Investigations on the accuracy of predicting methane emissions from Swiss, Brown Swiss dairy cows by either current equations based on milk mid-infrared spectra or by using laser methane detectors

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Interpretive Summary: Characteristics of low methane emitting cows as categorized by mid-infrared spectra and respiration chamber measurements. *Denninger et al.* Mid-infrared spectra (MIR) were used to identify low and high methane emitting dairy cows within the Swiss, Brown Swiss population. Thirty individuals were selected for methane measurements using respiration chambers and laser methane detectors. The MIR predictions were fairly persistent across different environments and differently developed equations. However, correlations with methane measurements were too weak to use MIR as a tool to select low emitting cows. Cows categorized as low emitters by respiration chamber data expressed distinct characteristics in digestion and efficiency.

ABSTRACT

Since heritability of methane (CH_4) emissions in ruminants was demonstrated, various attempts to generate large individual animal CH_4 data sets were initiated. Predicting individual CH_4 emissions based on equations using milk mid-infrared (MIR) spectra is currently considered promising as a low-cost proxy. However, the predicted CH_4 emission by MIR in individuals still has to be confirmed by measurements. In addition, it is still unclear how low CH_4 emitting cows differ in intake, digestion, and efficiency from high CH_4 emitters. In the current study, putatively low and putatively high CH_4 emitting Brown Swiss cows were selected from the entire Swiss herdbook population (176,611 cows), using a MIR-based prediction equation. Eventually, 15 low and 15 high CH_4 emitters from 29 different farms were chosen for a respiration chamber (RC) experiment, where all cows were fed the same forage-based diet. A number of traits related to intake, digestion, and efficiency were quantified over 8 d, and CH_4 emission was measured in 4 open circuit RC and daily CH_4 emissions were also estimated using data from 2 laser CH_4 detectors (LMD). The MIR-predicted CH_4 production (g/d) was quite constant in low and high emission categories, and individuals across sites (home farm, experimental station), and within equations (first available and refined versions). The variation of the MIR-predicted values was substantially lower using the refined equation. However, the predicted low and high emitting cows (n = 28) did not differ on average in daily CH_4 emissions measured either with RC or estimated using LMD, and there was no correlation between CH_4 predictions (MIR) and CH_4 emissions measured by RC measurements. When re-categorized based on CH_4 yield measured in RC, differences between categories of 10 low and 10 high CH_4 emitters were about 20%. Low CH_4 emitting cows had a higher feed intake, milk yield, and residual feed intake, but differed only weakly in eating pattern and digesta mean retention times. Low CH_4 emitters were characterized by lower acetate and higher propionate proportions of total ruminal volatile fatty acids. We concluded that the current MIR-based CH_4 predictions are not accurate enough to be implemented in breeding programs for cows fed forage-based diets. In addition, low CH_4 emitting cows have to be characterized in more detail using mechanistic studies to clarify in more detail the properties which explain the functional differences to other cows found.

Key words: digestion, feed efficiency, methane prediction, proxy

INTRODUCTION

Methane (**CH_4**) is a greenhouse gas with a more than 20 times greater global warming potential compared to carbon dioxide. The global livestock sector accounts for 18% of the anthropogenic greenhouse gas emissions, and CH_4 from ruminants is the main source (Steinfeld et al., 2006). There is an ongoing research effort towards CH_4 mitigation. Apart from the available set of efficient dietary interventions (Hristov et al., 2013), targeted animal breeding has emerged as a promising and, if successful, sustainable mitigation strategy (de Haas et al., 2017). Breeding progress is possible if a trait is sufficiently heritable and if phenotypic data are available from populations relevant for genetic selection purposes. The first is given as CH_4 emissions were found to be a heritable trait (e.g., Lassen and Løvendahl, 2016; Jonker et al., 2017), and by the observation that the phenotype seems to be persistent throughout lactation (Garnsworthy et al., 2012b). However, it has to be noted that Münger and Kreuzer (2008) did not find such a persistence. With respect to the need for individual animal data sets, the well-established techniques to measure CH_4 from cows, respiration chambers (**RC**) and sulfur hexafluoride (**SF_6**), are not fast and cheap enough. The laser CH_4 detector (**LMD**) has been used to make measurements of CH_4 concentrations in cow's breath over a short time period to estimate daily CH_4 emissions, and it has been suggested it might be used to allow a quick ranking of animals by CH_4 emission on farm (Chagunda et al., 2013; Sorg et al., 2018). Like RC, the LMD technique can be applied for all ruminant species and production purposes. One of the most promising proxies for CH_4 emission is that based on the mid-infrared (**MIR**) spectra of the milk (Vanlierde et al., 2016, 2018). This proxy is currently limited to lactating cattle where the milk recording scheme is in place and calibrated for this type of milk. The underlying equation has been, and is, continuously refined by extending calibration and reference CH_4 measurement data sets. MIR spectra are available from national milk recording schemes. Therefore, this proxy only requires electronic storage efforts.

The next logical step in validating the MIR approach consists in the backward approach, namely screening of entire cow populations for low CH_4 emitters and measuring the accuracy of the

corresponding CH_4 predictions. This was recently done with a single herd (Denninger et al., 2019), but not yet with cow population data. If this validation is successful, the proxy could be used in breeding programs. In addition, the data could be useful for national inventory purposes and also for potential payment or taxing regimens based on greenhouse gas emissions from dairy cows. As cows are exposed to a variety of farm-specific influences on CH_4 emissions including diet type, intake, feeding frequency, and physiological state of the animal (Garnsworthy et al., 2012b; Hristov et al., 2013; Goopy et al., 2014), this step also has to clarify whether the differences between cows in CH_4 prediction are of sufficient magnitude to be detected when cows are kept in the same housing and feeding environment. In the development of the equation, data from various breeds, sites, and feeding regimes were integrated, but they nonetheless originated from experimental herds. Finally, it is still unclear in which traits and at which levels low CH_4 emitters differ from high CH_4 emitters. Low CH_4 emitters might exhibit a greater feed efficiency (ECM/DMI), shown by a lower residual feed intake (**RFI**) (Hegarty et al., 2007; Alemu et al., 2017). Others reported that low CH_4 emitting sheep could have a proportionately smaller rumen (Goopy et al. 2014), and there are indications that low CH_4 emitting cattle have a low cell wall digestibility (Cabezas-Garcia et al., 2017). The latter differences, however, would be expected to result in a lower feed efficiency.

The objective of the current study was to test the feasibility of using CH_4 predictions from milk MIR spectra for the purpose of identifying truly low CH_4 emitting dairy cows on the basis milk recording data. The hypotheses tested were: (i) The MIR-based predictions of CH_4 production of individual cows on farm is recovered at the experimental farm on a uniform diet. (ii) The MIR predictions closely correlate with individual CH_4 emission measurements made with RC and LMD. (iii) Compared to high CH_4 emitters, low CH_4 emitting cows are superior in feed and digestive efficiency.

MATERIALS AND METHODS

Screening of the Swiss, Brown Swiss Dairy Cow Population and Selection of Low and High CH_4 Emitting Cows

A milk MIR prediction equation (Vanlierde et al., 2016; modified as described below) was used to predict the daily enteric production of CH_4 (**P_m**; g/d) of individual cows from MIR spectra stored from each test day, between January 2016 and July 2017, for 175,980 Brown Swiss and Braunvieh dairy cows. Only cow data that met the following criteria were included when identifying low and high CH_4 emitting cows: milk yield 5-60 kg/d, 4-306 DIM, 150-950 g/d $P_{m\ MIR}$, and availability of data from at least 5 milk recordings from cows. Records from summer alpine grazing periods and from farms located in the highest mountain regions were excluded. A linear mixed model considering log-transformed milk yield, log-transformed DIM, parity, and season within yr as fixed effects, as well as cow and farm as random effects was applied to model P_m by using the 'nlme' R package (Pinheiro et al., 2017). The conditional modes (difference between the average predicted response at population level for a given set of fixed-effect values and the response predicted for a particular individual) for the cow effect were used to select the extreme values (15% of cows in both directions), where the predicted P_m was either greater or lower than expected from the linear mixed model. For the first selection step all cows were used, but later selection was restricted to cows in second parity in order to exclude a further potential factor of influence. This screening procedure resulted in 318 candidate cows (159 low $P_{m\ MIR}$ cows, 159 high $P_{m\ MIR}$ cows). Out of these, 30 cows (15 low, 15 high $P_{m\ MIR}$ cows), preferably late lactating, were randomly selected for the experiment followed by getting the approval of the cow owner. The 30 cows originated from 29 different farms.

Although this categorization of the cows did not substantially change when analyzing MIR spectra obtained during the experiment and using different prediction equations, cows were later re-categorized because cow allocation to the categories was largely different when using the RC data (measurements described below). Therefore, new groups were formed based on their CH_4 yield (**Y_m**; here: g/kg DMI) as measured with RC. This adjusted trait was chosen to exclude the advantage small cows with low feed intake would have when DMI is not considered. To be able to distinguish clearly between categories and in response to the missing preselection of cows for $Y_{m\ DMI}$ in RC, only the 2 × 10 cows with either the lowest or the highest $Y_{m\ DMI}$, respectively, were used for the detailed comparison of the characteristics of low and high CH_4 emitters.

