Volatile fatty acids (VFA) in rumen samples collected pre-feeding had a stronger relationship with methane emissions in lambs fed graded levels of forage rape than VFAs in post-feeding samples

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Abstract

To evaluate the effect of sampling time on volatile fatty acid (VFA) concentrations and their ability to predict methane yield (g/kg DMI), wethers (n=14/treatment) were fed a ryegrass-based pasture substituted with 0, 25, 50, 75 and 100% forage rape (FR) on a dry-matter basis. Rumen samples were collected before morning feeding (pre-feeding) and 2 h after feeding (post-feeding) and VFA concentrations were measured. Methane emissions and dry-matter intake (DMI) were measured in respiration chambers. Two VFA ratios were calculated: **R1**= [acetate + butyrate] / [propionate + valerate], **R2** = [acetate + butyrate + caproate] / [propionate + valerate + (3 × propanol)].

The relationship of VFA-ratios and methane yield was determined using linear and quadratic regression. VFAs were statistically analysed including dietary treatment as fixed effect and sampling timing as repeated measurement. Pre-feeding R1 included in a quadratic regression was a better and simpler methane-yield predictor (RMSE 1.83) than was R2. In pre-feeding samples, acetate proportion was greater than that in post-feeding samples in all diets except in FR25% ($P<0.01$). Butyrate proportion before feeding was lower than the proportion after feeding in diets with 50% or more FR while propionate was lower in FR25% and higher in FR100% than in post-feeding ($P<0.01$). Considering sampling time can improve the predictive power of a methane proxy.

Keywords: brassica; methane yield; rumen fermentation

Introduction

Volatile fatty acid (VFA) concentrations in rumen can be used as a proxy to infer changes in rumen fermentation related to methane (CH_4) emissions (Williams et al. 2019) and rank individual sheep by their emissions (Jonker et al. 2021). VFAs from rumen samples collected pre- or post-feeding have been used indistinctly in several studies to make inferences about CH_4 emission as well as individual VFAs or ratios of different VFAs (Jonker et al. 2016; Williams et al. 2019). However, because VFA proportions change during the day (van Lingen et al. 2017), their value as a CH_4 proxy may depend on sampling timing relative to feeding.

Post-feeding rumen conditions are more dynamic over time, but production of more-reduced VFAs increases rapidly due to changes in rumen-liquid dissolved-hydrogen (H_2) pressure (van Lingen et al. 2016; van Lingen et al. 2017). Some of these electron sinks, such as caproate and alcohols, are not commonly included in stochiometric "H_2" balances to predict CH_4 emissions from rumen samples. Changes in rumen fermentation occur after feeding regardless of diet type because of an increase in fermentable substrates, but the extent of those changes is diet related. Some changes in VFA concentrations are related to microbial responses to excess H_2 (Janssen 2010).

Sheep fed forage rape were consistently found to produce less CH_4 compared to those fed ryegrass pasture (Sun et al. 2012; Sun et al. 2015a; Sun et al. 2015b), and in an earlier experiment, forage rape decreased methane emission in a linear fashion (Sun et al. 2015b). It is expected that feeding graded levels of forage rape will result in changes in VFA proportions and consequently CH_4 emissions, which would enable exploring the effect of time of feeding on VFA as proxies. The first aim of this study was to compare VFA proportions, including minor end-products, in rumen samples collected pre-feeding and post-feeding, and determine their relationship with CH_4 yield. The second aim was to evaluate the variation of individual end-products across diets and sampling times.

Materials and methods

Animals and diets

Seventy 10-month old Romney wether lambs (38.8±1.8 kg), and spare animals, were randomly assigned to one of five dietary treatments: (on dry-matter (DM) basis) 100% ryegrass-based pasture (FR0%), 75% ryegrass + 25% forage rape (FR25%), 50% ryegrass + 50% forage rape (FR50%), 25% ryegrass + 75% forage rape (FR75%) and 100% forage rape (FR100%). The animals were transitioned onto and adapted to grazing forage rape (*Brassica napus cv. Titan*) at AgResearch's Aorangi farm over a three-week period. The lambs were then transported to AgResearch Grasslands Research Centre (Palmerston North), for adaptation to indoor group housing and feeding of cut forages for at least three weeks prior to the measurement phase. The experiment was reviewed and approved by the Grasslands Animal Ethics Committee (Palmerston North, NZ; approval number 14990), and animals were cared for according to the AgResearch Code of Ethical Conduct.

