Genetic parameters of plasma and ruminal volatile fatty acids in sheep fed alfalfa pellets and genetic correlations with enteric methane emissions[1]

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ABSTRACT: Animal-to-animal variation in methane (CH_4) emissions determined in respiration chambers has a genetic basis, but rapid phenotyping methods that can be applied on-farm are required to enable increased genetic progress by the farming industry. Fermentation of carbohydrates in the rumen results in the formation of VFA with hydrogen (H_2) as a byproduct that is used for CH_4 formation. Generally, fermentation pathways leading to acetate are associated with the most H_2 production, less H_2 formation is associated with butyrate production, and propionate and valerate production are associated with reduced H_2 production. Therefore, VFA may constitute a potential correlated proxy for CH_4 emissions to enable high-throughput animal screening. The objective of the present study was to determine the genetic parameters for ruminal and plasma VFA concentrations in sheep fed alfalfa (*Medicago sativa* L.) pellets and their genetic (r_g) and phenotypic (r_p) correlations with CH_4 emissions. Measurements of CH_4 emissions in respiration chambers and ruminal (stomach tubing 18 h from last meal) and blood plasma (3 h post-feeding) VFA concentrations were made on 1,538 lambs from 5 birth years (2007 and 2009 to 2012) aged between 5 and 10 mo, while the animals were fed alfalfa pellets at 2.0 times maintenance requirements in 2 equal size meals (0900 and 1500 h). These measurements were repeated twice (rounds) 14 d apart. Mean (± SD) CH_4 production was 24.4 ± 3.08 g/d, and the mean CH_4 yield was 15.8 ± 1.51 g/kg DMI. Mean concentration of total ruminal VFA was 52.2 mM, with concentrations of acetate, propionate and butyrate of 35.97, 8.83, and 4.02 mM, respectively. Ruminal total VFA concentration had heritability (h^2) and repeatability estimates (± SE) of 0.24 ± 0.05 and 0.35 ± 0.03, respectively, and similar estimates were found for acetate, propionate, and butyrate. Blood plasma concentrations of VFA had much lower estimates of h^2 and repeatability than ruminal VFA. Genetic correlations with CH_4 yield were greatest for total concentrations of ruminal VFA and acetate, with 0.54 ± 0.12 and 0.56 ± 0.12, respectively, which were much greater than their corresponding r_p. The r_p and r_g of ruminal VFA proportions and blood VFAs with CH_4 emissions were in general lower than for ruminal VFA concentrations. However, minor ruminal VFA proportions had also moderate r_g with CH_4 yield. Pre-feeding concentrations of total VFA and acetate were the strongest correlated proxies to select sheep that are genetically low CH_4 emitters.

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INTRODUCTION

Globally, enteric methane (CH_4) emissions from ruminant livestock is the single most important source of emissions of this greenhouse gas (Steinfeld et al., 2006). In New Zealand, where pastoral livestock farming is an important economic activity, enteric CH_4 emissions represent the largest share of the country's total greenhouse gas emissions (Clark, 2013). Animal-to-animal variation in CH_4 emissions is well documented and has a genetic basis (Pinares-Patiño et al., 2011b, 2013). Therefore, genetic selection for lower CH_4 emissions may be a viable mitigation option, provided that the CH_4 emissions trait has no strong unwanted associations with animal production traits (Pinares-Patiño et al., 2013; Elmes et al., 2014). For this approach to progress, a high throughput and reliable method of ranking animals for their CH_4 emissions is required.

Fermentation of feed in the rumen by microbes results in the formation of VFA and hydrogen (H_2), and this H_2 is used by methanogens to form CH_4 (Van Nevel and Demeyer, 1996; Janssen, 2010). H_2 formation is a means of disposing of electrons derived from oxidation steps in fermentation. Overall, the formation of acetate is accompanied by the production of H_2, whereas the formation of butyrate is associated with less H_2 production. Production of propionate and valerate involves net uptake electrons that arise from fermentation and so reduce the total amount of H_2 formed (Czerkawski, 1986; Janssen, 2010). The relative amounts formed of these major VFAs therefore largely determine the amount of excess H_2 in the rumen ultimately available for CH_4 production. It was hypothesized that analyzing rumen or plasma VFA profiles would be a suitable correlated trait to screen animals for low CH_4 as an alternative to having to measure CH_4 in respiration chambers. The objective of the present study was to determine the genetic parameters for ruminal and plasma VFA concentrations in sheep fed alfalfa pellets and determine their genetic (r_g) and phenotypic (r_p) correlations with CH_4 production (g/d) and yield (g/kg DMI).

MATERIALS AND METHODS

A research program is being undertaken in New Zealand to determine the genetic parameters of CH_4 production and yield and their r_g with key production traits in sheep (Pinares-Patiño et al., 2013; Jonker et al., 2018a). Rumen and plasma samples are routinely collected from animals being measured for CH_4 emissions, and VFA concentrations analyzed. The animal experiments conducted adhere to the guidelines of the AgResearch Code of Ethical Conduct and were approved by the AgResearch Grasslands (Palmerston North, New Zealand) and AgResearch Invermay (Mosgiel, New Zealand) Animal Ethics committees (approval numbers 11930, 11975, 12206, 12233, 12241, 12324, and 12414).

Experimental Animals

The sheep, born in 2007, 2009, 2010, and 2011, used in the present study were the same as those used by Pinares-Patiño et al. (2013) with an additional cohort born in 2012 (Jonker et al., 2018a). In total, 1,538 lambs, 248 males and 1,290 females, of between 5 and 10 mo of age and weighing between 30 and 40 kg were used (Table 1).

Table 1. Summary of birth years, number of animals (and records) by sex, and number of sires for each trait

Parameter	Birth years	No. of males (no. of records)	No.of females (no. of records)	No. of sires
CH_4	2007, 2009–2012	248 (493)	1,290 (2,693)	112
Ruminal VFA	2009–2012	248 (492)	1,195 (2,377)	102
Plasma VFA	2009–2011	153 (305)	997 (1,985)	89

The animals born 2007 to 2011 were progeny of 112 maternal dual-purpose sires generated by the New Zealand industry Central Progeny Test (**CPT**) program (McLean et al., 2006), comprising Coopworth, Romney, Perendale, Texel, and composite breeds, where the latter breed consisted primarily of combinations of the former breeds with additional infusions of Finn and East Friesian. All rams were mated to composite ewe progeny. From these progeny, the CH_4 selection line flock was formed by screening CPT ewe lambs (and ram lambs born in 2009) for their CH_4 yield (g/kg DMI; Pinares-Patiño et al., 2013; Jonker et al., 2018a) with the most extreme low and high 10% retained for further breeding. Matings were conducted to generate divergent high and low CH_4 yield lines and generation 1 progeny were born in 2010, 2011, and 2012.