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143 ***Experimental Protocol***

144 The experiment was conducted at the research station Agrovet-Strickhof (Eschikon, Lindau,
145 Switzerland), from November 2017 to April 2018. The experimental protocol was approved
146 (ZH050/17) by the Committee on Animal Experimentation of the Cantonal Veterinary Office Zurich.
147 Owing to the 4 RC available, cows were transported in groups of 4 (2 predicted low P_m and 2 high
148 P_m; last time: 2 cows only), from the farms to the station and back after the experiment. This resulted
149 in 8 blocks of cows. During a 10-d period of adaptation to diet and management, the cows were kept
150 in a tie-stall barn and milked in a swing-over milking system. During this time the cows had access
151 to an outside area for 2 h every second d. In the following 8-d sampling period, cows were tethered
152 all the time, which allowed complete collection of feces and urine. On sampling d 9, the collection
153 devices were removed and rumen fluid was sampled. In the last 24 h cows were housed in a RC. All
154 cows received the same diet (Table 1), regardless of the diet they had received at their home farm.
155 The mixed ration was composed of 55% corn silage, 38% grass silage, 2% hay, and 5% dairy
156 concentrate (UFA-250, UFA, Sursee, Switzerland), offered at ad libitum access. In separate troughs,
157 cows daily received, per kg of milk, 250 g of an energy-rich concentrate (UFA-243, UFA) and 125 g
158 dried grass pellets. During morning feeding, 50 g/d NaCl and 100 g/d of a vitamin-mineral
159 supplement were provided. The latter contained (per kg) 160 g Ca, 80 g P, 100 g Mg, 32 g Na, 10 g
160 S, 8.0 mg Zn, 4.0 mg Mn, 1.0 mg Cu, 30 mg Se, 100 mg I, 30 mg Co, 1 200, 000 IU vitamin A,
161 200,000 IU vitamin D_3, 3,000 mg vitamin E, and 150 g biotin. The animals were milked at 0550 h
162 and 1645 h, and fed at the same time. Leftovers of the mixed ration were removed before each feeding
163 time and weighed. Energy concentrate and grass pellets were always eaten completely.

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165 ***Prediction of CH_4 Emissions from Milk Sampling Combined with MIR Spectra Analysis***

166 Analysis of MIR spectra was performed either on a stored data set (population) or on milk
167 sampled from the 30 cows either on their home farm or at the research station. The spectra were
168 obtained using Fourier transform infrared spectrophotometry (MilkoScan FT6000 Foss Electric,

Hillerød, Denmark). They were standardized according to the Grelet et al. (2017) procedure to avoid instrumental interference and ensure comparability of the spectra regardless the spectrometer used. From these spectra, $P_{m\ MIR}$ was first predicted using the lactation-stage-dependent prediction equation developed by partial least-square regression from Vanlierde et al. (2016). The published prediction equation was slightly modified for this purpose by including milk spectra and corresponding CH_4 measurements from 77 Swiss cows in the calibration data set for deriving the prediction equation. This added up to 225 RC-based CH_4 measurements in addition to the 532 SF_6-based CH_4 measurements in the original calibration data set of Vanlierde et al. (2016). The standard error of calibration (SEC) of this equation, later on called 'old equation', was 70 g/d, the calibration coefficient determination (R^2c) was 0.66, the standard error of cross-validation (SECV) was 73 g/d, and the cross-validation coefficient of determination (R^2cv) was 0.62. After the experiment had been completed, the prediction equation had been further refined. The 'new equation' was developed and calibrated in 2019 using 1089 RC- and SF_6-based CH_4 measurements (thereof 7% from Brown Swiss cows) originating from 299 cows (thereof 13% Brown Swiss cows). This new equation had a SEC of 58 g/d, a R^2c of 0.68, and a SECV of 61 g/d a R^2cv 0.64. Following Vanlierde et al. (2016) and considering the spectral dataset used to build the equation as the reference, Swiss spectra obtained during this study with a standardized Mahalanobis distance (global H distance; GH) of more than 3 were removed from the dataset. By following that 'GH' procedure, only 0.72 % of the current data set needed to be removed. With both equations, predicted $P_{m\ MIR}$ values <150 g/d or >950 g/d were excluded from further analyses and considered as outliers as described by Vanlierde et al. (2016, 2018). The new prediction equation was applied to evaluate whether cow allocation was robust when the P_m prediction equation changes and to determine whether correlations with measured CH_4 data were improved. In detail, $P_{m\ MIR}$ was predicted from MIR spectra for 5 times with both, the old and new equation (only the former was available at the time of the screening). Description and denominations are given in Table 2.

Measurement of Daily CH_4 Emissions Using Respiration Chambers

196 Four new RC (No Pollution, Industrial Systems Ltd., Edinburgh, UK) were used to measure CH_4
197 emissions from the individual cows at AgroVet-Strickhof. The chambers were 4.75 m wide, 3.25 m
198 deep and 2.5 m tall (38.0 m^3). Each chamber was fitted with 1 large back door for animal entrance, 1
199 smaller front door, safety opening devices and rubber seals around the whole perimeter. The animals
200 were tied in metabolism stalls (255 × 150 cm) equipped with water troughs and feed bins mounted
201 on an electronic balance. The doors were opened for a very short time twice daily at the same time
202 for milking and feeding. This was accounted for by interpolating about 2 × 20 min/d with values from
203 adjacent times where gas concentration had returned to the equilibrium. Fresh air was supplied
204 through a common duct through 2 shutters (SPI-F-160, Systemair AB, Buchs ZH, Switzerland and
205 LM 230, BELIMO Automation AG, Hinwil, Switzerland) to prevent backflow. The air was
206 exchanged about 12 times/h. Temperature was maintained at 16°C, relative humidity at 60%. Spent
207 air was removed by an extraction fan (K06-MS Blower, FPZ Blower Technology, Concorezzo, Italy),
208 coupled with a frequency controller (VLT 3,3 Kw, HWAC Drive, Danfoss GmbH, Offenbach,
209 Germany) maintaining an airflow between 19.0 and 23.0 L/s. The chambers were kept under a slight
210 negative pressure. The CH_4 concentration was determined with a MGA 3500 (ADC Gas Analysis
211 Ltd. Hertfordshire, UK) using nondispersive infrared absorption. This was done every 10 min in the
212 outgoing chamber air and in the fresh air collected on the roof of the building where the air pipe for
213 the RC was installed. Calibration was performed directly before and after each experimental run. At
214 first a pure N_2 gas (99.999%) was applied. Then a first standard gas mixture containing 0.1% H_2 and
215 99.9% N_2 was delivered for 3 min until H_2 level stabilized, followed by pure N_2 gas for 3 min. Then
216 a second standard gas mixture (0.08 % CH_4, 20.9% O_2, 0.4% CO_2, and 78.62% N_2) was delivered to
217 let the instrument return to the expected concentrations. A recovery test (total calibration) for CH_4
218 was performed on 3 times per chamber during the experiment. While the regular data collection was
219 performed, CH_4 (99.9%) was injected at 0.35 L/min via a tube through the outside wall for 4 h. The
220 measured concentration reached a plateau after 1 to 2 h. The flow rate was controlled by a Sierra mass
221 flow controller (MC-5SLPM-RD, Alicat Scientific, Tucson AZ, USA). The calibration of the

chambers and the gas analyzers provided a calibration factor for CH_4. The average recoveries in the 4 chambers were 88, 88, 90, and 89%, respectively.

Estimation of Daily CH_4 Emissions Using Laser Methane Detectors

Two LMD units (Mini-Green Lmm-g; Tokyo Gas Engineering Solutions, Tokyo, Japan) were used. Measurement principle (tunable diode laser absorption spectroscopy) and operation of the devices was described in detail by Chagunda et al. (2013) and Sorg et al. (2018). The duration of single measurements was set to 6 min/cow, and the distance between the LMD device and the cow's nostril was set to 1 m. The measurements were conducted on each cow during the last 3 d of the 8-d sampling period, and this before and after each feeding event while the animals were standing. From each LMD measurement, the CH_4 concentration (ppm × m; arithmetic mean of all peaks in a 6-min measurement) was calculated. Estimates of daily CH_4 emissions by the LMD technique ($P_{m\ LMD}$; g/d) were made as described by Sorg et al. (2018). The 3-d $P_{m\ LMD}$ values were averaged before feeding, after feeding, and overall.

Recordings, Sampling and Analysis of Feed, Feces, Urine and Rumen Fluid

Body weight was measured on a truck load scale (Waagen Döhrn GmbH & Co. KG, Wesel, Germany) upon arrival and directly before and after the sampling period. Milk yield was recorded automatically. During the sampling period, samples from each milking (50 mL) were separately conserved with Bronopol. Eating and ruminating behavior were recorded on 3 consecutive d per cow during the sampling period, using RumiWatch (Itin + Hoch GmbH, Liestal, Switzerland) halters equipped with pressure sensors detecting jaw movements, acceleration sensors detecting head position, and data loggers. Data were differentiated by the software into eating, ruminating, and other activities (Rombach et al., 2018). During the sampling period, feed intake was measured daily from supply and leftovers on flat troughs on electronic balances developed by Mettler-Toledo (Dübendorf, Switzerland) with separations between cows. The leftovers were pooled per animal. Forage samples were taken 1/wk in the adaptation period and twice in the sampling period. Grass pellets and

concentrate were sampled 3 times during the 23-d experiment. Samples were dried at 60°C to constant weight and ground to a particle size of 1 mm with either a cutting mill. For concentrate samples, a centrifugal mill was used.