Dry-matter intake and CH_4 emission measurements

The measurement phase consisted of two days of housing in individual crates for acclimatisation and two days in individual respiration chambers for CH_4 emission measurements. Dry-matter intake (DMI) was determined during the two days of measurements in the respiration

chambers by calculating the difference in the dry matter of feed offered and refused.

Twenty-four respiration chambers, connected to three SERVOMEX 4900 (Servomex Group Ltd., East Sussex, UK) analysers, i.e., eight chambers per cluster, were available for CH_4 measurements. The 70 trial animals were allocated to three batches of 24, 24 and 22 animals. The three batches went into chambers in three consecutive weeks. Full details of chambers and procedures were described by Jonker *et al.* (2016). Methane emission per day was divided by unit of DMI to calculate CH_4 yield.

Rumen sampling

Two rumen samples were collected by oesophageal stomach tubing, the first one approximately 2 h after morning feeding on the day before lambs entered the respiration chambers, and the second sample was collected two days later before morning feeding when the lambs left the respiration chambers. A subsample of 1.8 mL was placed in ice, taken back to the laboratory within ~1 hour, centrifuged at 21,000 × *g* for 10 min at 4°C, before 900 mL of the supernatant was transferred to a new tube containing 100 µL of internal standard (19.87 mM 2-ethylbutyric acid in phosphoric acid 20%, vol/vol) and stored at −20°C until VFA and alcohol analysis. After at least 24 h storage, samples containing the internal standard were thawed and centrifuged at 21,000 × *g* for 10 min at 4°C. Molar concentrations of VFAs (acetate, propionate, butyrate, valerate and caproate) and alcohols (ethanol and propanol) were determined by gas chromatography (Tavendale et al. 2005).

Data calculations

The proportion of each individual VFA and alcohol were expressed as mole percentage of total mol fermented hexoses (FH) equivalent. The mole hexose equivalents for each product are acetate, 0.5; propionate, 0.5; butyrate, 1.0; valerate, 1.0; caproate, 1.5; ethanol, 0.5; propanol, 0.5. Additionally, two different VFA ratios were calculated from the molar concentrations of the products:

R1 = [acetate + butyrate] / [propionate + valerate],

R2 = [acetate + butyrate + caproate] / [propionate + valerate + (3 × propanol)].

The ratios represent electron ("H_2") production/incorporation for each fermentation product, according to principles of rumen stoichiometry (Wolin 1960). The coefficients for acetate, butyrate and caproate are all 1.0, in accordance with long-established practice where every four electrons (two × "H_2") is given a value of 1.0 in the numerator, based on the product formation from hexoses. The coefficients for end products that result in net electron incorporation 1.0 for propionate and valerate, and 3.0 for propanol, again in accordance with established practice where every two electrons (one × "H_2") is given a value of 1.0 in the denominator. Ethanol formation does not result in net electron production/release, so was not included in the VFA ratio.

Statistical analyses

Data analyses were performed using the 'predictmeans', 'lme4' packages in statistical software R 4.0.3 (R Core Team, 2020). Methane yield (g/kg DMI) was analysed with dietary treatment as fixed effect, batch and chamber nested within cluster as random effects. Fermentation end-product data were analysed using a repeated-measurements model that included dietary treatment, time, and their interaction as fixed effects, and lamb ID nested within batch as a random effect. A multiple comparison was performed on the modelling results with P values adjusted by the "BH" method (Benjamini & Hochberg 1995). Significant mean differences were declared at $P<0.05$.

To evaluate relationship between R1or R2 calculated for pre-and post-feeding samples and CH_4 yield separately, a polynomial (up to quadratic level) regression with batch and chamber nested within cluster as random effects was used in the analysis. In this case, the CH_4 yield was averaged over two days of measurements.

Results

Relationship between end-product hydrogen balances and CH_4 yield

R1 ratios were slightly greater than those calculated with R2, regardless of sampling time. Pre-feeding R1 and R2 had stronger relationship with CH_4 yield than post-feeding R1 and R2 ratios (Table 1). Adding R1 or R2 ratios as quadratic terms in the model to predict CH_4 yield further reduced the RMSE, especially for pre-feeding samples (Table 1). CH_4 yield appeared to be overestimated when the measured CH_4 yield was less than 17 g/kg DMI and underestimated when measured CH_4 yield was greater than 17 g/kg DMI regardless of the VFA ratio or sampling time chosen.

