Validation strategy can result in an overoptimistic view of the ability of milk infrared spectra to predict methane emission of dairy cattle

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ABSTRACT

Because of the environmental impact of methane (CH_4), it is of great interest to reduce CH_4 emission of dairy cattle and selective breeding might contribute to this. However, this approach requires a rapid and inexpensive measurement technique that can be used to quantify CH_4 emission for a large number of individual dairy cows. Milk infrared (IR) spectroscopy has been proposed as a predictor for CH_4 emission. In this study, we investigated the feasibility of milk IR spectra to predict breath sensor-measured CH_4 of 801 dairy cows on 10 commercial farms. To evaluate the prediction equation, we used random and block cross validation. Using random cross validation, we found a validation coefficient of determination (R^2_{val}) of 0.49, which suggests that milk IR spectra are informative in predicting CH_4 emission. However, based on block cross validation, with farms as blocks, a negligible R^2_{val} of 0.01 was obtained, indicating that milk IR spectra cannot be used to predict CH_4 emission. Random cross validation thus results in an overoptimistic view of the ability of milk IR spectra to predict CH_4 emission of dairy cows. The difference between the validation strategies could be due to the confounding of farm and date of milk IR analysis, which introduces a correlation between batch effects on the IR analyses and farm-average CH_4 . Breath sensor-measured CH_4 is strongly influenced by farm-specific conditions, which magnifies the problem. Milk IR wavenumbers from water absorption regions, which are generally considered uninformative, showed moderate accuracy ($R^2_{\text{val}} = 0.25$) when based on random cross validation, but not when based on block cross validation ($R^2_{\text{val}} = 0.03$). These results indicate, therefore, that in the current study, random cross validation results in an overoptimistic view on the ability of milk IR spectra to predict CH_4 emission. We

suggest prediction based on wavenumbers from water absorption regions as a negative control to identify potential dependence structures in the data.

Key words: validation strategy, CH_4 emission, milk infrared spectroscopy, prediction

INTRODUCTION

Methane (CH_4) is emitted by dairy cattle during anaerobic fermentation of feed in the rumen (Gerber et al., 2013). Methane is a potent greenhouse gas and, in this way, dairy production contributes to global warming. In addition, the emitted CH_4 accounts for a loss of 2 to 12% of the gross energy intake of the cow (Johnson and Johnson, 1995). Therefore, there is a need to reduce CH_4 emission from dairy cattle and selective breeding might contribute to this (Wall et al., 2010). This approach would require the measurement of CH_4 or CH_4 indicators on a large number of individual dairy cows.

Several methods have been suggested to quantify CH_4 emission from individual dairy cows (Hammond et al., 2016; Negussie et al., 2017). Some studies investigated the possibility of predicting CH_4 emission based on milk infrared (IR) spectroscopy (Dehareng et al., 2012; Vanlierde et al., 2015, 2018; Shetty et al., 2017; van Gastelen et al., 2018). Milk IR spectroscopy is a method to analyze milk composition that is widely used to routinely quantify milk fat, protein, and lactose contents. The hypothesized relationship between CH_4 emission and milk composition is based on the observation that hydrogen and VFA produced during microbial fermentation in the rumen are both involved in the synthesis pathways of CH_4 and de novo milk fatty acids (short- and medium-chain fatty acids). Therefore, milk IR spectra might be used to predict CH_4 with the advantage—compared with alternative indicators for CH_4 emission—that it can be easily applied on a large scale without substantial cost. Results from studies predicting CH_4 emission based on milk IR spectra are not consistent; Vanlierde et al. (2015) reported a validation coefficient of determination (R^2_{val}) of 0.77 for CH_4 production, whereas Shetty et al. (2017) reported a R^2_{val} of 0.13. These differences warrant further re-

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search into the potential of quantifying CH₄ emission of individual cows using milk IR spectra.

