The relationship between milk metabolome and methane emission of Holstein Friesian dairy cows: Metabolic interpretation and prediction potential

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ABSTRACT

This study aimed to quantify the relationship between CH₄ emission and fatty acids, volatile metabolites, and nonvolatile metabolites in milk of dairy cows fed forage-based diets. Data from 6 studies were used, including 27 dietary treatments and 123 individual observations from lactating Holstein-Friesian cows. These dietary treatments covered a large range of forage-based diets, with different qualities and proportions of grass silage and corn silage. Methane emission was measured in climate respiration chambers and expressed as production (g per day), yield (g per kg of dry matter intake; DMI), and intensity (g per kg of fat- and protein-corrected milk; FPCM). Milk samples were analyzed for fatty acids by gas chromatography, for volatile metabolites by gas chromatography-mass spectrometry, and for nonvolatile metabolites by nuclear magnetic resonance. Dry matter intake was 15.9 ± 1.90 kg/d (mean $\pm$ SD), FPCM yield was 25.2 ± 4.57 kg/d, CH₄ production was 359 ± 51.1 g/d, CH₄ yield was 22.6 ± 2.31 g/kg of DMI, and CH₄ intensity was 14.5 ± 2.59 g/kg of FPCM. The results show that changes in individual milk metabolite concentrations can be related to the ruminal CH₄ production pathways. Several of these relationships were diet driven, whereas some were partly dependent on FPCM yield. Next, prediction models were developed and subsequently evaluated based on root mean square error of prediction (RMSEP), concordance correlation coefficient (CCC) analysis, and random 10-fold cross-validation. The best models with milk fatty acids (in g/100 g of fatty acids; MFA) alone predicted CH₄ production, yield, and intensity with a RMSEP of 34 g/d, 2.0 g/kg of DMI, and 1.7 g/kg of FPCM, and with a CCC of 0.67, 0.44, and 0.75, respectively. The CH₄ prediction potential of both volatile metabolites alone and nonvolatile metabolites alone was low, regardless of the unit of CH₄ emission, as evidenced by the low

CCC values (<0.35). The best models combining the 3 types of metabolites as selection variables resulted in the inclusion of only MFA for CH₄ production and CH₄ yield. For CH₄ intensity, MFA, volatile metabolites, and nonvolatile metabolites were included in the prediction model. This resulted in a small improvement in prediction potential (CCC of 0.80; RMSEP of 1.5 g/kg of FPCM) relative to MFA alone. These results indicate that volatile and nonvolatile metabolites in milk contain some information to increase our understanding of enteric CH₄ production of dairy cows, but that it is not worthwhile to determine the volatile and nonvolatile metabolites in milk to estimate CH₄ emission of dairy cows. We conclude that MFA have moderate potential to predict CH₄ emission of dairy cattle fed forage-based diets, and that the models can aid in the effort to understand and mitigate CH₄ emissions of dairy cows.

Key words: dairy cow, enteric methane production, milk metabolome

INTRODUCTION

Enteric CH₄ production is one of the main targets of greenhouse gas mitigation practices for the dairy industry (Hristov et al., 2013). Quantification of enteric CH₄ production is therefore important. Several CH₄ measuring techniques have been developed, but these are not yet suitable for large scale measurements (Hammond et al., 2016). Proxies (i.e., indirect traits or indicators correlated with CH₄ emission) might, therefore, serve as a good alternative.

Milk fatty acid (MFA) concentrations have been suggested as proxy to estimate CH₄ emission in dairy cattle, and many studies have evaluated this proposed relationship between MFA concentrations and CH₄ emission (Chilliard et al., 2009; Mohammed et al., 2011; Rico et al., 2016). However, the results of these studies are inconsistent, with some studies finding a clear and strong relationship between MFA and CH₄ emission (Chilliard et al., 2009; Rico et al., 2016), whereas other studies conclude that MFA alone might not be suitable to develop universal CH₄ prediction models

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(Mohammed et al., 2011). Recently, Castro-Montoya et al. (2017) concluded that MFA are not reliable predictors for specific amounts of CH₄ emitted by a cow. Furthermore, individual MFA selected in optimal models to predict CH₄ emission largely differ between studies, further hampering the applicability of MFA to predict CH₄ emission in various circumstances. Some of these inconsistencies can be explained by dietary composition and lactation stage, both being factors that can influence the relationship between MFA and CH₄ emission (Mohammed et al., 2011; Dijkstra et al., 2016; Vanrobays et al., 2016).