Feeding

For each year, measurements started in late-February and finished by mid-July. Animals were transported to the research facility in lots of 96 and CH_4 measurements were repeated twice (rounds), 14 d apart. Animals were housed in pens, with up to 10 animals per pen and gradually adjusted to the experimental alfalfa pellet diet with decreasing allowance of pasture forage over about 8 d, followed by another 14 d of pellet feeding before CH_4 measurements. Drinking water was available at all times. The alfalfa pellets (pressed through a die with 11-mm holes) were prepared from the first spring crop each year, which was artificially dehydrated (Dunstan Nutrition Ltd., Hamilton, New Zealand). Throughout the experiment, the feeding level was set at 2.0 times the animals' maintenance ME requirements ($\mathbf{ME_r}$; CSIRO, 2007) and feed was provided twice a day (0900 and 1500 h) in equal size meals. The alfalfa pellets were routinely analyzed for DM content after drying at 105 °C for 24 h and for chemical composition using near infrared spectroscopy (Corson et al., 1999). The pellets contained on average (± SD; %, DM basis) 15.3 ± 2.8 CP, 2.9 ± 1.2 lipids, 9.5 ± 1.9 soluble sugars, and 40.0 ± 5.7 NDF.

Methane Measurements

The CH_4 measurement protocol for selecting sheep with divergent CH_4 yield was previously described by Pinares-Patiño et al. (2013). Measurements of CH_4 and DMI were performed at the New Zealand Ruminant Methane Measurement Centre (AgResearch, Palmerston North, New Zealand), which comprises 3 clusters of 8 open-circuit respiration chambers. Construction, operation, maintenance, and calibration of the facility are described in full detail by Pinares-Patiño et al. (2012). Briefly, each chamber was 1.8 m^3 (1.8 m long × 0.85 m wide × 1.2 m high) with an air flow rate of 300 L/min, which was continuously monitored by measuring differential pressure within a Venturi flowmeter. Subsamples of outflow gases from a cluster of 8 chambers were directed to a gas analyzer and continuously sampled using a multiport gas switching unit (S.W. & W.S. Burrage, Ashford, Kent, UK), and the air stream of each individual chamber directed in sequence (over 5- to 6-min cycles) to a 4900C Continuous Emissions Analyzer (Servomex Group, East Sussex, UK; 1 analyzer per cluster of 8 chambers) to determine CH_4 and CO_2 concentrations by infrared technology. The CH_4 analyzer was tested daily using alpha standards (BOC, Auckland, New Zealand), and the analyzer was calibrated if required (Pinares-Patiño et al., 2012). The recovery of CH_4 from each chamber through the whole system was independently tested (twice yearly) by the National Institute of Water and Atmospheric Research (Wellington, New Zealand; Pinares-Patiño et al., 2012). Methane production was calculated from the difference between CH_4 inflow and outflow over time, adjusted for humidity, temperature, and pressure (Pinares-Patiño et al., 2012).

Measurements were conducted over 2 rounds (R1 and R2), and at each round, the sheep were randomly allocated to measurement groups (4 × 24 sheep) and individuals randomly allocated to 1 of the 24 respiration chambers. All 4 groups were measured consecutively within round (R1 and R2), which took approximately 9 d to complete. Two to 4 d before CH_4 measurements, sheep were moved into individual metabolism crates for individual feeding of the alfalfa pellets, followed by moving the metabolism crates (with sheep) into the respiration chambers for CH_4 emissions measurements over 2 (weekdays; Monday to Wednesday and Wednesday to Friday) or 3 (weekend; Friday to Monday) days. All chambers were opened twice daily (front and rear doors of each chamber) for approximately 30 min to allow collection of feed refusals (only a few cases), to provide fresh feed and to exchange excreta collection trays for clean ones. Gas emissions during these periods were extrapolated by taking the average of the last 12 values before opening the door. After completion of the respiration chambers measurements of R1, sheep were housed in group pens for 14 d before CH_4 measurements of R2 as described for R1. DMI was determined during the last 2 d in individual crates and while in respiration chambers. Animals with large refusals of feed DM offered on

d 1 in crates were removed from the experiment and replaced by spares. Animals were weighed before the morning feeding at arrival, before being moved into metabolism crates and weighed again after respiration chamber measurements.

Blood Sampling and Analysis

On the second day in metabolism crates in each round (1,150 animals; birth years 2009 to 2011), blood was collected from the jugular vein by venipuncture 3 h after morning feeding. Approximately 10 mL of blood was collected using heparin-coated vacutainer tubes, placed on ice, and immediately processed in the laboratory. Plasma was isolated by centrifugation (2,000 × *g*, 10 min) and stored frozen (−20 °C) until further processing and analysis.

Measurement of blood plasma VFA concentrations was carried out following the procedure described by Moreau et al. (2004). Thawed plasma (1 mL) was added to 100 µL of a 20% (w/v) aqueous 5-sulfosalicylic acid solution (deproteinizing agent) and 100 µL of internal standard mixture (D4 acetic acid was 200 µg, all other acids were 5 µg per sample), then vortexed, and frozen overnight. After thawing, the mix was centrifuged (19,000 × *g*, 45 min). Subsequently, 300 µL of supernatant was added to a flat bottomed insert and analyzed using a gas chromatograph–mass spectrometer (Shimadzu QP2010 with an AOC 5000 auto sampler; Shimadzu, Tokyo, Japan). An Agilent DB FFAP column (15 m length × 0.1 mm internal diameter × 0.1 µm film; Agilent, Santa Clara, CA) was used with helium as a carrier gas flowing at 0.59 mL/min (liner velocity 40.0 cm/s). Injection used a solid-phase microextraction (SPME) Carboxen/PDMS 1-cm fiber (Sigma-Aldrich, Auckland, New Zealand) for 5 min at room temperature. The inlet temperature was set to 280 °C and the fiber desorbed for 6 min. The inlet liner was an SPME liner with a 0.75 mm internal diameter. The ion source was set to 200 °C and the transfer line to 250 °C. The oven temperature was held at 80 °C for 1 min and then increased at a rate of 75 °C/min to 240 °C and was then held for 3 min. The detector gain was set at the voltage obtained during autotuning and then increased by 50% (140 s) after the elution of acetic acid. Mass spectrometry scanning was by selected ion monitoring mode using stable isotope internal standards according to Moreau et al. (2004), with an event time of 0.1 s.