A common strategy to evaluate the performance of prediction equations is random cross validation. The idea behind random cross validation is to randomly split the data into a part that is used to build the prediction equation (the calibration set) and a part used to evaluate the performance of the prediction equation (the validation set). When the validation set is independent from the calibration set, cross validation should provide a good estimate of prediction error of the equation when applied to new data. However, several studies in different fields of science have recently pointed at situations in which random cross validation resulted in overoptimistic estimations of predictive ability (Qin et al., 2016; Roberts et al., 2017). Problems with random cross validation might occur when there are dependence structures in the data; in these situations, block cross validation, where data are split strategically instead of randomly and dependence structures are accounted for, can address these problems. The issue of dependence structures might also be relevant for milk IR spectra prediction of traits relevant for dairy; for example, when milk samples on farms are collected and analyzed in batches. Therefore, in this study, we compared results from random cross validation and block cross validation using farms as blocks.

The objective of this study was to investigate the feasibility of milk IR spectra to predict breath sensor-measured CH₄ emission of individual dairy cattle on commercial farms using different validation strategies.

MATERIALS AND METHODS

Methane

Methane data were collected for 1,508 lactating dairy cows located on 11 commercial dairy farms in the Netherlands between November 2013 and March 2016. The number of cows per farm ranged from 62 to 224, and more than 85% of the cows were of the Holstein Friesian breed.

Methane was measured in the breath of cows during milking in automatic milking system (AMS; Lely Astronaut A4, Lely Industries NV, Maassluis, the Netherlands) with nondispersive infrared sensors (Acreo Swedish ICT, Kista, Sweden), a method commonly referred to as the “sniffer method.” Sensors were located in the front gate of the AMS, above the feeding trough and at the same level as the cow’s nose. In total, 4 different sensors were used during data collection and the sensors were moved from one farm to the other. The sensors measured CH₄ concentrations (expressed

in ppm) continuously twice per second and these measurements were averaged over the period that a cow visited the AMS. The average CH₄ concentrations were log₁₀-transformed to make the trait resemble a normal distribution; the transformed trait will be simply referred to as “CH₄” in the remainder of this article. More details about data collection and definition of the CH₄ phenotype are given in van Engelen et al. (2018).

Milk Infrared Spectra

Milk samples were collected during routine milk production recording for all lactating cows on the 11 commercial dairy farms involved in the current study. Milk recording was on farms with AMS, and milk samples were collected during a cows’ visit to the milking unit. Sample collection took place during routine milk recording as implemented by the cooperative cattle improvement organization CRV (Arnhem, the Netherlands). Milk IR spectra were obtained by analyzing the milk samples using Foss FT6000 instruments (Foss Analytical A/S, Hillerød, Denmark) by Qlip B.V. (Zutphen, the Netherlands). Milk IR-predicted fat, protein, and lactose percentages were based on the same milk samples and provided by CRV. Sampling and the performance of analyses are in accordance with the guidelines as set forth by the International Committee for Animal Recording (Rome, Italy).

Data Editing

The CH₄ and milk IR spectra data were combined to build the data set for IR prediction of CH₄. All CH₄ measurements of a cow for a period ranging from 2 d before and 2 d after the date of milk recording (a 5-d period) were averaged to represent CH₄ emission of a cow. Average CH₄ was based on 10 to 12 CH₄ measurements. Using a repeatability of 0.27 (van Engelen et al., 2018), we calculated that CH₄ emission of a cow based on the average of 11 repeated measurements can be estimated with 90% accuracy. Cows with fewer than 5 CH₄ measurements during the 5-d period were eliminated. Because of this restriction, several cows were eliminated from the data set, including all cows from 1 particular farm. The final data set consisted of 801 cows from 10 commercial farms.

Methane Prediction

The partial least squares (PLS) regression (Wold et al., 1983) procedure in SAS version 9.3 (SAS Institute, 2011) was used to develop CH₄ prediction equations. Milk IR spectra consist of 1,060 individual wavenum-

bers ranging from 925 to 5,008 cm^{-1} . The spectra were converted from transmittance to absorbance values. In total, 275 individual wavenumbers from 3 spectral regions of milk IR spectra were used to develop prediction equations: 925 to 1,584 cm^{-1} , 1,719 to 1,784 cm^{-1} , and 2,652 to 2,976 cm^{-1} . Wavenumbers in these regions are known to contain most of the information on milk composition (e.g., Capuano et al., 2014) and they will be referred to hereafter as “informative IR wavenumbers.”