These findings warrant further investigation of other proxies in milk to estimate CH₄ emission of dairy cattle, including volatile metabolites and nonvolatile metabolites. Antunes-Fernandes et al. (2016) and van Gastelen et al. (2017) evaluated the relationship between CH₄ emission and both volatile and nonvolatile metabolites in milk to better understand the biological pathways involved in CH₄ emission in dairy cattle as well as to determine the prediction potential of these milk metabolites. Antunes-Fernandes et al. (2016) concluded that CH₄ intensity (g/kg of fat- and protein-corrected milk; **FPCM**) may be related to lactose synthesis and energy metabolism in the mammary gland, as reflected by the significant relationship between both milk nonvolatile metabolites citrate and uridine diphosphate (**UDP**)-hexose B and CH₄ intensity. Methane yield (g/kg of DMI), on the other hand, may be related to glucogenic nutrient supply, as reflected by the milk nonvolatile metabolite acetone. In a recent review of CH₄ proxies, Negussie et al. (2017) concluded that no single proxy accurately predicts CH₄ emission, and that combining 2 or more proxies is the best way forward for the prediction of CH₄ emission. Van Gastelen et al. (2017) concluded that volatile metabolites and, in particular, nonvolatile metabolites in combination with MFA hold potential to predict CH₄ emission of dairy cows more precisely and accurately compared with MFA alone. The improved prediction potential was relatively small (i.e., the increase in adjusted R² and CCC is <0.18 and <0.12, respectively) for CH₄ production (g/d) and CH₄ yield (g/kg of DMI), whereas the prediction potential for CH₄ intensity (g/kg of FPCM) increased considerably (i.e., the adjusted R² and CCC increased with 0.36 and 0.24, respectively).

The analysis of both Antunes-Fernandes et al. (2016) and van Gastelen et al. (2017) was based upon a small range of diets (i.e., 4 forage-based diets in which grass silage was replaced partly or fully by corn silage) in one experiment. Therefore, the present study aims to quantify the relationship between CH₄ emission and the milk metabolome in dairy cattle fed a range of forage-

based diets with different qualities and proportions of grass silage and corn silage.

MATERIALS AND METHODS

Experiments and Data

Data on individual cows from 6 experiments, all designed as randomized block experiments, from Wageningen University & Research (Wageningen, the Netherlands) were used. These experiments were conducted in accordance with Dutch law, and approved by the Animal Care and Use Committee of Wageningen University & Research. Experiment 1 (Warner et al., 2015) involved 25 Holstein-Friesian dairy cows and 4 grass herbage diets (forage to concentrate ratio of 85:15 based on DM basis). The grass herbage was cut after 3 or 5 wk of regrowth, after receiving either a low (20 kg of N/ha) or a high (90 kg of N/ha) fertilization rate after initial cut. Experiment 2 (van Gastelen et al., 2015) involved 29 Holstein-Friesian dairy cows and 4 diets (forage to concentrate ratio of 80:20 on DM basis). The forage consisted of 1,000 g/kg of DM grass silage, 1,000 g/kg of DM corn silage, or a mixture of both silages (667 g/kg of DM grass silage and 333 g/kg of DM corn silage; 333 g/kg of DM grass silage and 667 g/kg of DM corn silage). Experiment 3 (Warner et al., 2016) involved 52 Holstein-Friesian dairy cows and 6 grass silage-based diets (forage to concentrate ratio of 80:20 on DM basis). The grass silage received low (65 kg of N/ha) or high (150 kg of N/ha) N fertilization level preceding its growth period, and there were 3 regrowth periods (28, 41, and 62 d of regrowth). Experiment 4 (Warner et al., 2017) involved 55 Holstein-Friesian dairy cows and 8 grass silage-based diets (grass silage, corn silage, and concentrate at a ratio of 70:10:20 on a DM basis). The grass silage was cut at 4 growth stages (leafy, boot, early heading, and late heading) and fed at 2 levels of DMI (15.5 and 16.6 kg of DM/d). Experiment 5 (Hatew et al., 2016) involved 25 Holstein-Friesian dairy cows and 4 corn silage-based diets with whole-plant corn harvested at a very early (25% DM), early (28% DM), medium (32% DM), and late (40% DM) stage of maturity, and with corn silage, concentrate, and wheat straw at a ratio of 75:20:5 (DM basis). Experiment 6 (Klop et al., 2017) involved 8 Holstein-Friesian dairy cows and 3 diets containing corn silage, grass silage, and concentrate at a ratio of 40:30:30 (DM basis). The concentrate was either a basal concentrate or contained a blend of essential oils or lauric acid. Repeated measures resulted in 32 observations.