Rumen sampling timing and diet interactions

There was an interaction between diet and sampling time ($P\leq0.01$) for all fermentation end-products, except valerate ($P=0.64$) (Table 2). Total calculated fermented hexoses were greater post-feeding than pre-feeding in diets which included 50% or less forage rape ($P<0.05$), while there was no difference between pre-feeding and post-feeding total fermented hexoses in lambs fed 75 and 100% forage rape ($P>0.05$).

The post-feeding acetate proportion was less than that pre-feeding for all dietary treatments ($P<0.05$). Post- and pre-feeding butyrate proportions were similar in diets containing 0 or 25% forage rape but were greater post-feeding in diets containing 50% or more forage rape (p$P<0.05$). The post-feeding caproate proportion was greater than pre-feeding only for 100% forage rape ($P<0.05$). The post-feeding propionate proportion was greater than that obtained pre-feeding when the diet contained 25% forage rape but was less if the diet contained 100% forage rape ($P<0.05$) without differences between sampling time for the other dietary treatments. The post-feeding valerate proportion was greater than that obtained pre-feeding

Table 1 Intercept (βo), linear (β1) and quadratic terms (β2) (coefficients ± s.e.) in regression equations to predict CH_4 yield using volatile fatty acid ratios as independent variables.

Sample	Ratio	Term	β_o	β_1	β_2	RMSE	AIC	BIC
Pre-feeding	R1	Linear	7.55 ± 1.21	2.73 ± 0.33		2.22	327.55	341.04
Pre-feeding	R1	Linear + Quadratic	-5.82 ± 3.73	11.23 ± 2.33	-1.26 ± 0.35	1.83	317.73	333.48
Pre-feeding	R2	Linear	7.41 ± 1.21	2.81 ± 0.34		2.18	326.79	340.29
Pre-feeding	R2	Linear + Quadratic	-5.86 ± 3.73	11.36 ± 2.35	-1.29 ± 0.35	1.83	317.01	332.75
Post-feeding	R1	Linear	11.39 ± 1.75	1.78 ± 0.53		2.96	362.56	376.06
Post-feeding	R1	Linear + Quadratic	-6.76 ± 6.62	12.85 ± 3.94	-1.61 ± 0.57	2.80	356.18	371.92
Post-feeding	R2	Linear	10.99 ± 1.77	1.93 ± 0.55		2.93	361.37	374.86
Post-feeding	R2	Linear + Quadratic	-8.23 ± 6.90	13.91 ± 4.21	-1.79 ± 0.62	2.77	354.60	370.34

RMSE: root mean square error, AIC: Akaike information criterion, BIC: Bayesian information criterion
R1: [acetate + butyrate]/ [propionate + valerate]
R2: [acetate + butyrate + caproate] / [propionate + valerate + (3 × propanol)]

Table 2 Mean methane and hydrogen yield (g/kg dry matter intake), and volatile fatty acids, ethanol and propanol as a proportion of total fermented hexoses (FH) in rumen samples collected pre-and post- feeding in sheep fed ryegrass-based pasture substituted with 0, 25, 50, 75 and 100% of forage rape (FR0%, FR25%, FR50%, FR75%, FR100%)