Wavenumbers from the water absorption regions are generally considered uninformative for predicting milk composition or any other trait. Therefore, we expected that prediction of CH_4 based on these wavenumbers would result in negligible prediction accuracy and we considered this prediction to be a negative control. Based on results from Wang et al. (2016), we selected wavenumbers from the water absorption regions, which contain negligible information on milk composition. In total, 114 wavenumbers from 1,623 to 1,670 cm^{-1} , 3,166 to 3,254 cm^{-1} , 3,285 to 3,463 cm^{-1} , and 3,547 to 3,659 cm^{-1} were selected. based on the following criteria: they should not be significantly affected by systematic environmental effects (except for date of IR analysis), they should not be significantly affected by *DGAT1* genotype, and more than 90% of the variation should be unexplained (residual variance).

A commonly used negative control is permutation of the data. To gain further insight into the IR prediction of CH_4 , we performed 2 additional permutation analyses. First, we randomly assigned the milk IR spectra to cows in the whole data set. Second, the data were permuted by randomly assigning milk IR spectra to another cow within the same farm. The permuted IR data were used to predict CH_4 based on selected wavenumbers from the water absorption regions or based on informative IR wavenumbers. Both permutation strategies were repeated 10 times.

The optimal number of latent variables for the PLS prediction equation was determined based on the lowest root mean squared error of a 10-fold cross validation procedure using the CVTEST option in PROC PLS in SAS 9.3 (SAS Institute, 2011). The optimal number of latent variables was 15 for the 275 informative IR wavenumbers and 4 for the 114 wavenumbers from the water absorption regions; these values were used as input for building the CH_4 prediction equations.

Fat, protein, and lactose percentages are milk composition traits that are routinely collected and predicted based on milk IR measurements and might contain information on CH_4 . Because there is interest in the additional information that might be available in the full milk IR spectra for predicting CH_4 , we also predicted

CH_4 based on these routinely collected milk composition traits.

Validation Strategies

For the described methane prediction, we applied 2 validation strategies: random cross validation and block cross validation with farms as blocks. The commonly used random cross validation refers to a strategy in which the samples are randomly assigned to a calibration or a validation set. This procedure is repeated and results are averaged. In the current study, 640 cows (~80%) were randomly assigned to the calibration set and 161 cows (~20%) to the validation set and the procedure was repeated 50 times. The averaged coefficient of determination based on the calibration samples ($\mathbf{R}^2_{\text{cal}}$) was calculated along with root mean squared error of calibration ($\mathbf{RMSEC}$). The validation samples were used to calculate $\mathbf{R}^2_{\text{val}}$ and the validation mean squared error of prediction ($\mathbf{RMSEP}$).

In the block cross validation strategy, samples were assigned to a calibration or validation set based on the farm from which the samples were taken. In the current study, the calibration set consisted of 9 farms and the validation set consisted of samples from the remaining farm. This procedure was repeated 10 times such that samples from each farm were validated based on the prediction equation that was calibrated based on data from the other 9 farms.

Repeated Observations

Several studies that predicted CH_4 emission based on milk composition used data that included repeated observations on the same cow (e.g., Dehareng et al., 2012). Consequently, when using random cross validation, different samples from the same cow might be present in both the calibration and the validation sets. This might affect the evaluation of the prediction equation because it creates dependencies between calibration and validation sets. Therefore, we also constructed CH_4 prediction equations based on cows with repeated observations. A second milk recording was available on 4 of the 10 farms. In total, 234 cows had repeated CH_4 and milk IR observations available and these 468 observations were used in this analysis. The repeated observations had the same data editing procedure as described above. The number of cows on each of the 4 farms ranged from 39 to 92. The time interval between 2 observations (2 routine milk recordings) of a cow was approximately 1 mo. Observations that were collected first were assigned to the calibration set and those that were collected later were assigned to the validation set.

Table 1. Evaluation of $\log_{10}$ -transformed CH_4 prediction using random cross validation and block cross validation¹

Predictor	Random cross validation ²				Block cross validation ³			
	R ² cal	R ² val	RMSEC	RMSEP	R ² cal	R ² val	RMSEC	RMSEP
Fat%+Prot%+Lact% ⁴	0.02	0.02	0.265	0.266	0.02	0.04	0.263	0.266
Infrared informative ⁵	0.55	0.49	0.180	0.192	0.58	0.01	0.171	0.408
Infrared water absorption ⁶	0.32	0.25	0.221	0.233	0.33	0.03	0.217	0.309

¹R²cal = coefficient of determination for calibration; R²val = coefficient of determination for validation; RMSEC = root mean squared error of calibration; RMSEP = root mean squared error of validation.