The experimental setup of these experiments was similar. After an adaptation period of 12 d, cows were

housed individually in open circuit, indirect climate respiration chambers for a 5-d period to determine CH₄ emission (expressed as production in g/d, as yield in g/kg of DMI, and as intensity in g/kg of FPCM). The climate respiration chambers are described by Heetkamp et al. (2015) and van Gastelen et al. (2015). Cows were milked twice daily and water was freely available, both during the adaptation period and in the climate respiration chambers. Diets were fed as a TMR twice daily and intake was restricted to 95% of the voluntary DMI of the cow consuming the least within a block.

Sample Collection and Analyses

Milk yield was recorded and 10-mL milk samples were collected at each milking in the climate respiration chambers. These milk samples were analyzed for fat, protein, and lactose content, and for milk urea N as described by the respective studies. In addition, a representative milk sample (i.e., 5 g/kg of milk production at each milking from each cow) was collected according to Antunes-Fernandes et al. (2016). We selected all observations from experiment 2 ($n = 29$), and we randomly selected another 94 observations from the other experiments based on complete blocks (i.e., all cows within the same block were selected; incomplete blocks, due to observations being removed from the experiment or statistical analysis, were excluded from the selection). The selected observations, a total of 123 observations, represented all dietary treatments without feed additives (i.e., 27 in total), and resulted in no repeated measurements from the same cows. The representative milk samples matching the selected observations were subsequently analyzed for MFA composition (g/100 g of total fatty acids) using GC according to van Gastelen et al. (2015), for volatile metabolites (peak area in arbitrary units) using GC-MS according to Antunes-Fernandes et al. (2016), and for nonvolatile metabolites (relative area in arbitrary units) using proton nuclear magnetic resonance according to Antunes-Fernandes et al. (2016).

Statistical Analyses

Linear Regression. The descriptive statistics of the feed intake, dietary composition, animal performance, and CH₄ emission are presented in Table 1. Descriptive statistics of the MFA, volatile metabolites, and non-volatile metabolites used for modeling are presented in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13334>). To determine the relationship between CH₄ emission (i.e., production, yield, and intensity) and individual MFA, volatile metabolites, and non-volatile metabolites, mixed model univariate regression

procedures (PROC MIXED of SAS; version 9.2, SAS Institute Inc., Cary, NC) were applied. These included a random experiment effect and individual MFA, volatile metabolites, and nonvolatile metabolites as fixed effects. Having the experiment effect as a random effect resulted in the equation parameter estimates to be estimated first within study, and then averaged to obtain overall estimates. To evaluate the influence of FPCM on the established relationships between individual MFA, volatile metabolites or nonvolatile metabolites and CH₄ intensity, FPCM was included as a covariate in the linear regressions.

Model Development. The PROC GLMSELECT procedure of SAS was used to develop multivariate models retaining the experiment effect in every step, with CH₄ emission (i.e., production, yield, and intensity) as the dependent variable, the milk metabolites (i.e., MFA, volatile metabolites, and nonvolatile metabolites) as independent variables, and stepwise selection as the selection procedure. The significance level for milk metabolites to enter or stay in the model was 0.01 and 0.05, respectively. The best models were selected based on the minimum Akaike's information criterion statistic. Adjusted independent variable values were calculated based on regression parameters of the final model to determine the adjusted R² corrected for experiment effect, as described by St-Pierre (2001). The selected models were evaluated with the PROC REG procedure in terms of multicollinearity (variation inflation factor >10), but no multicollinearity was observed for any of the CH₄ prediction models.