Measure[1]	FR0%	FR25%	FR50%	FR75%	FR100%	SED	diet	time	diet × time	*r* pre vs. post	*r* CH_4 yield vs end products
Dry matter intake [kg/d]	0.98^{c}	1.01^{bc}	1.08^{ab}	1.10^{a}	1.06^{abc}	0.03					
CH_4 yield [g/kg dry matter intake]	19.18^{a}	18.65^{a}	18.09^{ab}	17.02^{b}	12.67^{c}	0.66	<0.01				
Total hexoses fermented (FH) [mM][2]											
pre-feeding	26.00^{e}	29.08^{e}	34.18^{de}	40.97^{abc}	51.52^{a}	2.73	<0.01	<0.01	0.01	0.37	-0.41
post-feeding	38.73^{cd}	43.71^{abc}	47.68^{ab}	45.46^{abc}	51.44^{a}						-0.34
Acetate [% of FH]											
pre-feeding	62.89^{a}	59.07^{b}	56.07^{b}	53.27^{d}	44.69^{f}	1.10	<0.01	<0.01	<0.01	0.80	0.61
post-feeding	59.71^{b}	54.23^{cd}	48.54^{e}	45.76^{f}	41.97^{g}						0.66
Propionate [% of FH]											
pre-feeding	15.61^{e}	17.16^{de}	19.41^{bcd}	18.80^{bcd}	22.28^{a}	0.81	<0.01	0.89	<0.01	0.23	-0.71
post-feeding	17.08^{cde}	19.42^{bc}	20.28^{ab}	20.09^{ab}	16.08^{e}						-0.03
Butyrate [% of FH]											
pre-feeding	18.67^{f}	20.21^{ef}	21.37^{def}	24.08^{cd}	26.30^{bc}	0.99	<0.01	<0.01	0.01	0.61	-0.14
post-feeding	19.78^{ef}	22.64^{de}	27.18^{b}	28.89^{b}	32.22^{a}						-0.44
Valerate [% of FH]											
pre-feeding	1.77^{d}	2.09^{d}	1.94^{d}	2.14^{d}	3.97^{b}	0.33	<0.01	<0.01	0.64	0.54	-0.63
post-feeding	2.49^{cd}	2.41^{cd}	2.68^{cd}	3.25^{bc}	4.94^{a}						-0.68
Caproate [% of FH]											
pre-feeding	0.69^{cd}	0.92^{bcd}	0.87^{bcd}	1.37^{bc}	1.54^{b}	0.23	<0.01	0.48	<0.01	0.56	-0.15
post-feeding	0.59^{d}	0.81^{bcd}	0.77^{cd}	1.08^{bcd}	2.51^{a}						-0.42
Ethanol [% of FH]											
pre-feeding	0.31^{d}	0.40^{cd}	0.27^{d}	0.29^{d}	1.06^{b}	0.12	<0.01	<0.01	<0.01	0.72	-0.44
post-feeding	0.26^{d}	0.35^{d}	0.47^{cd}	0.73^{bc}	2.10^{a}						-0.53
Propanol [% of FH]											
pre-feeding	0.09^{e}	0.10^{de}	0.09^{e}	0.08^{e}	0.19^{b}	0.01	<0.01	<0.01	<0.01	0.36	-0.48
post-feeding	0.07^{e}	0.09^{e}	0.14^{cd}	0.16^{bc}	0.22^{a}						-0.56
R1											
pre-feeding	4.40^{a}	3.85^{b}	3.32^{df}	3.36^{cde}	2.59^{h}	0.18	<0.01	<0.01	<0.01	0.50	0.70
post-feeding	3.81^{bc}	3.21^{defg}	2.93^{egh}	2.86^{fgh}	3.33^{defg}						0.38
R2											
pre-feeding	4.33^{a}	3.81^{b}	3.30^{d}	3.34^{cde}	2.55^{g}	0.17	<0.01	<0.01	<0.01	0.51	0.73
post-feeding	3.78^{bc}	3.19^{def}	2.89^{efg}	2.81^{fg}	3.25^{def}						0.67

SED: average standard error of the differences
[1] **R1**: [acetate + butyrate]/ [propionate + valerate], **R2**: [acetate + butyrate + caproate] / [propionate + valerate + (3 × propanol)],
[2]FH = 0.5 × acetate + 0.5 × propionate + butyrate + valerate + 1.5 × caproate + 0.5 × ethanol + 0.5 × propanol
r pre vs. post: correlation coefficient between sampling time within each rumen fermentation end-product.
r CH_4 y vs end products: correlation coefficient between CH_4 yield and each fermentation end-product measured at each sampling time
^{abcdefgh} letter-based representation of pairwise comparisons at significant level '0.05'

(P<0.05) only when diet contained 100% forage rape. Post-feeding proportions of ethanol were greater than that obtained pre-feeding in diets containing >75% forage rape and for propanol when >50% forage rape in the diet. The pre-feeding VFA ratio calculated either with the R1 or R2 equation was less than the post-feeding VFA ratio in diets containing up to 75% of forage rape (P<0.05). However, the post-feeding VFA ratio was greater than the pre-feeding value (P<0.05) when the diet contained 100% forage rape.