²Results are averaged from 50 replicates.

³Results are averaged from 10 replicates, and in each replicate, samples from 1 farm were validated by samples from the other 9 farms.

⁴Fat, protein, and lactose percentages.

⁵Infrared informative: 275 individual wavenumbers from 3 spectral regions from 925 to 1,584 cm^{-1} , 1,719 to 1,784 cm^{-1} , and 2,652 to 2,976 cm^{-1} .

⁶Infrared water absorption: selected set of 114 wavenumbers from the water absorption regions from 1,623 to 1,670 cm^{-1} , 3,166 to 3,254 cm^{-1} , 3,285 to 3,463 cm^{-1} , and 3,547 to 3,659 cm^{-1} .

RESULTS

Descriptive Statistics

The average $\log_{10}$ -transformed CH_4 based on all 801 records from the first sampling period was 2.250 with a standard deviation of 0.267. The number of records per farm ranged from 34 to 116. We detected substantial differences between farms in average $\log_{10}$ -transformed CH_4 (ranging from 2.026 to 2.657), and the within-farm standard deviation (ranging from 0.072 to 0.188) was substantially smaller than that based on all records (0.267). The Pearson correlation coefficients between CH_4 and routinely recorded milk production traits were generally low: 0.03 for fat percentage, 0.07 for protein percentage, and -0.14 for lactose percentage. Pearson correlations between CH_4 and each of the 275 informative IR wavenumbers ranged from -0.17 to 0.25. Pearson correlations between CH_4 and each of the 114 wavenumbers from the water absorption regions ranged from -0.23 to 0.29.

Prediction of Methane

Performance of the different CH_4 prediction equations evaluated using random cross validation and block cross validation is shown in Table 1. Based on random cross validation, prediction of CH_4 based on the routinely recorded milk production traits fat, protein, and lactose percentages was poor ($R^2\text{val} = 0.02$). Prediction based on 275 informative IR wavenumbers and evaluation using random cross validation indicated moderate prediction accuracy ($R^2\text{val} = 0.49$). A surprisingly high prediction accuracy was found based on wavenumbers from the water absorption regions ($R^2\text{val} = 0.25$).

Block cross validation gave a completely different view of the ability to predict CH_4 based on milk IR spectra. Prediction based on 275 informative IR wavenumbers showed an $R^2\text{val}$ (averaged over 10 replicates) of 0.01, indicating that the predictive power of milk IR spectra for CH_4 is negligible. Results for each of the 10 replicates are shown in Table 2. In each replicate, samples from one farm were validated based on

Table 2. Descriptive statistics and evaluation of $\log_{10}$ -transformed CH_4 prediction based on informative infrared wavenumbers and block cross validation^{1,2}

No. of cows	Calibration				Validated farm	No. of cows	Validation			
	Mean	SD	R ² cal	RMSEC			Mean	SD	R ² val	RMSEP
733	2.264	0.273	0.57	0.178	A	68	2.095	0.115	0.00	0.298
767	2.254	0.272	0.56	0.181	B	34	2.160	0.072	0.01	0.222
702	2.209	0.258	0.54	0.175	C	99	2.542	0.105	0.00	0.363
733	2.253	0.275	0.67	0.159	D	68	2.216	0.156	0.01	0.516
729	2.262	0.272	0.57	0.178	E	72	2.128	0.174	0.03	0.266
721	2.205	0.235	0.60	0.149	F	80	2.657	0.188	0.00	0.671
698	2.281	0.269	0.62	0.166	G	103	2.037	0.126	0.03	0.467
749	2.222	0.253	0.62	0.155	H	52	2.653	0.080	0.03	0.714
685	2.288	0.269	0.53	0.183	I	116	2.026	0.094	0.00	0.438
692	2.264	0.283	0.57	0.184	J	109	2.160	0.093	0.00	0.128

¹Mean = averaged $\log_{10}$ -transformed CH_4 ; R²cal = coefficient of determination for calibration; R²val = coefficient of determination for validation; RMSEC = root mean squared error of calibration; RMSEP = root mean squared error of validation.