During the 8-d sampling period, the entire feces were collected on steel trays located below a grid at the end of the tie stall. Urine was collected separately from feces using urinals attached around the vulva and glued (Cyanolit 202, Panacol Elosol GmbH, Steinbach, Germany) onto hair and skin. Urine pH was maintained at < 3 by the addition of 5 *M* H_2SO_4 to prevent ammonia volatilization. Feces and urine were weighed daily, and representative samples proportional to the amounts excreted were taken and frozen at -20°C. For the quantification of digesta retention time, 100 g samples of feces were collected 4, 8, 12, 18, 22, 26, 30, 36, 42, 46, 52, 58, 66, 74, 82, 90, 98, 106, 114, 126, 138, and 150 h after application of a marker bolus. Baseline was determined by 3 samples taken on the d before bolus application. The markers used were Co-EDTA as solute marker and mordanted grass hay following Udén et al. (1980). For that, the hay was cut in a cutting mill (MM180S, Fuchs-Mühlen, Vienna, Austria) to pass a 8-mm screen and sequentially dry screened by shaking on sieves with mesh sizes of 3.55, 2, 1, and 0.5 mm to obtain 3 particle fractions of 8, 5, and 2 mm, mordanted with Ce, La, and Cr, respectively. For more details see Grandl et al. (2018). The marker-containing feces samples were dried at 60°C to constant weight and ground through a 1-mm screen with a centrifugal mill.

Rumen fluid was collected on d 9 of the sampling period at 4 h after morning feeding via a stomach tube (SELEKT Pump and Collector, Nimrod Veterinary Products Ltd, Gloucestershire, UK). Two duplicate samples of 10 mL were obtained. Trichloroacetic acid was added to 1 for ammonia analysis and sulfuric acid to the other for VFA analysis. Samples were stored at -20°C.

Feeds and feces were analyzed according to standard procedures (AOAC, 1995). Contents of DM and total ash were determined with a thermogravimetric device (TGA-701, Leco, St. Joseph, MI, USA, AOAC index no. 942.05). The OM was calculated as DM minus total ash. Nitrogen was assessed in feeds, non-dried feces, and acidified urine on a C/N analyzer (Type TruMac CN, Leco Cooperation, St. Joseph, MI; AOAC index No. 968.06). The CP was calculated as 6.25 × N. Ether

extract was determined with a Soxhlet extraction system (model B-811, Büchi, Flawil, Switzerland). Ash-corrected contents of NDF (AOAC index no. 2002.04; with heat-stable α-amylase (Sigma-Aldrich, St. Louis, USA)) and ADF (AOAC index no. 973.18) in feeds and feces were determined using the Gerhard Fibertherm FT 12 (Gerhardt GmbH and Co.KG, Köngswinter, Germany). Determination of ADL in feed items was performed sequentially after ADF analysis by incubation in sulfuric acid (72%) for 3 h. Gross energy (**GE**) contents were measured in feeds and feces with a bomb calorimeter (C7000, IKA-Werke GmbH & Co. KG, Staufen, Germany). The Bronopol-conserved milk was analyzed for contents of fat, protein, and lactose using a Fourier transform infrared spectrophotometer (MilkoScan FT6000 Foss Electric, Hillerød, Denmark) at SuisseLab AG (Zollikofen, Switzerland). The spectra obtained during this process were also used to determine $P_{m\ MIR}$. Milk protein was divided by 6.38 to calculate N content. The element concentrations in the Co-EDTA, the mordanted hay and the feces were analyzed after wet ashing using inductively coupled plasma optical emission spectrometry (Optima 8000, Pekin Elmer, Rodgau, Germany). The markers contained, per kg DM, 32.8 g Cr, 49.5 g La, 41.5 g Ce, and 151 g Co. Rumen fluid ammonium was measured with a potentiometer equipped with a corresponding glass electrode (6.0506.100, Metrohm AG, Herisau, Switzerland) calibrated by using NH_4Cl at 0.1, 1, and 10 m*M*/L. The VFA were analyzed by HPLC (LaChrom, L-7000 series, Hitachi Ltd., Japan) complete with an UV detector.

Calculations and Statistical Analysis

Feed conversion efficiency (ECM/DMI), milk production efficiency (ECM/BW), and RFI (difference between observed and predicted DMI) were calculated as measures of efficiency. For RFI, the predicted DMI was calculated using Equation 1 of Gruber at al. (2004), which was developed based on measured DMI data recorded in Switzerland, Austria and Germany thus reflecting similar farming systems. This equation considered breed, lactation number, DIM, BW, milk yield, concentrate amount, and forage composition. The ECM (kg/d) was calculated as milk (kg/d) × (0.38 × fat (%) + 0.24 × protein (%) + 0.17 × lactose (%))/3.14 (Agroscope, 2019).

Fecal baseline marker concentrations were used to correct for individual animal background levels. Mean retention time (**MRT**) in the gastrointestinal tract (**GIT**) was computed for each marker according to Thielemans et al. (1978), as MRT GIT = $(\Sigma C_i \times t_{i-1,i} \times dt_i)/(\Sigma C_i \times dt_i)$, where $t_{i-1,i}$ = mean time (h) after application of markers of 2 subsequent samplings i-1 and i calculated as $t_{i-1} + (t_i - t_{i-1})/2$, C_i = marker content in the fecal sample voided in the interval represented by time t_i and t_{i-1}, and dt_i = sampling interval [h] of the respective sample calculated as $((t_{i-1} - t_i) + (t_i - t_{i-1}))/2$. The MRT of Co-EDTA in the reticulorumen (**RR**) was calculated following Grovum and Williams (1973), that of the particles according to Huhtanen and Kukkonen (1995), as MRT RR particles = MRT GIT particles – (MRT GIT solute – MRT RR solute). Dry matter gut fill was calculated following Munn et al. (2015) and considering DMI, DM digestibility, and the MRT GIT of the particle marker (La, 5 mm).

All statistical analyses were performed with R version 3.3.1 (R Core Team, 2018). As a measure for accuracy, Lin's concordance correlation coefficients **(CCC)** were computed between the CH_4 emission data measured and predicted, and linear regressions as well. Pearson correlation coefficients were calculated between P_m and non-CH_4 variables. Data from the 10 low and 10 high $Y_{m\ RC}$ cows were subjected to ANOVA, performed with a linear mixed model using the 'nlme' R-package (Pinheiro et al., 2017). Emission category (low, high), experimental block (run 1 to 7 with 4 cows, run 8 with 2 cows) and their interaction were fixed effects, and cow was the random effect. The P_m data (MIR_1, $MIR_{3\ old\ and\ new}$, $MIR_{4\ old\ and\ new}$, RC, LMD) were subjected to ANOVA, performed with a linear mixed model using the 'nlme' R-package (Pinheiro et al., 2017). Emission category (low, high) was the fixed effect, and cow was the random effect. Homogeneity of variances was checked with the Bartlett test and normality of the residuals with the Shapiro-Wilks test. In order to evaluate the accuracy of the prediction of $P_{m\ MIR}$, the root mean square error of prediction (RMSEP) for predicted CH_4 (MIR_{3_old} and MIR_{3_new}) was also calculated according to Vanlierde et al. (2015).

RESULTS

Categorization of Cows by MIR CH_4 Predictions and its Recovery by Measured CH_4

Based on the population screening, the groups of 159 low and 159 high $P_{m\ MIR}$ cows differed in each month during the entire 1.5 years of assessment (Figure 1A), and this was also observed for 200 out of 318 individual cow predictions (Figure 1C). The 2 groups of 15 cows selected for the on-station experiment also differed in almost each month (Figures 1A and 1B). The characteristics of the latter cows are given in Table 3. Accordingly, the average difference (high in relation to low CH_4 emitting cows) in $P_{m\ MIR}$ was 16% (old equation) when determined directly before the start of the experiment on the home farm (MIR_1), and was 18% (old equation) and 10% (new equation) in spectra obtained on the d of arrival at the research station (MIR_2). The absolute $P_{m\ MIR}$ levels differed between the 3 assessments, especially in the high $P_{m\ MIR}$ cows. The 2 categories were similar in average DIM and milk yield.

There were close CCC ($P < 0.01$) in $P_{m\ MIR}$ across all time points ($MIR_{1,3,4}$) when using the old equation, and between the two time points assessed with the new equation ($P < 0.001$) (Table 4). This is illustrated as a result of the regression analysis for the MIR assessments on the home farm and during the 8-day collection period (Fig. 2A). The changes caused by using the new equation were moderate (Fig. 2B). By contrast, there were no significant correlations between individual cow data from either RC or LMD values with the MIR predictions, and also not between RC and LMD (Table 4, Fig. 2C-F). Accordingly, relating $P_{m\ RC}$ with $P_{m\ MIR3}$ ('old' and 'new') by means of a linear regression did not result in significant relationships (R^2 'old' = 0.014, $P = 0.23$; R^2 'new' = 0.026, $P = 0.19$). The RMSEP was smaller using RC data and MIR_{3_new} (30.6 g/d) compared to using RC data and MIR_{3_old} (48.1 g/d). There was no significant CCC between the three LMD-predicted P_m variables (Table 4).