Rumen Sampling and Analysis

Rumen contents from each animal were sampled by stomach tubing at the end of the respiration chamber measurement in each round (18 h after last feed offer; 1,443 animals; birth years 2009 to 2012).

The sample of approximately 50 mL was placed on ice and a subsample (1.8 mL) transferred into a 2-mL tube, maintained on ice and immediately transported to the laboratory for processing. Once in the laboratory, the rumen fluid samples were centrifuged (21,000 × *g*, 10 min) and 900 µL of the supernatant was transferred to a 1.5-mL tube and acidified with 100 µL 20% (v/v) phosphoric acid containing ethylbutyric acid as an internal standard and frozen overnight (−20 °C). The tubes were thawed and recentrifuged under the same conditions as above to remove any remaining particles. Afterwards, 0.8-mL of the supernatant was transferred to 1.5-mL crimp top vial with a silicone septum aluminum cap for VFA analysis.

Analysis of ruminal VFA was conducted using a gas chromatograph (Hewlett Packard HP6890 series; Agilent) fitted with a flame ionization detector. An Agilent J&W HP-PLOT molesieve capillary column (30.0 m length × 0.53 mm internal diameter × 25 µm film) was used for separation, with helium as a carrier gas flowing at 5.5 mL/min. Samples (1.0 µL) were injected into the column inlet (cool on-column inlet). The inlet temperature was set to track the oven temperature and the detector was at 240 °C. A program altered oven temperature from 85 to 180 °C at 10 °C/min and then held it at 180 °C for 5 min, giving a run time of 14.5 min per sample.

Calculations and Statistical Analyses

Methane from each animal was expressed as CH_4 production (g/d) and CH_4 yield (g/kg DMI). Daily CH_4 records with gaps of more than 1 h (e.g., in cases of power failure, computer failure, etc.) were excluded. Similarly, records where the daily DMI (DM offered − DM refused) was <75% of the feed offered were omitted. Altogether, approximately 7% of records were excluded or missing. For the present study, the CH_4 data recorded daily were averaged per animal within each round to give a single value corresponding to parallel VFA measures for that round. Data for plasma VFA concentrations were expressed as millimolar. Major (i.e., acetate, propionate, and butyrate) and minor (i.e., *iso*-butyrate, valerate, *iso*-valerate, and caproate) ruminal VFA were expressed on the basis of absolute concentrations (mM) and molar proportions (mol/100 mol total VFA). Ruminal acetate and butyrate formation are associated with H_2 formation, whereas increased propionate and valerate

formation are associated with decreased H_2 formation (Demeyer and Van Nevel, 1975; Janssen, 2010). Therefore, ratios of acetate/propionate and (acetate + butyrate)/(propionate + valerate) [(A + B)/(P + V)] were calculated. To improve the fit to normality or homoscedasticity, VFA concentrations and molar proportions of major VFA were transformed as $\log_{10}(x + 1)$, where x is the concentration of a VFA.

Data analysis models were configured for each trait separately. Fixed effect models were determined using the general linear model procedure, fitting a repeatability model, which assumes a common variance for the repeated measures and a nonzero covariance between them (SAS, 2011). Fixed effects fitted included flock (flk), birth year (byr), sex, birth, and rearing rank combination (brr; 6 levels for born as a single, twin or triplet, and reared as one of those types up to the birth level, e.g., level 3/2 refers to an animal born as a triplet but reared as a twin), recording year (ryr), round within year (1 or 2), lot within round (1 to 4), group (of up to 24, within each lot), system (8 chambers to a system, 1 to 3 within each group), and chamber (1 to 24 within each group). Birthday deviation from mean of the animal's sex by weaning mob group and age of dam as linear and quadratic effects, were fitted as covariates. These main effects and their interactions between these effects were tested and, by a process of backward elimination, parsimonious models were selected (Table 2). Pedigree records, containing up to 12 generations, were obtained from animals born between 1990 and 2012 in the 5 birth flocks that the CH_4 yield selection line animals were derived from. Two-trait models were subsequently fitted using ASREML 3.0 (Gilmour et al., 2009) with full animal random effects and permanent environmental effects to obtain r_p and r_g between CH_4 emissions (both production and yield) and VFA concentrations (both ruminal and plasma). Repeated records of each CH_4 and VFA trait on the same animal were accounted for by fitting repeatability (permanent environmental) effects for each type of repeat interval, namely an animal effect, and an animal by year effect (for methane traits only). Repeatability estimates relate to repeatability of measurements across rounds within the same year and therefore included all permanent environmental effect variances. Heritability and repeatability estimates of rumen and plasma VFA traits were calculated by averaging their estimates from the 2-trait runs with CH_4 production and yield.

The efficiency (Q) of indirect selection for 24-h CH_4 yield using a single measure of a VFA parameter was calculated as $Q = |r_g| (h_{VFA}/h_{CH4})$, where h is the square root of the heritability (Searle, 1965). The efficiency with CH_4 yield was calculated because this was the selection criterion in the sheep breeding program and because feeding level was fixed.

RESULTS

Feed Intake and CH_4 Emissions

Across the entire data set, the mean DMI was 1.56 kg/d, CH_4 production was 24.4 g/d, and CH_4 yield was 15.8 g/kg DMI. Heritability (h^2) for CH_4 production was 0.31 with repeatability between measurement rounds of 0.60 (Table 3). Heritability for CH_4 yield was 0.15 with repeatability between rounds of 0.36.