²Results are shown for each of the 10 replicates where records from one of the farms are used for validation.

the prediction equation that was calibrated based on samples from the other 9 farms. The R^2_{val} ranged from 0.00 to 0.03, whereas R^2_{cal} ranged from 0.53 to 0.67. These results show that the small R^2_{val} was not due to specific conditions on just one or a few farms.

Block cross validation also resulted in low prediction accuracy based on wavenumbers from the water absorption regions (Table 1, R^2_{val} = 0.03). The R^2_{val} based on block cross validation for routinely recorded milk production traits fat, protein, and lactose percentages was negligible (R^2_{val} = 0.04) and in line with results obtained using random cross validation.

When IR spectra were permuted and IR spectra were randomly assigned to cows in the data set, random cross validation resulted in R^2_{val} < 0.01, for prediction equations based on both informative IR wavenumbers and wavenumbers from the water absorption regions. However, when IR spectra were randomly assigned to another cow on the same farm, random cross validation resulted in an average R^2_{val} of 0.44 based on informative wavenumbers and R^2_{val} of 0.23 based on wavenumbers from water absorption regions. When using block cross validation, R^2_{val} was negligible for all combinations studied; that is, informative IR wavenumbers or wavenumbers from the water absorption regions and random or within herd permutation.

Repeated Observations

Results for validation of CH₄ prediction based on repeated observations of the same cow are shown in Table 3. The predictions were based on 275 informative IR wavenumbers and validation was based on 1 replicate. Results are presented for each farm separately as well as based on all 234 cows. For each prediction, we found a large discrepancy between R^2_{val} and corresponding R^2_{cal} . For individual farms, the R^2_{val} for CH₄ ranged from 0.10 to 0.21. Based on data from all 234 cows, the R^2_{val} for CH₄ was 0.07.

Table 3. Prediction of repeated observations on the farms with 2 measurements of log₁₀-transformed CH₄ and milk infrared spectra^{1,2}

Farm	No. of cows	R^2_{cal}	R^2_{val}	RMSEC	RMSEP
A	58	0.77	0.10	0.057	0.526
C	45	0.72	0.10	0.047	0.277
H	39	0.78	0.21	0.037	0.850
J	92	0.63	0.19	0.066	0.094
Total	234	0.81	0.07	0.109	0.364

¹ R^2_{cal} = coefficient of determination for calibration; R^2_{val} = coefficient of determination for validation; RMSEC = root mean squared error of calibration; RMSEP = root mean squared error of validation.

²Results are based on 1 replicate. Prediction equations were developed on the first collected samples and validated on the later collected samples.

DISCUSSION

In this study, we investigated the feasibility of milk IR spectra for predicting breath sensor-measured CH₄ emission of individual dairy cows on commercial farms. The CH₄ prediction was evaluated using different validation strategies. Results from random cross validation suggested a moderate prediction accuracy (R^2_{val} = 0.49), whereas block cross validation with farms as blocks indicated that milk IR spectra cannot be used to predict CH₄ emission (R^2_{val} = 0.01). Wavenumbers from water absorption regions are commonly assumed to contain no information on milk composition; however, these wavenumbers resulted in a R^2_{val} of 0.25 for CH₄ based on random cross validation, whereas a R^2_{val} of 0.03 was obtained based on block cross validation. These results indicate that random cross validation leads to an overoptimistic view of the ability of milk IR spectra to predict CH₄ emission of individual dairy cows.

Validation Strategy

The current study showed large differences in results between random cross validation and block cross validation. Random cross validation has been commonly applied in studies that use milk IR spectra to predict CH₄ emission (e.g., Dehareng et al., 2012; Shetty et al., 2017) or other traits of dairy cows (e.g., Soyeurt et al., 2006; Rutten et al., 2009). However, in the current study, we showed that random cross validation can lead to misleading conclusions. Qin et al. (2016) indicate that random cross validation underestimates the error rate of the prediction equation when predictors are analyzed in batches and there are systematic differences between batches. In the current study, CH₄ measurements were taken by installing breath sensors consecutively on different farms. Milk samples were collected on these farms during routine milk production recording. Consequently, milk IR analyses of all milk samples collected from a farm were performed on the same day and probably using the same spectrometer, whereas milk samples from different farms were analyzed on different days and possibly using different spectrometers. Consequently, systematic differences between the date of milk IR analysis, which might include differences between spectrometers, are confounded with farm. Grelet et al. (2015) showed the importance of differences between spectrometers. Furthermore, Wang et al. (2016) showed significant effects for date of milk IR analysis, and the time trend in milk IR wavenumbers indicates instability of the spectrometer over time. Confounding of farm with the date of milk IR analysis results in errors associated with the date of milk IR analysis being