The categories established before the experiment (MIR_1) and those based on spectra obtained in the 8-d sampling period (MIR_3) all were mostly different on average ($P < 0.05$ to 0.01) in $P_{m\ MIR}$ with any equation (old, new; Table 5). Using the new equation for MIR-based predictions largely reduced SE of the category means. Re-categorization resulted a certain regrouping of the 28 cows. This was 1 cow each from low to high $P_{m\ MIR}$ and vice versa when moving from $Categorization_{MIR1}$ to $Categorization_{MIR3\_old}$. When changing either from $Categorization_{MIR1}$ to $Categorization_{MIR3\_new}$ or

355 from Categorization$_{MIR3_old}$ to Cagetorization$_{MIR3_new}$, 3 cows each were regrouped. Other than
356 predicted, $P_{m\ RC}$ and $P_{m\ LMD}$ levels measured in low and high $P_{m\ MIR}$ cows did not differ ($P > 0.10$)
357 (Table 5).
358
359 ***Characteristics of Cows Categorized by the Respiration Chamber Results***
360 The animals were re-categorized into low and high CH_4 emitters based on their $Y_{m\ DMI}$ as
361 measured by RC (Table 6). Group differences (high in relation to low $Y_{m\ RC}$) accounted for 18%,
362 21%, 19% and 19% when CH_4 was related to intakes of DM, digestible OM, digestible NDF, and
363 GE, respectively ($P < 0.01$ to 0.001). The difference ($P = 0.001$) was even larger with 21% for CH_4
364 emission intensity (**I_m**; CH_4/ECM), but not for CH_4/BW. The $P_{m\ RC}$, in contrast, was not different
365 between the categories, similar to the $P_{m\ MIR}$ averages obtained at 2 different time points and with 2
366 different equations. When relating $P_{m\ MIR}$ to measured DMI ($Y_{m\ MIR\ DMI}$), $Y_{m\ RC}$ categorized cows
367 differed (16%; $P < 0.05$) with MIR_1, and trends for such differences (13 and 18%; $P < 0.10$) were
368 found using MIR_3 ('old' and 'new' equations). Group differences in LMD results before and after
369 feeding were reversed leading to almost the same average $P_{m\ LMD}$ in the 2 categories. Also $Y_{m\ DMI}$ did
370 not differ between groups when measured with LMD.
371 Compared to the high $Y_{m\ RC}$ cows, the low $Y_{m\ RC}$ cows were characterized by a higher DMI
372 (+11%; $P < 0.01$) and a higher ECM yield (+27%; $P < 0.05$) (Table 7). The diets as consumed
373 contained 10.0 ± 1.5% and 7.4 ± 0.6% concentrate for low and high $Y_{m\ RC}$ cows, respectively. The
374 low $Y_{m\ RC}$ cows were higher ($P < 0.05$) in ECM/BW compared to the high $Y_{m\ RC}$ cows. The RFI was
375 higher ($P < 0.05$) for low compared with high $Y_{m\ RC}$ cows. Cow categories did not differ in daily
376 eating and ruminating times, total tract apparent digestibility of nutrients, and balance, losses and
377 utilization of N for milk protein formation (milk N, % of N intake or digested N intake). The low $Y_{m\ RC}$ cows had a different VFA pattern compared to the high $Y_{m\ RC}$ cows, with higher proportions of
379 propionate ($P < 0.01$) and lower proportions of acetate ($P < 0.05$) with a consequently lower acetate-
380 to-propionate ratio ($P < 0.001$). Ruminal ammonia concentration and most variables describing MRT
381 of the digesta in RR and GIT did not differ between categories. Only GIT MRT of small particles

was shorter ($P < 0.05$) in low compared to high $Y_{m\ RC}$ cows, associated with a slightly higher ($P < 0.05$) DM gut fill.

The data on $P_{m\ RC}$ correlated with DMI and N intake, digestibility of OM and NDF, urinary N losses, propionate, iso-butyrate and iso-valerate proportions of total VFA as well as acetate-to-propionate ratio ($P < 0.05$ to 0.01) (Table 7). In addition, there were trends for Pearson correlations ($P < 0.10$) in RFI, CP digestibility, ($P < 0.05$-0.01) and milk N relative to intake of digested N. Only few correlations were found between $P_{m\ MIR}$ and these variables ($P < 0.05$-0.01). These included ECM per unit of DMI and milk N proportion of intake of N, and digested N (MIR_{2_old} only).

DISCUSSION

The current study attempted to confirm the usefulness of predictions of daily methane emissions as made based on MIR spectra of milk samples or estimates of daily methane emissions made using LMD detectors to predict actual daily methane emissions of dairy cows. Our study showed that both the predictions based on currently available equations derived from MIR spectra and estimates based on short term measurements made with the LMD devices did not have a close phenotypical correlation with the measured values. This does not necessarily exclude the presence of genetic correlations, and further developed MIR spectra based equations might perform better. An in-depth investigation of potential factors of influence from ingestion and digestion, which may cause the differences in CH_4 emission among animals, was carried out. In addition, the utility of the LMD technique was assessed. For both, MIR-based prediction and LMD spot-sampling measurements, the RC technique was used as a reference method as it captures total CH_4 emissions and is generally considered highly accurate. Indeed, the current results found with RC are highly plausible. This can be concluded from comparing levels of $P_{m\ RC}$, $Y_{m\ RC\ DMI}$ and $Y_{m\ RC\ GE}$ with published data and from the expected significant positive relationships between $P_{m\ RC}$ and DMI, apparent fiber digestibility and ruminal acetate-to-propionate ratio (e.g., Niu et al., 2018).

Robustness and Accuracy of the CH_4 Prediction from Milk Mid-Infrared Spectra

Among the MIR-based prediction methods intended to be implemented into breeding schemes, the Belgian approach (Vanlierde et al., 2015, 2016, 2018) is probably the most developed as a high number of CH_4 measurements and different diets are considered. Shetty et al. (2017) also attempted to predict CH_4 via $P_{m\ MIR}$ by using reference data obtained from non-dispersive infrared analyzer installed (sniffer) in an automated milking system. There was a good agreement between this sniffer method and RC measurements (Garnsworthy et al., 2012a). However, when using the full milk MIR spectra and including DIM, the correlations were clearly lower using the sniffer method compared to those described by the Belgian group (Shetty et al., 2017). The Belgian equation was at first based on data derived from the SF_6 method, but RC data were included later. This reduced the R^2c from 0.74 to 0.66 ('old equation' used in the present study), but enhanced the applicability by generating additional variability (Vanlierde et al., 2016, 2018). Including more RC-based P_m data in the most recent equation ('new equation') improved R^2c to 0.68.

The MIR predictions developed by Vanlierde et al. (2015), based on SF_6 data, had shown a good correlation to a reference data set ($R^2c = 0.75$), and when relating predicted CH_4 emissions to RC-based measurements from an external data set, there was still a moderate correlation ($r = 0.48$). The SECV of a refined equation using SF_6 and RC was 61 g/d. As the correlation is highly dependent of the distribution of the considered data set, the error of prediction also needs to be considered when evaluating the performance of a method. Additionally, prediction equations have known errors to consider. For the equation of Vanlierde et al. (2018), the SECV was 47 g/d for a RC-based prediction and 70 g/d for a SF_6-based prediction. As a lower SECV indicates that the equation is closer to actual values (Vanlierde et al., 2018), there was an improvement of the 'new equation' compared to the SF_6-based equation. The RMSEP can be used to evaluate the predictive ability of the obtained calibration models (Shetty et al., 2017). Indeed, the RMSEP decreased when using MIR_{3_new} instead of MIR_{3_old} from 48 to 31 g/d. This RMSEP of the 'new equation' is even lower than the known errors established during calibration and cross-validation processes.