Rumen Volatile Fatty Acids

The mean (back transformed; $y = 10^x - 1$) total VFA concentration was 50.3 mM. The concentrations of acetate, propionate, and butyrate were 34.6, 8.4, and 3.8 mM, respectively, and their molar proportions were 68.8, 16.6, and 7.5 mol/100 mol VFA, respectively (Table 3). Overall, the variations of molar proportion of the major VFA were much smaller than variations of their concentrations. Mean acetate/propionate ratio was 4.15 and the mean (A + B)/(P + V) ratio was 4.32. Minor VFAs made up less than 6.5% of total VFA with mean proportions of 0.11, 1.12, 2.43, and 2.81 mol/100 mol

Table 2. Final mixed-models and fixed effects used for trait analysis

Parameter	Fixed effects[1]	Random effects[2]
CH_4, g/d	byr.flk.sex, ryr.lot.group.round, brr, bdev	animal, eperm, eperm.ryr,
CH_4, g/kg DMI	byr.flk.sex, ryr.lot.group.round	animal, eperm, eperm.ryr,
VFA, mM or molar proportion	byr.flk.sex, ryr.lot.group.round	animal, eperm

[1]brr = birth rearing rank; bdev = birth day deviation; flk = birth flock; ryr = recording year; lot = mob of 96 animals; group = sub-mob of up to 24 animals within a lot measured contemporaneously; round = measurement time 14 d apart; byr = birth year, where "." indicates an interaction. The average numbers in each byr.flk.sex level were 62 (4 classes; range 13 to 96) for male groups and 68 (19 classes; range 13 to 96) for female groups.

[2]eperm = permanent environmental effects.

Table 3. Means of methane emission traits, ruminal and plasma VFA [$\log_{10}(x + 1)$ transformed], and corresponding heritability and repeatability values across rounds 14 d apart[1]

Parameter	Mean [$\log_{10}(x + 1)$]	Mean[2]	σ_p	Heritability	Repeatability
CH_4, g/d		24.4	3.08	0.31 ± 0.05	0.60 ± 0.02
CH_4, g/kg DMI		15.8	1.51	0.15 ± 0.03	0.36 ± 0.02
Rumen VFA concentrations, mM					
Total VFA	1.71 ± 0.09	50.3	0.09	0.24 ± 0.05	0.35 ± 0.03
Acetate	1.55 ± 0.09	34.6	0.09	0.23 ± 0.05	0.35 ± 0.03
Propionate	0.97 ± 0.10	8.40	0.10	0.25 ± 0.05	0.34 ± 0.03
Butyrate	0.68 ± 0.11	3.79	0.11	0.20 ± 0.04	0.32 ± 0.03
Caproate	0.03 ± 0.01	0.06	0.02	0.03 ± 0.02	0.15 ± 0.03
Valerate	0.20 ± 0.01	0.57	0.04	0.15 ± 0.04	0.28 ± 0.03
Iso-butyrate	0.35 ± 0.01	1.22	0.06	0.07 ± 0.03	0.25 ± 0.03
Iso-valerate	0.38 ± 0.01	1.41	0.07	0.06 ± 0.03	0.25 ± 0.03
Rumen VFA proportions, mol/100 mol VFA					
Acetate	1.84 ± 0.01	68.8	0.01	0.07 ± 0.03	0.24 ± 0.03
Propionate	1.25 ± 0.04	16.6	0.04	0.11 ± 0.03	0.24 ± 0.03
Butyrate	0.93 ± 0.06	7.45	0.06	0.09 ± 0.03	0.31 ± 0.03
Caproate	0.05 ± 0.01	0.11	0.03	0.05 ± 0.03	0.14 ± 0.03
Valerate	0.33 ± 0.01	1.12	0.03	0.02 ± 0.02	0.14 ± 0.03
Iso-butyrate	0.54 ± 0.01	2.43	0.09	0.15 ± 0.04	0.28 ± 0.03
Iso-valerate	0.58 ± 0.01	2.81	0.10	0.13 ± 0.04	0.27 ± 0.03
Rumen VFA ratios					
Acetate/propionate	0.71 ± 0.04	4.15	0.05	0.11 ± 0.03	0.25 ± 0.03
(A + B)/(P + V)[3]	0.73 ± 0.02	4.32	0.04	0.11 ± 0.03	0.24 ± 0.03
Plasma VFA concentrations, mM					
Total VFA	0.34 ± 0.06	1.20	0.06	0.12 ± 0.04	0.25 ± 0.03
Acetate	0.33 ± 0.06	1.16	0.06	0.12 ± 0.04	0.25 ± 0.03
Propionate	0.012 ± 0.005	0.028	0.01	0.13 ± 0.04	0.20 ± 0.03
Butyrate	0.007 ± 0.003	0.015	0.01	0.12 ± 0.04	0.16 ± 0.03

[1]Mean ± phenotypic SD for concentration and molar proportions. Mean ± average SE for heritability and repeatability.

[2]Back transformed for the traits ($y = 10^x - 1$).

[3](A + B)/(P + V) = ratio of (acetate + butyrate)/(propionate + valerate).

VFA for caproate, valerate, *iso*-butyrate and *iso*-valerate, respectively.

The h^2 of concentrations of total VFA [as $\log_{10}(x + 1)$] was 0.24, and the repeatability was 0.35, with similar h^2 and repeatability for concentrations of the 3 major VFAs (Table 3). Concentrations of major VFAs had greater estimates of h^2 and repeatability than when these VFAs were expressed as molar proportions. Heritability and repeatability of minor VFAs were lower than for major VFAs, especially when expressed as concentrations. Heritability and repeatability of rumen VFA ratios were in the same range as VFA proportions.

Blood Plasma VFAs

The mean (back transformed) total concentration of plasma VFA was 1.20 mM, and the concentrations of acetate, propionate, and butyrate were 1.16, 0.03 and 0.02 mM, respectively (Table 3). Acetate was the predominant VFA in plasma, representing approximately 95% of total VFA concentrations.

The h^2 of total VFA concentrations [as $\log_{10}(x + 1)$] in plasma was 0.12, and the repeatability was 0.25. Plasma acetate and propionate concentrations had h^2 and repeatability values similar to those for total VFA, whereas the corresponding h^2 and repeatability value for butyrate was lower (Table 3).

Phenotypic and Genotypic Correlations of Ruminal VFAs with CH_4 Production and Yield

Rumen concentrations of total VFA, concentrations, and molar proportions of VFAs and VFA ratios had mostly positive r_p with CH_4 production (g/d), except molar proportion of propionate had a negative r_p (Table 4). The r_g for VFA concentrations, molar proportions, and ratios with CH_4 production were mostly nonsignificant due to large SEs.