correlated with average CH_4 emission of a farm. The problem is illustrated by prediction of CH_4 based on wavenumbers from the water absorption regions, which resulted in a R^2 val of 0.25. These wavenumbers show highly significant effects for the date of milk IR analysis (Wang et al., 2016) and contain negligible information on the actual milk composition. Because of this confounding, wavenumbers from the water absorption regions explained part of the between-herd variation in CH_4 emission. Correlations due to confounding of farm and date of milk IR analysis will not be transmitted to new data points and are spurious. These correlations are broken down in block cross validation with farms as blocks; however, they remain undetected when using random cross validation and thus random cross validation will lead to wrong conclusions. Similar discrepancies between validation strategies have been reported in other fields of science (Gasch et al., 2015; Roberts et al., 2017; Meyer et al., 2018). Burman et al. (1994) conducted simulations on dependent observations and showed that classical leave-one-out cross validation can be misleading. They also demonstrated that blocking adapts cross validation to dependency by allowing near independence between calibration and validation sets.

Based on largely the same data as used in the current study, van Engelen et al. (2018) determined that 56% of the total variation in CH_4 can be explained by the interaction of day of CH_4 measurement and AMS, which reflects mainly variation between farms. The sniffer method used in the current study to quantify CH_4 is known to be affected by specific farm conditions such as airflow patterns and barn management (Wu et al., 2016). Differences in R^2 val between the validation strategies are directly related to the magnitude of the between-farm variation in CH_4 emission.

Prediction of CH_4 based on fat, protein, and lactose percentages suggests that no information of breath CH_4 emission is captured by routinely recorded milk composition traits. Interestingly, there was no discrepancy between the validation strategies, as both R^2 val were negligible. This indicates that batch effects on IR analyses do not affect IR-predicted fat, protein, and lactose percentages; the signal-to-noise ratio of “date of milk IR analysis” does not affect all IR wavenumbers to the same extent.

Negative Control

Situations might exist in which confounding effects of batch analyses are not immediately obvious. To identify potential problems, we therefore recommend prediction based on wavenumbers from the water absorption regions as a negative control. This assumes that wavenumbers from the water absorption regions

contain negligible information on milk composition. Previously, Wang et al. (2016) showed that some of the wavenumbers from the water absorption regions are significantly affected by the *DGAT1* polymorphism and by lactation stage, suggesting that these wavenumbers do contain information regarding milk composition. When we included these wavenumbers in the prediction, we obtained a random cross validation R^2 val of 0.34, which is considerably higher than the value of 0.25 (Table 1). This underlines the importance of carefully selecting wavenumbers from the water absorption regions when used as a negative control.

We performed different permutation analyses where milk IR spectra were randomly assigned to cows in the data set or to another cow within the same farm. The second permutation strategy did not affect herd CH_4 averages; therefore, these results confirm that errors associated with date of milk IR analysis are correlated with average farm CH_4 emission. Batch effects on the IR analyses explain differences in CH_4 between farms. The permutation analysis provides further insight into the problem but will not necessarily be able to identify the problem; that is, confounding. Including prediction based on wavenumbers from the water absorption regions is a way to detect potential problems.

Literature

Several studies have investigated the feasibility of predicting CH_4 emission for individual cows based on milk IR spectra. The reported random cross validation R^2 val ranged from 0.13 (Shetty et al., 2017) to 0.77 (Vanlierde et al., 2015). These large differences between studies can be partly explained by differences in methods used to quantify CH_4 emission: some studies used respiration chambers (van Gastelen et al., 2018; Vanlierde et al., 2018), whereas others used sulfur hexafluoride (SF_6) tracer (Dehareng et al., 2012; Vanlierde et al., 2015) or the sniffer method (Shetty et al., 2017). Shetty et al. (2017) used the sniffer method, which is similar to the CH_4 measuring method used in the current study, and reported a R^2 val based on random cross validation of 0.13, considerably lower than the value of 0.49 we found using the same validation strategy. Therefore, the spurious results obtained based on random cross validation in the current study seem less severe in the study by Shetty et al. (2017). This might be because Shetty et al. (2017) averaged the IR spectra over multiple milk samples. It is expected that averaging milk IR spectra, when analyzed in different batches, will reduce “date of IR analysis” effects. In addition, differences in CH_4 emission between farms might be smaller in Shetty et al. (2017) than in the current study. Shetty et al. (2017) used data from 3