To be useful for breeding purpose, the genetic variability of a trait among cows has to persist over time and different feeding regimens (Pinares-Patiño et al., 2011). The prediction equation indeed

436 turned out to be robust over different countries, feeding regimens, and measurement techniques
437 (Vanlierde et al., 2016, 2018). Persistence was also observed for the cohort of 318 cows of the present
438 study when MIR spectra were followed across 1.5 yr. A suitable equation also has to consider that
439 the P_m of dairy cows is changing over the course of the lactation (Garnsworthy et al., 2012a). The $P_{m\ MIR}$
440 equation developed by Vanlierde et al. (2016) therefore considers DIM. Indeed, a biologically
441 reasonable change in predicted $P_{m\ MIR}$ with DIM was found for both the 318 and the 30 cows. This
442 change was also observed by Garnsworthy et al. (2012a) in an automated milking system fitted with
443 an infrared sensor. To further exclude bias caused by DIM, we considered only cows with $\geq$ 5 milk
444 performance records obtained at different DIM when selecting the experimental cows.
445 Despite the good performance of the Belgian prediction equation (Vanlierde et al., 2018),
446 especially in its newest version (unpublished), with respect to RMSEP and the biological meaningful
447 change in CH_4 emission over the course of the lactation (Figure 1 C), the relationship (CCC) with the
448 RC data in the present study was weak. Different from a previous assessment on a single herd using
449 GreenFeed instead of RC as standard method (Denninger et al., 2019), this also concerned
450 categorization and not only the prediction of individual cow values. The latter was better when the 2
451 × 10 cows were categorized retrospectively by using RC data. When relating $P_{m\ RC}$ and $P_{m\ MIR}$ to
452 DMI, the differences went into a similar direction, and $P_{m\ MIR}$ was consistent across different stages
453 of refinement of the prediction equation. However, none of the anticipated correlations of $P_{m\ MIR}$ with
454 DMI, NDF digestibility and ruminal VFA pattern was apparent. Also, the average $P_{m\ MIR}$ level
455 predicted was too high (amounting to 9.9 and 9.3% of GE in high $Y_{m\ MIR}$ cows, predicted with MIR_1
456 and MIR_{3_old}). This clearly exceeded the default value of the IPCC (2006) of 6.5%. This may be the
457 result of including data from cows beyond the range of P_m of 350 to 450 g/d in the reference data set
458 for the equation by Vanlierde et al. (2016). With further refinement of the equation (MIR_{3_new}) the
459 predicted Y_m was lower with 8.5% of GE. The number of animals used for the development of the
460 equation whereof CH_4 was measured by RC was still limited, but this cohort only included cows
461 where a strong scrutiny or preselection against unusual animals/measurements had been practiced.
462 This could also be a reason why the difference in $P_{m\ MIR}$ did not exceed 20% between low and high

463 $P_{m\ MIR}$ cows and why the MIR prediction equations did not clearly discriminate between the categories
464 distinguished by RC. However, it has to be noted that the goal of the present study had been a different
465 one, namely to recover the variability observed by MIR spectra on farm ($P_{m\ MIR}$) by means of RC
466 measurements where we did not succeed. The findings based on the present data set support the claim
467 of van Gastelen and Dijkstra (2016) that MIR data alone might be insufficient for a reliable prediction,
468 at least in order to distinguish between animals not considered as extremes in their CH_4 emission
469 level. In this context, MIR spectra might not sufficiently predict indicative milk fatty acids related to
470 processes associated with CH_4 formation. However, van Gastelen and Dijkstra (2016) suggested that
471 the MIR-based prediction might be improved by implementing more factors like milk yield, DMI and
472 others. However, care has to be taken that correlated factors included do not get too much weight in
473 the equation thus diminishing the weight of the milk spectral information. Improvements by
474 continuing with further developing the equation by adding new data of interest can also be expected.
475 One other important drawback of the MIR-based prediction is that they currently only aim at
476 absolute CH_4 production and not at CH_4 yield or CH_4 emission intensity. However, the latter might
477 quite easily be implemented, because the milk recording events provide also data on milk yield.
478 Currently, the knowledge of genetic correlations between different CH_4 proxies and reference CH_4
479 values is extremely limited.
480
481 ***Accuracy of the CH_4 Measurement with the Laser Methane Detector***
482 The LMD has been shown under some circumstances to be potentially useful in fast phenotyping
483 the P_m of cows, as measurements need only a few min per cow (Sorg et al., 2018). The circadian
484 pattern of P_m is mainly driven by feed intake as determined by Bell et al. (2018) in a freestall barn.
485 We therefore measured $P_{m\ LMD}$ before and after feeding, and found the expected lower P_m before
486 feeding, but only in the low $Y_{m\ RC}$ group. It could be speculated that, for a short time, low CH_4 emitters
487 have greater post-feeding emissions due to particularly effective fermentation, whereas greater 'basal'
488 (pre-feeding) emissions lead to the overall high emissions in high CH_4 emitters. This indicates that
489 the LMD technique may indeed be able to detect differences in P_m caused by feeding events as shown

earlier by Sorg et al. (2017), but also that no reliable categorization is possible with LDM values obtained after feeding. Chagunda (2013) reported a positive relationship between LMD and RC data and concluded that the LMD would rank cows for P_m in a very similar way. When compared to the GreenFeed system and 2 different infrared sensors installed in an automatic milking system, the LMD method ranked cows similarly with respect to their P_m (Sorg et al. 2018). Nevertheless, we found no correlation of any of the LMD measurements with the RC data (and not with any MIR prediction, either) despite repeating measurements over 3 d. This coincides with the report of a low agreement by Ricci et al. (2014). The level of $P_{m\ LMD}$ was high compared to $P_{m\ RC}$ and in the range found with MIR_{3_new}, and categorization for $Y_{m\ DMI}$ with RC was only weakly recovered with LMD. It was especially puzzling that the categories were reversed in $P_{m\ LMD}$ rank before and after feeding. The LMD operates indirectly and relies on an assumed relationship between breath CH_4 concentration and other parameters. As such it is subject to greater variance and uncertainty compared to quantitative direct measurements. Besides that, the accuracy is affected by proximity of other animals, the distance to the cows' head or the angle of the laser beam and, as shown, the time point in relation to feeding. All of this was controlled in the present study, but may be difficult to control on farm and thus add further uncertainty.

Characteristics of Low Emitting Cows Identified by CH_4 yield in Respiration Chambers

Re-categorizing cows for low and high emission by the RC CH_4 data was limited to the 28 remaining cows with complete data sets. It can, therefore, be expected that the difference in CH_4 yield between the 2 categories in the Swiss, Brown Swiss population is clearly larger as in the 2 × 10 cows eventually selected. Still a number of clear differences between these 2 groups were found. These included higher RFI and ECM/BW ratios in low vs. high $Y_{m\ RC}$ cows, whereas differences in feed efficiency were not statistically significant despite similar DMI and different ECM. A low RFI is an indicator for a good feed utilization. Hegarty et al. (2007) demonstrated that beef cattle selected for low RFI have a lower P_m, but not a decreased $Y_{m\ DMI}$. The RFI itself is also a heritable trait (h^2 = 0.40), and genetic correlations in the range of 0.18 to 0.84 between RFI and predicted P_m indicate

517 that selection for lower RFI might also reduce P_m (de Haas et al., 2011). It has to be noted that the
518 indirect effect of differences in feed efficiency of ruminants fed on the same diet explains at least half
519 of the heritability of $Y_{m\ DMI}$ (Pinares-Patiño et al., 2013). This is independent of the side-effect of
520 breeding for ECM yield on $I_{m\ ECM}$ (-15% per kg ECM; Knapp et al., 2014). Here, $I_{m\ ECM}$ is mainly
521 declining because of the lower dilution by maintenance and more concentrate (less fiber) associated
522 with higher ECM yield (Grandl et al., 2016). The lower dilution of maintenance also explains the
523 higher ECM/BW ratio of the low $Y_{m\ RC}$ cows. In addition, the re-categorization also slightly increased
524 ECM difference (on farm: 22 vs. 20 kg/d in low and high $P_{m\ MIR}$ cows; re-categorized on station: 23
525 vs. 19 kg/d in low and high $Y_{m\ RC}$ cows) and, thus, minimally the allocation of concentrate (10 vs.
526 7.5% of diet).

527 Flay et al. (2019) showed that $Y_{m\ DMI}$ was smaller in high RFI animals (consistent with our
528 findings) and hypothesized that this might be due to a decreased ruminal NDF digestibility which is,
529 regarding the RFI. Accordingly, Cabezas-Garcia et al. (2017) found that a reduced $Y_{m\ DMI}$ was
530 associated with a reduced diet and cell wall digestion. The fiber degrading microbes produce less
531 hydrogen, the main substrate for CH_4 formation, possibly as a consequence of a faster ruminal digesta
532 passage rate. Indeed, MRT in RR or GIT was suggested to be a main contributor leading to differences
533 in CH_4 emissions in ruminants, because a shorter MRT leaves less time for CH_4 formation from the
534 same amount of feed (Goopy et al., 2014). Goopy et al. (2014) showed that low $Y_{m\ DMI}$ sheep have a
535 lower rumen particulate content and a proportionately smaller rumen. However, only part of these
536 findings were recovered in the current study. Indeed the proportion of ruminal acetate was declining
537 at cost of propionate in low compared to high $Y_{m\ RC}$ cows, a clear sign of a shift in fermentation from
538 fiber towards starch (Hristov et al., 2013). This might have been slightly supported by the concomitant
539 small difference in concentrate allocation. However, total tract fiber digestibility was not different in
540 the present study. The same is true (with 1 exception) for RR and GIT MRT of all particle fractions,
541 where only the smallest particles hade a shorter GIT MRT in the low $Y_{m\ RC}$ cows. The estimated gut
542 DM fill was even higher for the low $Y_{m\ RC}$ cows. The present results therefore point towards a shift
543 of fiber fermentation from the rumen to the hindgut in low compared to high $Y_{m\ RC}$ cows. In the

hindgut, CH_4 formation per unit of fiber degraded is lower due to the absence of the protozoa and the higher competitiveness of the reductive acetogens (Fievez et al., 1999). In case these category differences were caused by genetics, the genotype indeed appears to have some control over the gut microbial community. Accordingly, transcription of methanogenesis pathway genes was found by Shi et al. (2014) to be lower in low CH_4 producing sheep even though methanogen abundance was unaffected. Goopy et al. (2014) also described that host genetics may be able to influence the rumen ecosystem, which itself might affect ruminal CH_4 production.