Genetic and phenotypic correlations of CH_4 yield (g/kg DMI) with concentrations of major

Table 4. Phenotypic (r_p) and genotypic (r_g) correlations of ruminal and plasma VFA [$\log_{10}(x + 1)$ transformed] and CH_4 production and yield[1]

	CH_4, g/d		CH_4, g/kg DMI	
Parameter	r_p	r_g	r_p	r_g
Rumen VFA concentrations, mM				
Total VFA	0.14 ± 0.02	0.15 ± 0.13	0.34 ± 0.02	0.54 ± 0.12
Acetate	0.17 ± 0.02	0.19 ± 0.13	0.36 ± 0.02	0.56 ± 0.12
Propionate	−0.01 ± 0.02	0.09 ± 0.13	0.12 ± 0.02	0.41 ± 0.14
Butyrate	0.13 ± 0.02	0.15 ± 0.14	0.34 ± 0.02	0.49 ± 0.13
Caproate	0.07 ± 0.02	−0.31 ± 0.27	0.15 ± 0.02	−0.06 ± 0.28
Valerate	0.19 ± 0.02	0.12 ± 0.15	0.37 ± 0.02	0.53 ± 0.14
Iso-butyrate	0.27 ± 0.02	0.09 ± 0.19	0.37 ± 0.02	0.36 ± 0.20
Iso-valerate	0.25 ± 0.02	0.12 ± 0.19	0.34 ± 0.02	0.28 ± 0.22
Rumen VFA proportions, mol/100 mol VFA				
Acetate	0.16 ± 0.02	0.28 ± 0.18	0.15 ± 0.02	0.19 ± 0.20
Propionate	−0.33 ± 0.02	−0.16 ± 0.16	−0.38 ± 0.02	−0.14 ± 0.17
Butyrate	0.06 ± 0.02	0.08 ± 0.18	0.18 ± 0.02	0.22 ± 0.19
Caproate	0.04 ± 0.02	−0.34 ± 0.22	0.06 ± 0.02	−0.51 ± 0.28
Valerate	0.11 ± 0.02	−0.26 ± 0.31	0.14 ± 0.02	−0.13 ± 0.32
Iso-butyrate	0.10 ± 0.02	−0.21 ± 0.15	0.06 ± 0.02	−0.34 ± 0.15
Iso-valerate	0.11 ± 0.02	−0.19 ± 0.16	0.07 ± 0.02	−0.35 ± 0.16
Rumen VFA ratios				
Acetate/propionate	0.30 ± 0.02	0.24 ± 0.16	0.35 ± 0.02	0.17 ± 0.17
(A + B)/(P + V)[2]	0.30 ± 0.02	0.22 ± 0.16	0.36 ± 0.02	0.20 ± 0.14
Plasma VFA concentrations, mM				
Total VFA	0.18 ± 0.02	0.01 ± 0.18	0.20 ± 0.02	0.16 ± 0.19
Acetate	0.18 ± 0.02	0.02 ± 0.17	0.20 ± 0.02	0.16 ± 0.19
Propionate	−0.02 ± 0.02	−0.26 ± 0.17	−0.04 ± 0.02	0.04 ± 0.19
Butyrate	0.12 ± 0.02	−0.22 ± 0.19	0.13 ± 0.02	0.02 ± 0.19

[1]Correlation ± SE.

[2](A + B)/(P + V) = ratio of (acetate + butyrate)/(propionate + valerate).

VFA were much greater than the corresponding correlations with CH_4 production, and r_g estimates were greater than for r_p (Table 4). For example, r_g and r_p between CH_4 yield and concentration of acetate were 0.56 and 0.36, respectively. In contrast, r_g of CH_4 yield with molar proportions of major VFAs and VFA ratios were nonsignificant, whereas the corresponding r_p were similar but significant due to much lower SEs.

The size of r_g of CH_4 yield with concentrations (mM) of total and major VFA were medium to high, whereas the corresponding r_g with molar proportions of major VFA were low and nonsignificant (Table 4). The greatest r_g of CH_4 yield was with concentration of acetate, followed by total concentrations of VFA, concentration of valerate, proportion of caproate, and concentration of butyrate. In terms of VFA proportion, *iso*-buyrate and *iso*-valerate also had a moderate (negative) r_g with CH_4 yield. The greatest r_p of CH_4 yield was with the molar proportion of propionate (r_p = −0.38) and concentrations of valerate, *iso*-butyrate, acetate, total VFA, butyrate, and *iso*-valerate.

Correlations of Blood Plasma VFAs with CH_4 Production and Yield

Plasma concentrations of total and major VFA, except propionate, had positive r_p with CH_4 production (g/d) and CH_4 yield (g/kg DMI; Table 4). Total and major plasma VFA concentrations had low genetic correlations, with high SE, with CH_4 production (r_g = −0.26 ± 0.17 to 0.01 ± 0.18) and CH_4 yield (r_g = 0.02 ± 0.19 to 0.16 ± 0.19).

Efficiency of Using VFAs as an Indirect Selection for CH_4 Yield

The efficiency for indirect selection of CH_4 yield was greatest for concentration of rumen acetate and total VFA (70% to 71%) followed by rumen concentrations of butyrate, propionate, and valerate (54% to 57%; Table 5). Rumen VFA proportions with the greatest efficiency to indirectly select for CH_4 yield were *iso*-butyrate and *iso*-valerate (−35% and −34%, respectively). Plasma VFA and rumen VFA ratios had low efficiencies for indirect selection for CH_4 yield.

Table 5. Efficiency[1] of using rumen and plasma VFA as an indirect selection for CH_4 yield (g/kg DMI)

	Rumen VFA		Plasma VFA
Parameter	mM	mol/100 mol	mM
Total VFA	70%	—	14%
Acetate	71%	13%	14%
Propionate	54%	12%	3%
Butyrate	57%	17%	2%
Caproate	3%	29%	—
Valerate	54%	5%	—
Iso-butyrate	24%	35%	—
Iso-valerate	18%	34%	—
Rumen VFA ratios		15%	
Acetate/propionate			—
(A + B)/(P + V)[2]		17%	—

[1]The efficiency of indirect selection for 24-h CH_4 yield (g/kg DMI) using a single measure of a VFA parameter was calculated as follows: $|r_g|$ (h_{VFA}/h_{CH4}), where r_g is from Table 4 and h is the square root of the heritability (Table 3).