Model Evaluation. The CH₄ prediction models were evaluated using 2 methods. First, the mean square error of prediction (**MSEP**), calculated as

$$\text{MSEP} = \sum_{i=1}^n (O_i - P_i)^2 / n,$$

where n is the total number of observations, O_i is the observed value, and P_i is the predicted value. The square root of the MSEP (**RMSEP**), expressed in the same unit as the observed mean or as percentage of the observed mean, gives an estimate of the overall prediction error. Second, concordance correlation coefficient analysis (**CCC**; Lin, 1989) was performed, where CCC is calculated as

$$\text{CCC} = r \times C_b,$$

where r is the correlation coefficient providing a measure of precision, and C_b is a bias correction factor providing a measure of accuracy. The C_b variable is calculated as

$$C_b = \frac{2}{\left[v + 1 / v + \mu^2 \right]},$$

where

$$v = \frac{S_o}{S_p},$$

$$\mu = \frac{\bar{O} - \bar{P}}{(S_o \times S_p)^{0.5}},$$

where v provides a measure of scale shift, whereas μ provides a measure of location shift, S_o and S_p are the observed and predicted standard deviations, and $\bar{O}$ and $\bar{P}$ are the observed and predicted means.

Cross-Validation. We performed a random cross-validation with 10 splits and 10 iterations as recommended by Rodriguez et al. (2010) for all prediction models to calculate the performance parameters of the models (i.e., root mean square error of cross-validation; **RMSECV**, and the coefficient of determination of cross-validation; **R²CV**). For each iteration, a model was developed as described above using 9 splits of the data set, and the selected model was subsequently

evaluated as described above on the remaining part of the data set (i.e., one split). The cross-validation performance values represent the average of the 10-fold cross-validation.

RESULTS

Relationship Between Individual Milk Metabolites and CH₄ Emission

Milk Fatty Acids. In the present study, 43 MFA were identified. The relationships between each individual MFA and CH₄ production, CH₄ yield, and CH₄ intensity are shown in Supplemental Tables S2, S3, and S4, respectively (<https://doi.org/10.3168/jds.2017-13334>). Several short- and medium-straight even-chain MFA (**SMCFA**; ≤16 carbon fatty acids), some odd- and branched-chain fatty acids (**OBCFA**; C13:0, C15:0, *iso* C15:0, and *iso* C17:0), and C20:4n-3 were positively related to CH₄ production, whereas another OBCFA (i.e., C17:0), all C18:1 and C18:2 isomers, and other long-chain fatty acids (>16 carbon fatty acids) were negatively related to CH₄ production. The SMCFA C16:0 was positively related to CH₄ yield, similar to some OBCFA and several long-chain fatty acids, whereas the SMCFA C4:0, and all C18:1, C18:2,

Table 1. Descriptive statistics of DMI, dietary composition, animal characteristics, and CH₄ emissions (n = 123) [data from van Gastelen et al. (2015), Warner et al. (2015, 2016, 2017), Hatew et al. (2016), and Klop et al. (2017)]

Item	Mean	Median	SD	Minimum	Maximum
DMI (kg/d)	15.9	15.9	1.90	10.8	19.8
Forage content diet (g/100 g of DM)	80	80	3.0	70	85
Dietary characteristic (in g/kg of DM, unless stated otherwise)					
DM (g/kg)	507	519	113.4	306	797
Ash	76	76	14.7	53	103
CP	170	158	43.2	82	251
NDF	386	386	53.4	242	501
ADF	225	219	26.9	183	291
ADL	14	15	4.7	6	26
Crude fat	31	28	7.2	21	46
Starch	111	79	92.6	5	326
Sugar	95	76	66.4	21	265
Gross energy (MJ/kg of DM)	18.6	18.7	0.41	17.6	19.3
NDF to starch ratio	8.8	5.0	16.03	1.0	86.2
Lactation characteristic					
Milk production (kg/d)	22.6	22.5	3.87	14.6	33.7
FPCM ¹ (kg/d)	25.2	24.7	4.57	15.0	38.4
Milk fat content (g/100 g of milk)	4.64	4.65	0.635	2.94	6.44
Milk protein content (g/100 g of milk)	3.37	3.33	0.359	2.62	4.53
Milk lactose content (g/100 g of milk)	4.58	4.59	0.219	3.80	5.03
Urea (mg/dL)	19.6	18.8	6.79	8.4	41.4
DIM	176	191	70.9	70	403
Parity	2.6	2.0	1.29	1.0	7.0
CH ₄ emission					
CH ₄ production (g/d)	359	358	51.1	234	469
CH ₄ yield (g/kg of DMI)	22.6	22.9	2.31	17.2	28.0
CH ₄ intensity (g/kg of FPCM)	14.5	14.6	2.59	8.5	24.0

¹Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g of milk) + 0.06 × protein (g/100 g of milk)] × milk yield (kg/d) (CVB, 2012).