CONCLUSIONS

The present study demonstrated that the mid-infrared spectra based predictions of CH_4 production of individual cows on farm is recovered at the experimental farm on a uniform diet thus confirming hypothesis (i). However the CH_4 production of individuals or categorized groups as predicted with MIR did not correspond to that measured in respiratory chambers, even though with the most refined equation a slight improvement was noted (numerical increase in the concordance correlation coefficient to 0.16). This disproves hypothesis (ii) It indicates that, at least with forage-based diets and with this range of variation in CH_4 values, the proxy is not yet accurate enough to be implemented for selection purposes in Brown Swiss breeding, an assessment which applies to the laser CH_4 detector technology, too. The current study also provided detailed information about the characteristics of low CH_4 emitting cows in terms of intake, efficiency and digestion. Compared to high CH_4 emitters, low CH_4 emitting cows are superior in some variables describing feed and digestive efficiency, which partially confirms hypothesis (iii). Still, cows with low CH_4 yield will have to be further characterized by mechanistic studies to understand the relative importance of different physiological aspects contributing to the lower CH_4 emissions and to clarify the extent to which these are under genetic control.

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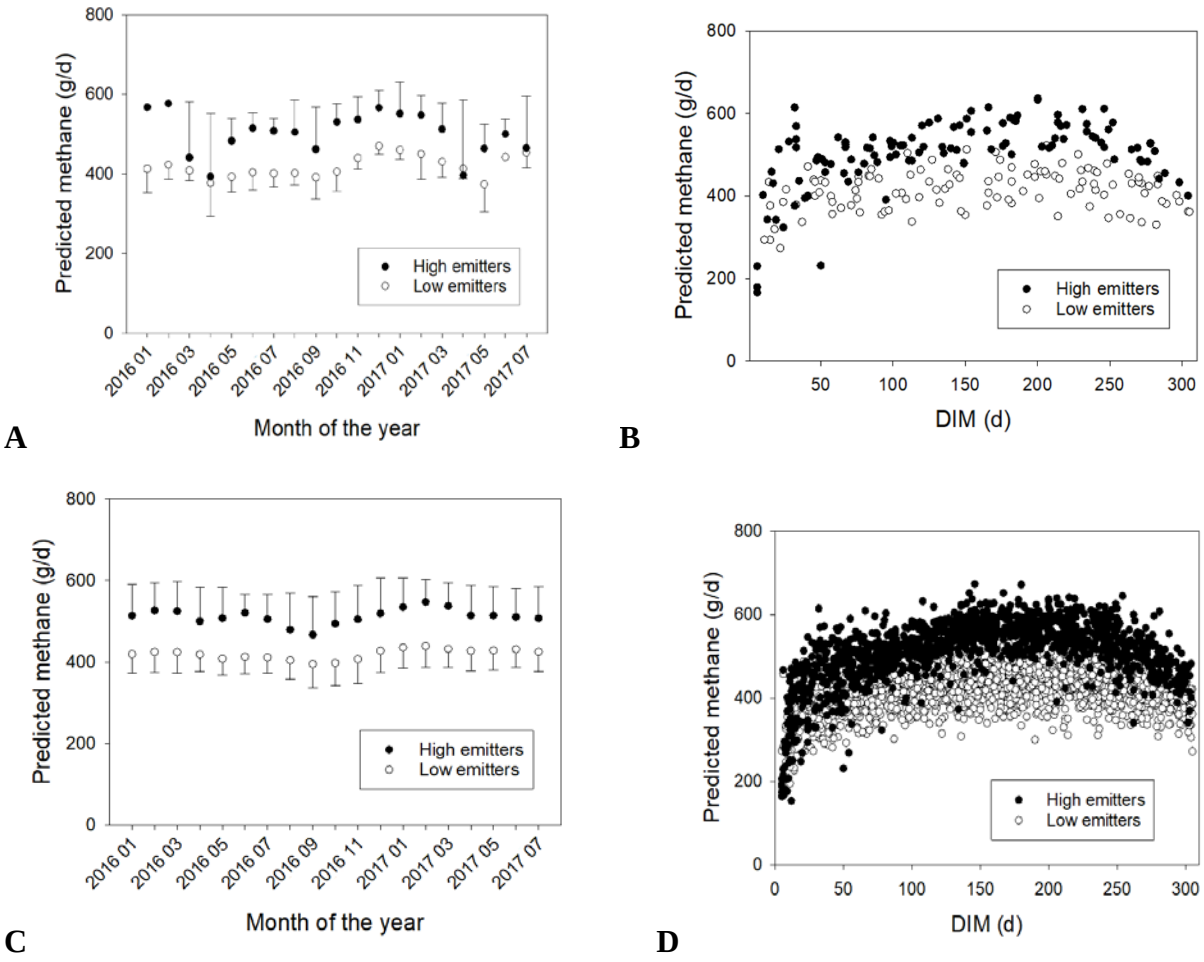

Figure 1. Methane production (g/d) before the experiment predicted from milk MIR spectra obtained in the 18 mo from January 2016 to July 2017 (means ± SE; A and B: changes with calendar time; C and D: changes with progressing DIM) of either all lactating Brown Swiss cows identified as low and high methane emitters (n = 2 × 159; upper and lower quartile of preselected cows; C and D) or the cows selected for the present experiment (n = 2 × 15; A and B).

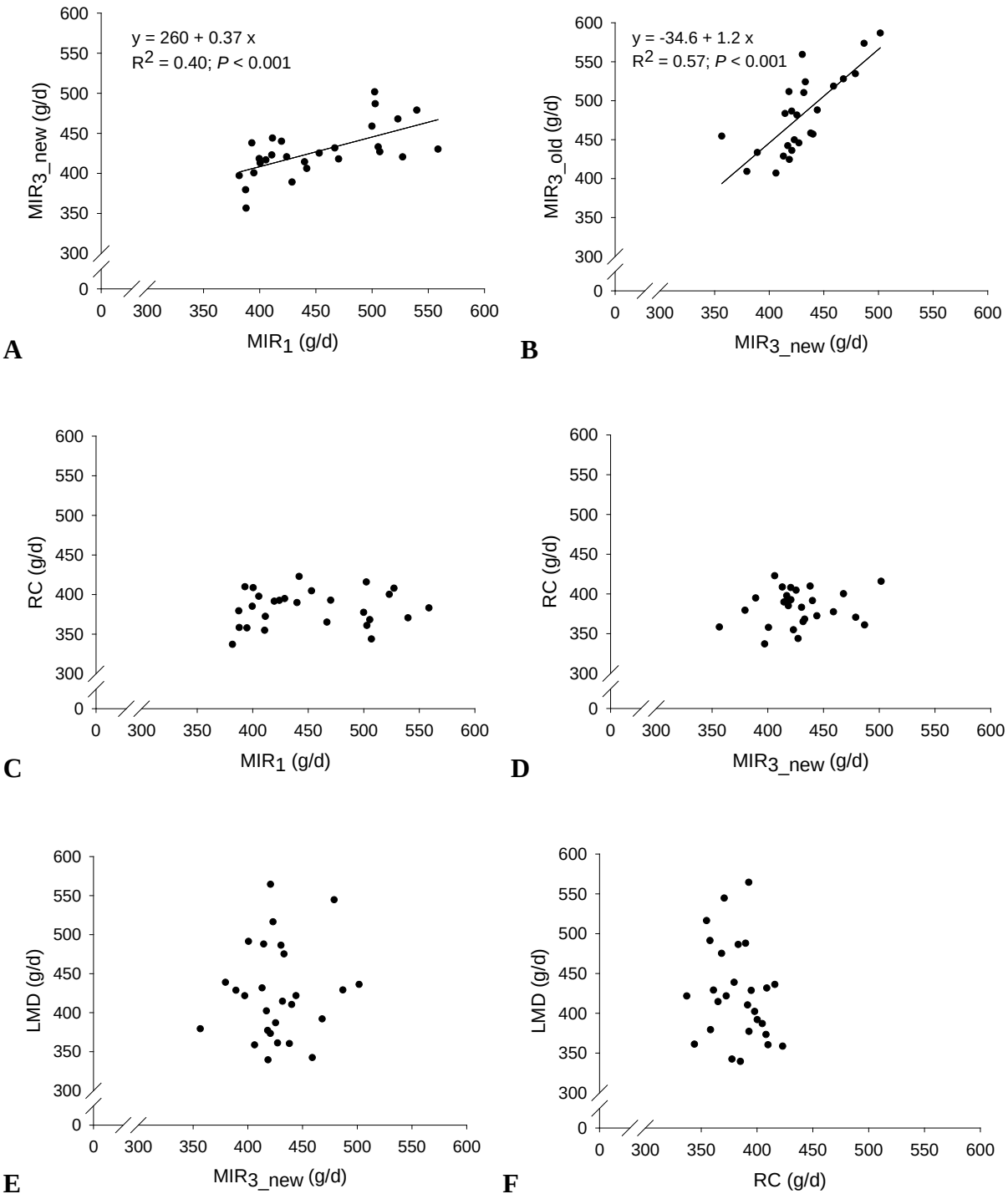

Figure 2. Relationships between individual cow CH_4 production data (g/d) predicted by the milk MIR spectra obtained from the last milking before the experiment (MIR_1; July 2017) and across the 8 d of sampling (A: MIR_{3_new} and B: MIR_{3_old}; using the newest and the first available equation, respectively) and those measured in respiration chambers (RC; on sampling d 9; C, D) and with the laser methane detector (LMD; across the last 3 d within 8 d of sampling; E) and those measured in RC and with LMD (F).