[2](A + B)/(P + V) = ratio of (acetate + butyrate)/(propionate + valerate).

DISCUSSION

Genetic Control of Rumen and Plasma VFAs and Relationship with CH_4 Production and Yield

In the present study, the rumen and blood VFA and CH_4 data were generated using a large population of sheep managed under the same feeding and measurement protocol, with feed in general consumed within 1 h of meal delivery, and ruminal and blood samples collected at fixed times. The h^2 and repeatability values for CH_4 production and yield in the present study were in a similar range to those previously found in other studies with sheep (Robinson et al., 2010b; Robinson and Oddy, 2016) and beef cattle (Hayes et al., 2016; Robinson and Oddy, 2016). These indicate that it is possible to selectively breed animals with lower CH_4 production and yield including taking advantage of any genomic information, which is obtained and used within the New Zealand sheep evaluation system (Auvray et al., 2014). Measuring CH_4 from a large number of animals in respiration chambers is, however, slow and different from the animal production environment. Finding indirect selection criteria associated to CH_4 is therefore required. Hydrogen utilized by methanogens for CH_4 formation is formed during the steps in fermentation to acetate and butyrate, and less so for other VFAs (Janssen, 2010). Ruminally produced VFA, in the rumen contents or blood plasma, are therefore potential correlated proxies to select for CH_4 production and yield.

The estimates of h^2 for ruminal concentrations of total and major VFAs (in samples collected just before morning feeding) were in a similar range as for CH_4 production and yield in the present study, with estimates being greater for VFA concentrations than for VFA proportions (0.20 to 0.25 vs. 0.07 to 0.11, respectively). The breed mixes used in this study are typical of New Zealand's sheep industry and selection is performed across these breed types. When breed proportions were fitted in the models for the main traits of interest (rumen total VFA and acetate concentrations and CH_4 yield), there was a small decrease (up to 2.5% higher) in heritability estimates. This is similar to Pickering et al. (2012) who found that fitting breed had a minimal effect on estimates of genetic variance for a range of traits for a similar breed mix as used in the present study.

Heritability and repeatability for rumen VFAs in the present study were much greater than previously reported for sheep fed wheaten hay with rumen samples taken about 2 h after feeding (Robinson et al., 2010b). Robinson et al. (2010a) found that r_p between CH_4 production and rumen VFAs was stronger pre-feeding than 2 to 3 h after feeding. In Angus cattle, r_p of CH_4 yield and rumen VFA proportions and concentrations was stronger pre-feeding than at 3 h after feeding (weak phenotypic correlation between pre- and post-feeding rumen VFAs); h^2 and repeatability estimates were not calculated (Herd et al., 2013; Smith et al., 2015). Concentrations of VFA sharply increase within 2 h post-feeding, followed by a decline to the lowest concentration before the next feeding (Moss et al., 1995; Robinson et al., 2010a; Sun et al., 2012, 2015). This pattern is also mirrored in the CH_4 production rates (Robinson et al., 2010a; Jonker et al., 2014; Brask et al., 2015), which suggests that it should be possible to use either pre- or post-feeding rumen samples. However, the pattern of VFA proportions and A + B/P + V ratio followed a reversed pattern relative to CH_4 production, with the ratio reaching the highest value pre-feeding and a sharp decrease to the lowest value after feeding (Brask et al., 2015).

Phenotypic correlations between CH_4 production and VFA concentrations were positive (0.07 to 0.27) in the present study, except the r_p for propionate (−0.01). Similarly, Robinson et al. (2010b) reported r_p of 0.16 to 0.19 between CH_4 production and VFA concentrations sampled 2 h post-feeding in sheep. Genetic and phenotypic correlations were greater between VFA concentrations and CH_4 yield than with CH_4 production in the present study, which is similar to findings of Herd et al. (2013; pre-feeding rumen sample), but opposite to findings of Smith et al. (2015;

3 h post-feeding rumen sample), both with cattle. These suggest that rumen sampling time will affect the relationship between VFAs and CH_4 production and yield, which requires further investigation.

The r_g between VFA concentrations and CH_4 yield was in general more than double the r_g between VFA proportions and CH_4 yield (>0.40 vs. <0.23, respectively) in the present study. A common finding of the present study and the study of Herd et al. (2013) was that molar proportions of VFA were associated with smaller variation than their corresponding concentrations. Consequently, the molar proportions of major VFA have weaker potential to account for variation in CH_4 yield than their corresponding concentrations. Both total VFA and acetate concentration had the greatest r_g with CH_4 yield and both had the greatest h^2 and repeatability of all parameters tested. These resulted in total VFA and acetate concentrations having the greatest efficiencies as indirect selection proxies for CH_4 yield (70% to 71%) in sheep, at least when fed alfalfa pellets at 2.0 × ME_r. Acetate was also the predominant VFA in plasma, which is consistent with findings by others (Oba and Allen, 2003; Kristensen and Harmon, 2004). However, h^2, repeatability, and r_g with CH_4 yield was much lower for plasma acetate, resulting in much lower efficiency as an indirect proxy (14%) than for rumen acetate concentration (71%).

The efficiencies were calculated at the same selection intensity, with the rationale that these proxy traits are cheaper and more practically measured. Using these VFA proxies would allow for a higher selection intensity, which would likely more than overcome the reduced efficiency compared with selecting directly for CH_4 yield. As an example of a breeding scenario, for the same cost, all animals could be measured for total concentration of rumen VFAs compared with 5% of the animals measured in respiration chambers for 48 h. In this scenario, using the Q value for total VFAs of 70%, the expected response to selection would be 4 times greater using total rumen VFAs than it would be to selection through respiration chambers for the same budget. In practice, however, the selection response will be lower because on-farm feeding conditions are more variable than in the present study, as discussed below.