and C18:3 isomers were negatively related to CH₄ yield. Furthermore, many SMCFA, OBCFA, and long-chain fatty acids were positively related to CH₄ intensity, whereas mostly C18:1, C18:2, and C18:3 isomers were negatively related to CH₄ intensity. The relationships between each MFA and CH₄ intensity including FPCM as a covariate are shown in Supplemental Table S4 (<https://doi.org/10.3168/jds.2017-13334>). Including FPCM as a covariate in the regression model resulted in several changes. Many MFA remained significantly related to CH₄ intensity, whereas the relationship between CH₄ intensity and 2 MFA (i.e., C4:0 and C22:5n-3) disappeared. In total, 8 relationships appeared or strengthened, including C6:0, C8:0, C17:1 *cis*-9, several C18:1 isomers, and C18:2 *cis*-9, *trans*-11, that became significantly related upon including FPCM as a covariate.

Volatile Metabolites. In the present study, a total of 14 volatile metabolites were identified, including ketones, aldehydes, organic acids, alcohols, esters, and sulfur compounds. The relationships between each individual volatile metabolite and CH₄ production, CH₄ yield, and CH₄ intensity are shown in Supplemental Tables S5, S6, and S7, respectively (<https://doi.org/10.3168/jds.2017-13334>). The volatile metabolites 1-pentanol, acetone, and hexanal were negatively related to CH₄ production, whereas no positive relationship between volatile metabolites and CH₄ production were observed. Ethyl butanoate and 2 free fatty acids (FFA; butanoic and hexanoic acid) were positively related, whereas hexanal was negatively related to CH₄ yield. Many volatile metabolites were related to CH₄ intensity, with 2-heptanone, ethyl butanoate, and all FFA being positively related to CH₄ intensity, and 1-pentanol, acetone, and dimethyl sulfone being negatively related to CH₄ intensity. The relationships between each volatile metabolite and CH₄ intensity including FPCM as a covariate are shown in Supplemental Table S7 (<https://doi.org/10.3168/jds.2017-13334>). Including FPCM as a covariate in the regression model resulted in most volatile metabolites remaining to be related to CH₄ intensity, with a few exceptions. Some relationships disappeared or weakened, such as acetic acid ethyl ester, which no longer showed a tendency to be positively related to CH₄ intensity, and the significant positive relationship of ethyl butanoate, which became a tendency upon including FPCM as covariate. Another relationship strengthened; the tendency for a negative relationship of hexanal became significant upon including FPCM as covariate.

Nonvolatile Metabolites. In the present study, 41 nuclear magnetic resonances could be assigned either to a nonvolatile compound or to a member of a class of nonvolatile compounds. The relationships between each

individual nonvolatile metabolite and CH₄ production, CH₄ yield, and CH₄ intensity are shown in Supplemental Tables S8, S9, and S10, respectively (<https://doi.org/10.3168/jds.2017-13334>). The nonvolatile metabolites acetylcarnitine and UDP-hexose D were negatively related, whereas 11 nonvolatile metabolites, including acetate, 3 *N*-acetylsugars, and succinate, were positively related to CH₄ production. Only one nonvolatile metabolite, UDP-hexose C, was negatively related, and no single nonvolatile metabolite was positively related to CH₄ yield. Similarly, with respect to CH₄ intensity, only citrate was negatively related. In contrast, 14 nonvolatile metabolites, including acetate, methylmalonate, and succinate, were positively related to CH₄ intensity. The relationships between each nonvolatile metabolite and CH₄ intensity including FPCM as a covariate are shown in Supplemental Table S10 (<https://doi.org/10.3168/jds.2017-13334>). Including FPCM as a covariate in the regression model resulted in several changes. Many nonvolatile metabolites remained significantly related to CH₄ intensity, whereas the relationship between CH₄ intensity and 5 nonvolatile metabolites (including citrate and ethanol) disappeared. Other relationships, however, appeared or strengthened, including acetylcarnitine and the 3 *N*-acetylsugars C, D, and E that became significantly positively related upon including FPCM as covariate.

Prediction Models for CH₄ Emission

Four sets of test variables were used to develop CH₄ prediction models; (1) MFA alone, (2) volatile metabolites alone, (3) nonvolatile metabolites alone, and (4) all 3 types of metabolites combined. In total, 11 prediction models were obtained: 4 for CH₄ production, 3 for CH₄ yield (no model was obtained with nonvolatile metabolites only), and 4 for CH₄ intensity (Table 2). The observed and residual (observed minus predicted) versus predicted CH₄ production, CH₄ yield, and CH₄ intensity plots are shown in Figures 1, 2, and 3, respectively. The evaluation results (i.e., adjusted R², RMSEP, and CCC analysis) of the 11 obtained CH₄ prediction models are shown in Table 3.