Acetate, butyrate and ethanol proportions had medium-high (r ~0.70) correlations between pre- and post-feeding samples, while propionate and propanol had weak (r <0.40) correlations (Table 2).

Discussion

The main finding of the current study was that VFA ratios (R1 and R2) in rumen liquid collected pre-feeding were a stronger predictor of CH_4 yield in lambs fed graded levels of forage rape than were VFA ratios (R1 and R2) from rumen samples collected post-feeding. Propionate proportion behaved unpredictably in post-feeding rumen samples, while the other VFAs changed in the direction that was reported in previous studies in sheep fed forage rape (Sun et al. 2012; Sun et al. 2015a). The finding that the pre-feeding VFA ratio was a stronger predictor of CH_4 yield than in post-feeding samples is consistent with findings of a recent meta-analysis of data from sheep fed with a range of brassica crops in New Zealand (He et al. 2020). This meta-analysis showed that pre-feeding VFA ratio explained 42% of variability in CH_4 yield, while post-feeding VFA ratio was not selected in the model to explain CH_4 yield.

The low predictive value of post-feeding VFA ratios may be a consequence of a greater variability in VFA concentrations after feeding due to rapid changes in fermentation rate and consequently rumen environment after feeding (van Lingen et al. 2017). The main VFA produced in all diets was acetate, as its production is thermodynamically more efficient than production of other fermentation end-products (van Lingen et al. 2016). However, H_2 concentrations in the rumen increase after feeding due to rapid feed fermentation. This results in an increased concentration of soluble hydrogen that forces alternative electron-accepting pathways to maintain re-oxidation of reduced electron carriers (van Lingen et al. 2016). Propionate is generally the main electron sink, but also other electro-accepting pathways increase when CH_4 yield decreases, resulting in formation of products such as butyrate, valerate, caproate, ethanol and propanol (van Lingen et al. 2017; Ungerfeld, 2020). Propionate was the most variable VFA across the two sampling times and diets which could indicate that the threshold for switching to this less-energy-yielding pathway is relatively higher and a more-permanent switch than the condensation of existing VFAs to produce butyrate, caproate and valerate to remove some of the hydrogen created. Feeding FR100% resulted in the lowest CH_4 yield across diets and in the greatest propionate proportion in pre-feeding samples. However, lambs fed FR100% had the lowest propionate proportion post-feeding, which greatly affected the electron balances (as R1 and R2 VFA ratios) calculated post-feeding. These results were different from previous findings, where propionate proportion increased post-feeding in sheep fed forage rape (Sun et al. 2012; Sun et al. 2015a). The other electron sinks that appear in the R1 and R2 VFA-ratios calculations, valerate and propanol, represent together roughly between 2 and 6% of fermented hexoses equivalent across the diets, with the greatest level for FR100%. Including propanol in the R2 electron balance (VFA ratio) calculation generated numerically smaller values, but this change did not result in a better prediction of CH_4 yield.

Inclusion of VFA ratio as quadratic term in the model did improve the CH_4 yield prediction accuracy in pre- and post-feeding samples, but pre-feeding samples were still more accurate than post-feeding samples. In a previous study, the addition of VFA ratio as linear and quadratic terms to explain CH_4 yield in sheep fed lucerne silage substituted with graded level of maize grain and silage (Jonker et al. 2016) was also stronger than only the linear term, indicating the factors other than simple diet composition are playing a major role in the hydrogen balance of the rumen. In both our study and the study of Jonker et al. (2016), CH_4 yield changed non-linearly with increasing substitution of one feed with another feed, which might explain the improvement in CH_4 yield prediction when the VFA ratio is added as a quadratic term.

Conclusions

Pre-feeding VFA ratios had a stronger relationship with CH_4 yield in lambs fed graded levels of forage rape than did VFA ratios in post-feeding rumen samples. Propionate behaved unpredictably in post-feeding rumen samples with the smallest proportion in lambs fed 100%FR, while being greatest pre-feeding. Including caproate, ethanol and propanol in R2 did not improve its relationship with CH_4 yield compared to R1. Time of sampling relative to feeding and type of diet evaluated is a key factor to consider for developing CH_4 proxies from rumen metabolite concentrations.

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