Potential Mechanisms of the Observed Correlations for CH_4 Production and Yield with Ruminal VFAs

Fermentation of carbohydrates by rumen microbes results in the formation of VFA and CH_4. Concentrations and profiles of VFA produced are determined by ruminal microbial community composition and activity, which in turn is dependent on rumen conditions and diet (Henderson et al., 2015; Bannink et al., 2016). Diet and feeding level were similar for all sheep in the present study and should therefore not have affected rumen VFA concentrations and profile. The evidence of a genetic basis for animal-to-animal differences in CH_4 yield (Pinares-Patiño et al., 2013; Jonker et al., 2018a) as well as VFAs (present study), and the r_g between these traits in the present study suggest that there are common host animal factors involved. Host animal factors that may affect rumen VFA concentrations include VFA production rate, rumen liquid volume, VFA absorption rate through the rumen wall, and passage rate of VFA with the liquid phase (Hall et al., 2015; Bannink et al., 2016), whereas rumen VFA proportions in the rumen are influenced by rumen conditions such as pH and dissolved H_2 concentration (Janssen, 2010; Bannink et al., 2016), differential absorption rates of VFAs (Bannink et al., 2016; Dieho et al., 2016), and microbial community composition and activity (Russell and Rychlik, 2001).

Animals selected to have a low CH_4 yield were previously found to have a shorter rumen retention time (**RRT**) of feed particles (Pinares-Patiño et al., 2003; Hegarty, 2004; Pinares-Patiño et al., 2011a) and smaller rumen size (Goopy et al., 2014; Bain et al., 2014; Elmes et al., 2014) than their counterparts with high CH_4 yield. Shorter RRT has been linked with reduced CH_4 yield in sheep in a mechanistic modeling approach (Huhtanen et al., 2016) and in vivo (Pinares-Patiño et al., 2003; Hammond et al., 2014). Shorter RRT was predicted to be related to reduced substrate digestibility in the rumen, that is, less substrate for rumen fermentation, and improved efficiency of microbial synthesis (acting as an H_2 sink; Huhtanen et al., 2016). In combination with faster passage of VFAs with rumen liquid, this could lead to lower VFA concentrations in the rumen and therefore might explain the positive correlations of rumen VFA concentrations and CH_4 yield. Rumen volume might affect VFA absorption rate, with reducing rates at increasing volume (Dijkstra et al., 1993). Low CH_4 yield sheep were previously found to have a smaller rumen size and volume (Goopy et al., 2014; Bain et al., 2014) and had anatomical features associated with increased ruminal VFA absorption (e.g., papillae density and structure; McEwan et al., unpublished data) and increased rumen wall gene expression related to VFA absorption (Xiang et al., 2016, 2018) compared with high CH_4 yield sheep.

All of these features might indicate increased rates of VFA absorption through the rumen wall, which would consequently lead to lower rumen VFA concentrations in low CH_4 yield sheep, as found in the present study. Differences in rumen contents and conditions (e.g., pH) might lead to differential absorption rates of the 3 major VFAs (Dijkstra et al., 1993; Dieho et al., 2016). Rumen contents were previously found to be less stratified in low CH_4 yield sheep compared with high CH_4 yield sheep (Goopy et al., 2014; Bain et al., 2014), but little has been documented in regards to other rumen conditions. These might, however, have led to differential absorption of the different VFAs and therefore lead to differential VFA concentrations and proportions between low and high CH_4 yield sheep.

Stoichiometrically, ruminal acetate and butyrate formation are associated with H_2 formation, and therefore CH_4 production, whereas increased propionate and valerate formation are associated with decreased H_2 formation, and therefore decreased CH_4 production (Demeyer and Van Nevel, 1975; Janssen, 2010). This is apparent in the negative r_g for CH_4 yield with molar proportions of propionate and valerate; and the positive associations with molar proportions of acetate and butyrate, acetate/propionate ratio and (A + B)/(P + V) ratio. Most r_g were, however, weak and nonsignificant (large variation), as was also the case in cattle (Herd et al., 2013; Smith et al., 2015; Cabezas-Garcia et al., 2017). Relationships between CH_4 production and their associated VFA concentrations and molar proportions from in vivo experiments have generally been weak (Moss et al., 1995; Robinson et al., 2010a; Alemu et al., 2011). Including diet digestibility together with VFA proportions in empirical models resulted, however, in satisfactory predictions of CH_4 production (Ramin and Huhtanen, 2013; Brask et al., 2015). Brask et al. (2015) concluded that the amount of feed fermented was the primary factor determining variation in CH_4 production between animals and diets, but animal-to-animal variation was also identified as factor complicating the accuracy of CH_4 predictions. A meta-analysis of dairy cow data by Cabezas-Garcia et al. (2017) indicated that variation in organic matter digestibility and efficiency in microbial N synthesis due to variation in RRT contribute more to variation in CH_4 yield between animals than rumen VFA profile.

Rumen microbial community composition (Kittelmann et al., 2014; Rowe et al., 2015) and microbial metabolic function, including gene expression related to VFA metabolism (Shi et al., 2014; Kamke et al., 2016), were previously found to differ between sheep with low and high CH_4 yield. Rumen microbial community composition in the low CH_4 yield cohort could be further separated in 2 ruminotypes having a greater abundance of either *Quinella* spp. or *Sharpea* spp. (Kittelmann et al., 2014). Both ruminotypes are thought to be associated with lower H_2 formation in the rumen, but through different mechanisms. In the animals dominated by *Quinella*, this might be via increased formation of propionate (Vicini et al., 1987) and in those with *Sharpea* via formation of lactate and consequent metabolism into butyrate by *Megasphera* spp. (Kamke et al., 2016). It might therefore be that sheep within the low CH_4 yield cohort show a variable phenotype in ruminal VFA concentrations and proportions, which would weaken the correlations for rumen VFAs and CH_4 yield considerably and might explain the greater standard errors for the VFA proportions in the present study. This also reopens the debate regarding the results found in the present study. For example, the (A + B)/(P + V) ratio had relatively low h^2 and a weak r_g with CH_4 yield in the present study, but selection for low (A + B)/(P + V) ratio, which is logical from a stoichiometry point, probably still result in selection of a low CH_4 yield phenotype as suggested by the analysis of cattle data by Palarea-Albaladejo et al. (2017). Interestingly, propionate proportion, also driving acetate/propionate and A+B/P+V ratios, had moderate r_p with CH_4 yield suggesting that environmental factors were more important determinant of rumen propionate proportion independent of animal genetic background.