farms and performed an additional calibration based on data from an experimental farm while validating based on commercial farm data, which is identical to the block cross validation we propose here. This block validation strategy showed negligible R^2_{val} and the authors concluded that variation in commercial farm data is not included in the experimental data (Shetty et al., 2017). Most studies have used random cross validation to evaluate the prediction of CH_4 emission based on milk IR spectra (Dehareng et al., 2012; Vanlierde et al., 2015, 2018; van Gastelen et al., 2018). Results from the current study indicate that random cross validation results in an overoptimistic view of the ability of milk IR spectra to predict CH_4 emission; therefore, results presented in the aforementioned studies might be too optimistic. However, this is only the case under specific conditions. The description of the data in most studies does not allow conclusions to be drawn about whether, and to what extent, the results reported are affected by the validation strategy. In several situations, however, confounding might be an issue but not immediately obvious; for example, confounding of batch effects of IR analyses and CH_4 measured in respiration chambers. Respiration chambers are regarded as the gold standard and one might conclude that CH_4 measurements from respiration chambers are not influenced by location- or experiment-specific conditions, as is the case for farms in the current study. However, results from respiration chambers in a study in the UK showed large between-chamber variation (Gardiner et al., 2015). Therefore, confounding of respiration chamber and milk IR analysis might also lead to an overoptimistic view of the ability of milk IR spectra to predict CH_4 emission. To identify potential problems, wavenumbers from the water absorption regions can be used as a negative control.

Several studies have shown that milk IR spectra can be used to predict a wide variety of traits such as milk fat composition (e.g., Soyeurt et al., 2006; Rutten et al., 2009), milk protein composition (Bonfatti et al., 2011; Rutten et al., 2011), milk coagulation, and ketone bodies and energy status of dairy cows (McParland et al., 2011; De Marchi et al., 2014). All of these studies based their conclusions on random cross validation and, as shown in the current study, this might lead to overoptimistic conclusions under certain conditions. Studies by Rutten et al. (2009, 2011) involved almost 400 farms, and milk samples from approximately 20 farms were analyzed on the same day. Therefore, 20 farms were confounded with date of milk IR analysis. Random cross validation might result in overoptimistic R^2_{val} if the averaged milk fat or milk protein composition for these farms differed between dates of milk IR

analysis. This is unlikely and was confirmed by analysis of the negative control (results not shown).

Predicting on Repeated Observations

The differences between random and block cross validations indicate that we obtained an overoptimistic result when both calibration and validation data sets contained samples from the same farms. To investigate whether the presence of the same cows in both calibration and validation sets affects evaluation of the prediction equation, we performed additional analyses using the repeated observations. As shown in Table 3, the R^2_{val} were between 0.10 and 0.21 for individual farms and 0.07 when based on all cows with repeated observations. These values were low but higher than the block cross validation R^2_{val} of 0.04. Although these results are based on a small number of observations and only one replicate, they suggest that having repeated observations on the same cow is a lesser problem than the issue related to confounding of farm and date of milk IR analysis. However when multiple observations per cow are available, the validation strategy should take this into account (Shetty et al., 2017; Vanlierde et al., 2018).

CONCLUSIONS

We investigated the feasibility of milk IR spectra to predict breath sensor-measured CH_4 emission of dairy cows on commercial farms in the Netherlands using different validation strategies. We showed that random cross validation could result in an overoptimistic view on the ability of milk IR spectra to predict CH_4 emission. This is due to confounding of farm and date of milk IR analysis. Whether adjusting for date of milk IR analysis can avoid these issues requires further investigations. To identify dependence structures in the data, we recommend prediction based on wavenumbers from the water absorption regions as a negative control. The negligible prediction accuracy of CH_4 emission based on block cross validation with farms as blocks indicated that milk IR spectra cannot be used to predict breath sensor-measured CH_4 of dairy cows.

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