might be due to a lag in the growth of methanogen numbers and/or activity after the

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Table 6 *Proposed relationship between CH_4 emissions and rumen microbial communities and ruminal pH value in sheep*

Period	Rumen microbial community favouring CH_4 formation in the SC group compared to the control	Ruminal pH value in the SC group compared to the control	CH_4 yield in the SC group compared to the control
P01, P02	−	−	−
P03	−	+	−
P04, P05, P06	+	+	+
P07	+	−	−

SC, Na_2CO_3 = sodium carbonate.
+ = increase or more; − = no change.

suboptimal pH for methanogenesis present in sheep fed forage rape is manipulated to a more optimal pH for methanogens (Table 6).

Once SC supplementation was stopped, CH_4 emissions and ruminal pH value immediately returned to levels similar to the control group. We would not expect a dramatic change in rumen microbial community immediately after the removal of Na_2CO_3 as methanogens have a slow growth rate. For example, it took more than a week for rumen microbes to recover after the cessation of chloroform supplemented in the diet of cows (Knight *et al.*, 2011). Ruminal pH dropped to below 5.8 after SC was removed, which would not favour for CH_4 formation (Van Kessel and Russell, 1996), and consequently CH_4 emissions per unit of feed intake became similar to those from sheep given the control treatment. Thus, rumen microbial community not favouring CH_4 formation and low rumen pH could be a mechanism explaining low CH_4 emissions per unit of intake in sheep fed forage rape.

Addition of sodium bicarbonate ($NaHCO_3$) to the diet has been reported to increase rumen passage rate (Russell and Chow, 1993), and increased rumen passage rate has been identified as a factor decreasing CH_4 emissions per unit of intake (Pinares-Patiño *et al.*, 2003; Goopy *et al.*, 2014; Hammond *et al.*, 2014). We used SC (Na_2CO_3) in this study, which might have increased rumen passage rate in the SC supplemented sheep, but the passage rate was not measured in this study. However, this mechanism is not supported by the evidence obtained in our study. The change of rumen passage rage by carbonate supplementation is via increased water intake (Russell and Chow, 1993). This would occur in a short time after the supplementation, and thus CH_4 emissions would be affected rapidly after supplementation, which contrasts with our results. While we speculate that the effects of SC on CH_4 emissions measured in our study were not mediated by changes in passage rate, future studies are needed to confidently confirm that was the case.

Supplementation of the diet with SC affected, to varying degrees, the rumen fermentation parameters such as total SCFA concentration, molar proportions of individual SCFA and ratios of acetate to propionate, or acetate and butyrate to propionate and valerate in each period. These results are consistent with previous findings from a summary of 41 animal experiments with neutralising agents supplemented in the diet of dairy cows (Staples and Lough, 1989). Increasing the ratio of acetate to propionate, especially the ratio of acetate and butyrate to propionate and valerate is considered to be associated with increasing CH_4 emissions (Ramin and Huhtanen, 2013). Such changes in SCFA ratios were observed in all periods, but in the opposite direction to that expected from the CH_4 measurements. The contradiction suggests the complexity of the relationship between SCFA and CH_4 emissions.

The present results with sheep fed forage brassica and effects on ruminal pH and methane emissions are consistent with previous results in sheep (Sun *et al.*, 2012a, 2015a, 2015b) and in dairy cows (Williams *et al.*, 2016). In our previous study (Sun *et al.*, 2015a), the daily average ruminal pH was 6.02 for forage rape-fed sheep and 6.71 for perennial ryegrass-fed sheep (i.e. a mean difference of 0.69 pH units) in rumen fluid samples collected via the rumen cannula. Using the same sampling technique, the daily average ruminal pH for forage rape-fed sheep was 5.87 in the present study and 6.34 for sheep fed forage rape supplemented with SC (i.e. a mean difference of 0.46). In previous studies, CH_4 emissions per unit of forage DM intake from ryegrass-fed sheep were 1.3 (Sun *et al.*, 2012a), 1.3 to 1.4 (Sun *et al.*, 2015a), 1.5 to 1.6 (Sun *et al.* unpublished) and 2.7 (Sun *et al.*, 2015b) times greater than emissions from sheep fed forage rape. In the current experiment, in which the pH value in the rumen of forage rape-fed sheep was increased by SC supplementation to levels similar to sheep fed ryegrass, CH_4 emissions per unit of forage DM intake were 1.3 to 1.5 times larger when the pH value was raised relative to the unsupplemented group. This suggested that ruminal pH, at least in part, is mediating the effect of feeding forage rape to sheep on CH_4 emissions, and this effect can be partially counteracted by supplementing SC.

Conclusion

The dietary supplementation of SC to sheep fed forage rape at 5% and 8% of forage DM increased ruminal pH. The change in pH with SC supplementation did not have an immediate effect on CH_4 emissions, however, over time, the greater pH was associated with higher CH_4 emissions. After the removal of the SC supplementation, ruminal pH and CH_4 emissions returned to a lower level similar to that from the sheep fed the control diet. These results suggest that lower CH_4 emissions previously reported for sheep fed forage rape might, at least partially, be related to lower ruminal pH when this crop is fed.

Acknowledegments

We thank the Pastoral Greenhouse Gas Research Consortium (New Zealand), the New Zealand Agricultural Greenhouse Gas Research Centre and the financial support received for

https://doi.org/10.1017/S1751731119002799 Published online by Cambridge University Press

XueZhao Sun from the Jilin Agricultural Science and Technology University (start-up fund no. 2018:5001) and the Jilin Provincial Department of Science and Technology (grant no. 20180201041NY).

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Declaration of interest

The authors declare no conflicts of interest.

Ethics statement

The animal experiment was conducted under approval No.12982 granted by the Grasslands Animal Ethic Committee (AgResearch, Palmerston North, New Zealand).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119002799

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