Minor VFA concentrations and/or proportions had a wide range of estimates for r_g with CH_4 yield but in general had relatively large SE and relatively low h^2. These minor VFA were also found to be increased when methanogen inhibitors were fed to cattle (Haisan et al., 2014; Lopes et al., 2016). *Iso*-butyrate and *iso*-valerate are in general associated with protein fermentation, but were also found to be formed via isomerization of butyrate and valerate, respectively, when methanogens were inhibited (Lovley and Klug, 1982). Supplementation of low-protein diets with *iso*-butyrate and *iso*-valerate fed to cattle were found to decrease rumen protozoa and methanogen numbers and decrease CH_4 yield (Liu et al., 2014; Wang et al., 2015). It is, however, not clear if these points are relevant to the negative r_g between *iso*-butyrate and *iso*-valerate with CH_4 yield found in the present study.

Ørskov et al. (1967) stated that excess H_2 in the rumen could be used to reduce acetate to ethanol plus longer chain acids, such as valerate

and caproate, instead of being used for CH_4 formation. Decreasing RRT increases ruminal concentrations of dissolved H_2 resulting in a thermodynamic feedback that makes H_2 production less favorable (Janssen, 2010), which leads to increased electron flow to alternative sinks such as propionate, valerate, and caproate. Valerate and caproate can be produced by bacterial species of *Clostridium* and *Megasphera*, which were abundant in one of the low CH_4 yield ruminotypes (Kamke et al., 2016). The greater caproate proportion found in the rumen of sheep with a low CH_4 yield might therefore be an indication of rumen conditions associated with low CH_4 production.

Selection of minor VFAs might thus result in selection of animals with a low CH_4 yield phenotype, but h^2 and repeatability of these minor VFAs were lower than for major VFA concentrations. In terms of VFA proportions, however, *iso*-butyrate and *iso*-valerate, followed by caproate, were the most efficient correlated proxies for CH_4 yield. Further research is required since the low CH_4 yield phenotype seems to comprise several ruminotypes that potentially result in different VFA profiles.

Relevance of the Observed Correlations for CH_4 Production and Yield with Ruminal VFAs to Other Feeding Systems

The present study constitutes the first report of h^2 and repeatability of ruminal VFAs and correlations with both CH_4 production and yield. The VFA and CH_4 data were, however, generated under highly controlled experimental conditions involving feeding a completely pelleted diet at a fixed level fed twice daily, in general consumed within 1 h of meal delivery. This is different to the majority of common on-farm feeding systems, especially pastoral systems prevalent in countries such as New Zealand. The physical form of the diet used in the present study may reduce chewing activity and salivation and increase passage of particles from the rumen, which can modify the rumen environment (Khafipour et al., 2009) and reduce diet digestibility (Hironaka et al., 1996; Zhao et al., 2016). These probably explain the low CH_4 yield observed in the present study compared with values commonly found with forage-fed sheep (Jonker et al., 2018b; Swainson et al., 2018), as also found by others feeding pelleted hay (Hironaka et al., 1996; Pinares-Patiño et al., 2011b; Zhao et al., 2016). There was, however, a moderate genetic correlation (r_g = 0.52) between CH_4 yield measured in respiration chambers on alfalfa pellets and $CH_4/(CH_4 + CO_2)$ ratio (proxy for CH_4 yield) measured using portable accumulation chambers (2 × 1 h spot-samples) with the same sheep grazing pasture (Jonker et al., 2018a) and a similar difference in CH_4 yield was found between CH_4 yield selection line sheep when fed alfalfa pellets and cut pasture in respiration chambers (Jonker et al., 2017b).

The mean concentrations of total VFA observed in samples obtained 18 h after the last feeding in the present study were in close agreement with concentrations reported by other authors for collections corresponding to 18 to 24 h post-feeding of a wide range of forages to sheep (Moss et al., 1995; Robinson et al., 2010a; Sun et al., 2012). The VFA proportions were also in a range similar to those reported by Sun et al. (2012) for sheep fed a range of fresh forages. Rumen VFA concentrations were also found to be lower, as in the present study with alfalfa pellets, in low compared with high CH_4 yield sheep fed cut pasture (Jonker et al., 2017a).

The anatomical features of a smaller rumen with less stratified contents found in the low CH_4 yield selection line sheep selected on alfalfa pellets in New Zealand (Bain et al., 2014; Elmes et al., 2014) were also found in an independent screening program with sheep fed alfalfa/oaten chaff in Australia (Goopy et al., 2014). Ranking of animals on the basis of RRT are consistent across diets and feeding levels in sheep (Faichney, 1993) and cattle (Campling et al., 1961; Ørskov et al., 1988), and RRT is a repeatable physiological trait (repeatability of approximately 0.45; Smuts et al., 1995; Cabezas-Garcia et al., 2017). These data suggest that parameters related to rumen size and RRT are probably repeatable when animals are fed either alfalfa pellets or pasture.

There is, therefore, a body of evidence that suggests that the correlations of VFA concentrations and profiles with CH_4 yield observed under the feeding conditions of the present study would be repeatable under other feeding conditions, including prevailing pasture feeding conditions in New Zealand. However, the suitability of using rumen total VFA and acetate concentrations to screen individual sheep for differential CH_4 yield should be confirmed for different feeding conditions before it can be recommended for use as a correlated proxy. Furthermore, genetic selection using ratio traits, CH_4 yield in this case, are well known to act unpredictably, but the denominator (feed intake) was predetermined prior to the CH_4, and VFA, measurement in the present trial. However, CH_4 production may be favored as a trait for genetic selection in industry because it can be directly converted

into an economic cost if trading scheme charges for CO_2-eq emissions are implemented. It also can be combined in any selection index that includes production and environmental emission breeding values as it transparently includes both genetic and phenotypic correlations between these traits.

CONCLUSIONS

This highly controlled study conducted with a large population of sheep of diverse genetic background provides evidence that VFA concentrations in the rumen are heritable and repeatable, with greater r_g estimates with CH_4 yield (g/kg DMI) than with CH_4 production (g/d). The high h^2 and repeatability for ruminal concentrations of total VFA and acetate, and their high r_g with CH_4 yield may constitute a proxy to identify individuals that are extreme for CH_4 yield, which can be used in breeding programs.

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