Table 1. Composition (% of DM; DM: % of wet weight) of the individual experimental feeds and amounts offered and refused (arithmetic means ± SD)

	Diet component					
	Mixed ration (MR)					
Item	Hay[1]	Grass silage[1]	Corn silage[1]	Protein concentrate[2]	Energy concentrate[2]	Grass pellets[2]
Composition						
DM	88.6 ± 1.5	35.2 ± 4.0	37.0 ± 2.8	93.1 ± 0.0	88.8 ± 0.0	90.3 ± 0.4
OM	91.0 ± 1.1	88.0 ± 1.9	96.8 ± 0.4	91.0 ± 0.0	93.7 ± 0.0	87.4 ± 0.4
CP	9.8 ± 2.3	13.9 ± 1.6	6.7 ± 0.9	31.7 ± 0.3	22.8 ± 0.3	17.3 ± 0.6
NDF	49.3 ± 4.6	39.9 ± 3.6	45.2 ± 4.0	14.5 ± 1.5	27.1 ± 1.2	40.2 ± 2.5
ADF	35.8 ± 3.8	31.9 ± 3.1	27.8 ± 3.4	11.0 ± 0.1	10.8 ± 0.1	26.3 ± 3.0
ADL	4.80 ± 0.91	4.55 ± 1.52	3.49 ± 0.95	5.78 ± 0.04	3.65 ± 0.03	4.50 ± 0.35
EE[3]	1.68 ± 0.28	2.88 ± 0.42	3.41 ± 0.25	2.03 ± 0.01	7.41 ± 0.16	3.15 ± 0.46
DM amounts, kg/d						
Offered	0.64 ± 0.19	4.94 ± 0.47	7.34 ± 0.52	1.75 ± 0.25	1.27 ± 0.43	0.63 ± 0.22
Refused	0.13 ± 0.05	0.86 ± 0.28	0.49 ± 0.15	0.11 ± 0.35	0	0

[1]n=19.
[2]n=3.
[3]EE = ether extract.

Table 2. Overview and definitions of MIR assessments, categorization of cows into low and high CH_4 emitters and MIR-based equations used

Item	Acronym[5]	Duration	Number of spectra per cow[1]	Site	Number of cows
MIR	Screening	18 mo	18	Home farm	175,980
assessments	MIR_1	1 day[2]	1	Home farm	30
	$MIR_{2\ (old/new)}$	1 day[3]	2 (averaged)	Station barn	30
	$MIR_{3\ (old/new)}$	8 days	16 (averaged)	Station barn	28[4]
	$MIR_{4\ (old/new)}$	1 day	2 (averaged)	Respiration chamber	28[4]
Categorization	$Categorization_{MIR1}$	1 day	1	Home farm	30
	$Categorization_{MIR3\_old}$	1 day	16 (averaged)	Station barn	28[4]
	$Categorization_{MIR3\_new}$	1 day	16 (averaged)1	Station barn	28[4]

[1]In case, morning and evening milk was collected or collection was performed across several d, the spectra were averaged proportionately to milk yield to 1 spectrum following Vanlierde et al. (2015).
[2]From last routine performance recording before the experiment (July 2017).
[3]At arrival at station.
[4]Two high $P_{m\ MIR}$ cows had severe diarrhea and were excluded.
[5]Old equation = equation available at the time of the experiment; new equation = further developed equation now available.

Table 3. Description of the experimental animals selected for presumed low and high CH_4 emissions (P_m) based on milk mid-infrared (MIR) spectra predictions before the experiment (arithmetic means ± SD or ranges)

	MIR-CH_4 emission category		Overall characterization of the cows
	Low	High	
Item	n=15	n=15	n = 30
Methane (P_m; g/d)[1]			
MIR_1[2]	409 ± 23	507 ± 36	455 ± 57
MIR_{2_old}[3]	450 ± 42	529 ± 28	487 ± 61
MIR_{2_new}[3]	411 ± 26	443 ± 34	426 ± 54
BW[1]	656 ± 39	637 ± 39	651 ± 42
DIM[2]	244 ± 29	238 ± 31	241 ± 30
Milk yield, kg/d[4]	20.2 ± 2.62	20.7 ± 2.8	20.3 ± 5.3
BW range[4], kg	570 – 740	550 – 680	570 – 740

[1]MIR = methane emission predicted from mid-infrared spectra of milk samples analyzed. For further explanations please see Table 2.
[2]Measured during last monthly milk performance recording before the start of the experiment (July 2017).
[3]Measured on the first d at the experimental station.
[4]Measured during the experimental period.

Table 4. Lin's concordance correlation coefficients between different predictions and measurements of methane production (g/d) (n = 28) and, in brackets, CI

	MIR_1	MIR_{3_old}	MIR_{4_old}	MIR_{3_new}	MIR_{4_new}	RC	LMD_{avg}	LMD_1
MIR_{3_old}	0.70** (0.37; 0.84)							
MIR_{4_old}	0.65** (0.36;0.83)	0.89*** (0.77;0.95)						
MIR_{3_new}	0.36^{ns} (0.21;0.57)	0.32^{ns} (0.13;0.48)	0.41^{ns} (0.17;0.60)					
MIR_{4_new}	0.31^{ns} (0.05;0.53)	0.28^{ns} (0.09;0.44)	0.37^{ns} (0.14;0.57)	0.90*** (.81;0.94)				
RC	0.05^{ns} (-0.15;0.08)	0.05^{ns} (-0.13;0.04)	0.03^{ns} (-0.12;0.07)	0.11^{ns} (-0.06;0.28)	0.16^{ns} (-0.05;0.37)			
LMD_{avg}	0.02^{ns} (-0.01;0.02)	0.003^{ns} (-0.004;0.12)	0.01^{ns} (-0.03;0.15)	-0.01^{ns} (-0.01;0.01)	0.001^{ns} (-0.01;0.01)	-0.004^{ns} (-0.01;0.001)		
LMD_1	0.11^{ns} (-0,16;0.37)	0.13^{ns} (-0.09;0.34)	0.12^{ns} (0.12;0.35	0.06^{ns} (-0.14;0.24)	0.01^{ns} (-0.20;0.23)	0.05^{ns} (-0.18;0.08)	0.05^{ns} (0.02;0.08)	
LMD_2	-0.18^{ns} (-0.51;0.19)	-0.10^{ns} (-0.38;0.20)	-0.02^{ns} (-0.31;0.35	-0.09^{ns} (-0.39;0.22)	0.04^{ns} (-0.28;0.35)	-0.05^{ns} (0.20;0.11)	0.01^{ns} (-0.01;0.02)	-0.28^{ns} (-0.53;0.01)

MIR = CH_4 values predicted by mid-infrared analysis (for further explanations see Table 2). RC = respiration chamber. LMD = laser methane detector, '1' applied before feeding, '2' after feeding and 'avg' as daily average. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; †$P < 0.10$¸ ^{ns}not significant.

Table 5. Methane production (P_m, g/d) of cows categorized into low (n = 15) or high (n = 13) emitters based on values predicted from milk MIR spectra obtained before or during the experiment (LSM ± SE)

Milk spectra database	Categorization based on	MIR emission category		*P*-value
		Low	High	
MIR_1	MIR_1	407 ± 14.1	501 ± 14.5	0.002
	MIR_{3_old}	417 ± 11.1	492 ± 10.7	0.010
	MIR_{3_new}	422 ± 17.1	479 ± 13.7	0.14
MIR_{3_old}	MIR_1	486 ± 55.6	563 ± 55.2	0.002
	MIR_{3_old}	477 ± 48.2	561 ± 47.8	0.007
	MIR_{3_new}	526 ± 85.5	587 ± 84.6	0.023
MIR_{4_old}	MIR_1	550 ± 87.4	622 ± 86.2	0.004
	MIR_{3_old}	535 ± 83.7	611 ± 82.9	0.020
	MIR_{3_new}	556 ± 103.2	603 ± 102.1	0.057
MIR_{3_new}	MIR_1	448 ± 7.9	411 ± 7.0	0.029
	MIR_{3_old}	447 ± 7.6	411 ± 7.9	0.050
	MIR_{3_new}	405 ± 6.5	451 ± 6.3	0.002
MIR_{4_new}	MIR_1	441 ± 9.5	403 ± 8.5	0.080
	MIR_{3_old}	440 ± 9.2	404 ± 9.5	0.14
	MIR_{3_new}	391 ± 8.7	447 ± 7.2	0.005
RC	MIR_1	384 ± 5.7	382 ± 6.4	0.24
	MIR_{3_old}	386 ± 6.2	385 ± 5.9	0.56
	MIR_{3_new}	380 ± 6.5	385 ± 6.6	0.72
LMD	MIR_1	423 ± 16.5	426 ± 18.5	0.95
	MIR_{3_old}	416 ± 18.4	428 ± 17.7	0.60
	MIR_{3_new}	429 ± 17.5	426 ± 18.0	0.47

MIR = values predicted by mid-infrared analysis. For further explanations see Table 2. RC = Respiration chamber. LMD = laser methane detector.

[1]Measurements in ppm × m were converted to g/d using the regression of Sorg et al. (2018).

Table 6. Methane production of cows categorized into low or high CH_4 emitters (n = 2 × 10) based on methane yield per unit of DMI (Y_m) measured in the respiration chambers (RC; LSM ± SE)

	Emission category		
Item	Low $Y_{m\ RC}$	High $Y_{m\ RC}$	*P*-value
Respiration chamber			
Methane, g/d (P_m)	379 ± 6.9	391 ± 6.5	0.22
Methane yield (Y_m)			
g/kg DMI ($Y_{m\ DMI}$)	21.7 ± 0.35	25.6 ± 0.33	<0.001
g/kg digestible OM	34.6 ± 0.71	41.7 ± 0.66	<0.001
g/kg digestible NDF	104 ± 3.8	124 ± 3.6	0.003
% of gross energy ($Y_{m\ GE}$)	6.77 ± 0.129	8.04 ± 0.134	<0.001
Methane emission intensity (I_m)			
per ECM, g/kg ($I_{m\ ECM}$)	16.6 ± 1.15	21.1 ± 1.09	0.001
per BW, g/kg	0.60 ± 0.022	0.59 ± 0.023	0.81
MIR predictions			
MIR_1 g/d (P_m)	473 ± 20.0	483 ± 18.8	0.97
MIR_{3_old}, g/d (P_m)	453 ± 19.0	450 ± 17.9	0.58
MIR_{3_new}, g/d (P_m)	412 ± 23.5	415 ± 24.0	0.66
MIR_1 g/kg DMI (Y_m)	27.2 ± 1.45	31.6 ± 1.37	0.044
MIR_{3_old},g/kg DMI ($Y_{m\ DMI}$)	26.1 ± 1.39	29.5 ± 1.32	0.090
MIR_{3_new}, g/kg DMI ($Y_{m\ DMI}$)	23.5±1.04	27.8 ± 1.08	0.058
Laser methane detector[1]			
Before feeding, g/d	384 ± 39.2	445 ± 37.7	0.48
After feeding, g/d	455 ± 17.1	412 ± 17.4	0.051
Average, g/d (P_m)	422 ± 22.0	414 ± 20.7	0.77
Average, g/kg DMI ($Y_{m\ DMI}$)	24.3 ± 1.63	27.0 ± 1.54	0.26

MIR = values predicted by mid-infrared analysis. For further explanations see Table 2. Average = Averages of measurements before and after feeding using the laser methane detector

[1]Measurements of methane in ppm × m were converted to g/d using the regression of Sorg et al. (2018).

Table 7. Variables characterizing intake, performance and digestion of cows categorized into low or high CH_4 emitters (n = 2 × 10) based on CH_4 yield per unit of DMI (Y_m) measured in the respiration chambers (RC; LSM ± SE) and correlations of these variables (n = 28) with methane production (g/d) measured with RC and predicted with MIR based on first available (old) and further developed equation (new)

	Emission category			Pearson correlation coefficients		
Item	Low $Y_{m\ RC}$	High $Y_{m\ RC}$	*P*-value	RC	MIR_{3_old}	MIR_{3_new}
DMI, kg/d	17.5 ± 0.47	15.7 ± 0.44	0.004	0.55**	-0.21^{ns}	0.06^{ns}
Feeding behavior						
Eating time, min/d	371 ± 34.8	351 ± 38.1	0.70	-0.22^{ns}	0.16^{ns}	-0.09^{ns}
Ruminating time, min/d	412 ± 19.1	394 ± 20.9	0.54	-0.32^{ns}	0.06^{ns}	0.02^{ns}
BW, kg	634 ± 17.9	665 ± 16.9	0.25	0.27^{ns}	-0.22^{ns}	-0.09^{ns}
ECM, kg/d	23.8 ± 1.58	18.8 ± 1.49	0.041	0.29^{ns}	0.27^{ns}	0.33†
Efficiency						
ECM, kg/kg DMI	1.35 ± 0.073	1.22 ± 0.061	0.17	0.08^{ns}	0.49**	0.43*
ECM, kg/100 kg BW	3.73 ± 0.241	2.85 ± 0.232	0.020	0.21^{ns}	0.32^{ns}	0.35†
Residual feed intake, kg/d	-1.58 ± 0.224	-2.47 ± 0.201	0.012	0.33†	-0.16^{ns}	0.01^{ns}
Apparent digestibility, %						
OM	69.5 ± 0.82	68.2 ± 0.78	0.29	0.51**	-0.12^{ns}	0.16^{ns}
NDF	51.9 ± 1.46	51.1 ± 0.84	0.84	0.41*	-0.02^{ns}	0.16^{ns}
ADF	49.1 ± 2.10	50.3 ± 1.98	0.69	0.25^{ns}	-0.31^{ns}	0.36†
CP	60.8 ± 2.91	57.4 ± 2.80	0.83	0.34†	-0.12^{ns}	0.03^{ns}
N balance, g/d per cow						
N intake	433 ± 25.1	446 ± 56.3	0.39	0.52**	-0.15^{ns}	0.09^{ns}
Fecal N	166 ± 10.1	175 ± 21.4	0.48	0.30^{ns}	-0.10^{ns}	0.07^{ns}
Urinary N	136 ± 8.0	135 ± 8.3	0.57	0.59**	-0.31^{ns}	-0.10^{ns}
Milk N	127 ± 9.0	110 ± 17.0	0.26	0.18^{ns}	0.27^{ns}	0.29^{ns}
N losses, % of N intake						
Fecal N	38.8 ± 1.51	40.8 ± 1.53	0.26	-0.34†	0.12^{ns}	-0.03^{ns}
Urinary N	34.2 ± 1.56	38.9 ± 1.57	0.14	-0.11^{ns}	-0.22^{ns}	-0.23^{ns}
Milk N	30.9 ± 1.14	28.7 ± 1.44	0.83	-0.24	0.57**	0.37†
Milk N; % of dig. N	50.8 ± 1.92	48.5 ± 1.94	0.96	-0.34†	0.57**	0.32
Urine N, % of total N loss	48.7 ± 1.62	46.9 ± 1.60	0.50	0.30^{ns}	-0.22^{ns}	-0.15^{ns}
VFA in rumen fluid, mol % of total VFA						
Acetate	73.1 ± 3.14	86.8 ± 2.97	0.013	0.19^{ns}	0.02^{ns}	-0.06^{ns}
Propionate	22.6 ± 0.59	19.3 ± 0.56	0.002	-0.47**	0.11^{ns}	-0.14^{ns}
n-butyrate	15.5 ± 0.88	14.4 ± 0.83	0.42	-0.19^{ns}	0.19^{ns}	0.09^{ns}
iso-buryrate	1.46 ± 0.11	1.33 ± 0.10	0.38	-0.38*	-0.12^{ns}	-0.18^{ns}
n-valerate	1.85 ± 0.08	1.84 ± 0.07	0.92	-0.23^{ns}	-0.07^{ns}	-0.29^{ns}
iso-valerate	1.64 ± 0.13	1.86 ± 0.12	0.23	-0.50**	0.03^{ns}	-0.25^{ns}
Acetate-propionate ratio	3.26 ± 0.19	4.51 ± 0.18	<0.001	0.42*	-0.08^{ns}	0.07^{ns}
Ruminal ammonia, mM	10.8 ± 1.41	13.8 ± 1.32	0.16	0.09^{ns}	0.08^{ns}	-0.02^{ns}
Reticuloruminal retention time, h						
Small particles (2 mm)	26.6 ± 1.58	29.0 ± 1.88	0.93	0.09^{ns}	0.00^{ns}	0.01^{ns}
Medium particles (5 mm)	34.1 ± 2.46	35.7 ± 2.60	0.88	0.03^{ns}	-0.21^{ns}	-0.18^{ns}
Large particles (8 mm)	34.9 ± 2.47	36.3 ± 2.61	0.95	0.03^{ns}	-0.21^{ns}	-0.13^{ns}
Solute	16.5 ± 1.61	17.0 ± 1.69	0.42	-0.04^{ns}	-0.05^{ns}	-0.12^{ns}
Gastrointestinal (GIT) retention time, h						
Small particles (2 mm)	37.3 ± 1.83	40.5 ± 1.94	0.03	-0.09^{ns}	0.28^{ns}	0.25^{ns}
Medium particles (5 mm)	44.7 ± 2.09	47.1 ± 2.21	0.11	-0.18^{ns}	0.09^{ns}	0.08^{ns}
Large particles (8 mm)	45.7 ± 2.18	47.7 ± 2.30	0.16	-0.17^{ns}	0.09^{ns}	0.12^{ns}
Solute	29.9 ± 1.34	28.1 ± 1.42	0.84	-0.30^{ns}	0.35^{ns}	0.24^{ns}
GIT fill, kg DM	35.0 ± 1.64	29.9 ± 1.55	0.042	-0.15^{ns}	0.10^{ns}	0.24^{ns}

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; †$P < 0.10$, ^{ns}not significant.