Milk Fatty Acids Alone. The RMSEP of the MFA-based CH₄ prediction models was 34 g of CH₄/d, 2.0 g of CH₄/kg of DMI, and 1.7 g of CH₄/kg of FPCM, respectively. Additionally, the adjusted R² and CCC of the MFA-based CH₄ prediction models ranged from 0.38 to 0.75, and from 0.44 to 0.75, respectively, with the prediction model for CH₄ yield performing the worst and the prediction model for CH₄ intensity performing the best. This is also evident by the lower *r* and *C_b* values for the MFA-based prediction model for CH₄ yield relative to the MFA-based prediction model for CH₄

Table 2. The prediction equations developed for CH₄ production (g/d), yield (g/kg of DMI), and intensity (g/kg of FPCM¹) based on milk fatty acids, volatile or nonvolatile metabolites alone, or all metabolites combined

Metabolites included	CH ₄ production (g/d)				CH ₄ yield (g/kg of DMI)				CH ₄ intensity (g/kg of FPCM)			
	Item	Estimate	SE	P-value	Item	Estimate	SE	P-value	Item	Estimate	SE	P-value
MFA ²	Intercept	522	19.2	<0.001	Intercept	28.4	0.99	<0.001	Intercept	13.7	2.46	0.003
	C18:1 <i>trans</i> -15	-98.0	18.91	<0.001	C18:1 <i>cis</i> -12	-18.79	2.586	<0.001	C4:0	-1.33	0.465	0.005
	+ C18:1 <i>cis</i> -11											
	C18:2 <i>cis</i> -9, <i>trans</i> -11	-116.7	21.31	<0.001	C18:3n-3	-5.44	1.296	<0.001	<i>iso</i> C15:0	32.00	4.183	<0.001
V ³	C18:3n-3	-80.9	23.59	0.008					C18:3n-3	-4.12	1.108	<0.001
	Intercept	372	15.0	<0.001	Intercept	22.2	0.53	<0.001	C20:1 <i>cis</i> -11	-35.44	9.579	<0.001
	Hexanal	-5.4 × 10 ⁻⁷	1.76 × 10 ⁻⁷	0.003	Ethyl butanoate	1.56 × 10 ⁻⁷	5.250 × 10 ⁻⁸	0.004	C22:5n-3	27.62	9.292	0.004
									Intercept	14.4	0.726	<0.001
NV ⁴	Intercept	420	26.6	<0.001	No model obtained				2-Heptanone	1.08 × 10 ⁻⁷	2.070 × 10 ⁻⁸	<0.001
	Uridine diphosphate-hexose D	-1,438.8	495.01	0.005					1-Pentanol	-1.38 × 10 ⁻⁷	3.320 × 10 ⁻⁸	<0.001
	Acetylcarbitine	-442.4	99.94	0.002					Intercept	12.9	0.76	<0.001
	Oxaloacetate	535.7	158.14	<0.001					Butyrate	3.52	0.708	<0.001
All ⁵	Intercept	522	19.2	<0.001	Intercept	28.4	0.99	<0.001	Intercept	7.7	1.38	<0.001
	+ C18:1 <i>cis</i> -11	-98.0	18.91	<0.001	C18:1 <i>cis</i> -12	-18.79	2.586	<0.001	Acetyl-carnitine	9.93	3.788	0.008
	C18:2 <i>cis</i> -9, <i>trans</i> -11	-116.7	21.31	<0.001	C18:3n-3	-5.44	1.296	<0.001	<i>iso</i> C15:0	23.63	4.946	<0.001
	C18:3n-3	-80.9	23.59	0.008					C18:3n-3	-4.95	1.093	<0.001
									C20:1 <i>cis</i> -11	-34.11	8.999	0.004
									C22:5n-3	31.93	8.768	0.003
									C24:0	37.27	12.425	0.008
									2-Heptanone	5.14 × 10 ⁻⁸	1.500 × 10 ⁻⁸	0.004

¹Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g of milk) + 0.06 × protein (g/100 g of milk)] × milk yield (kg/d) (CVB, 2012).²Milk fatty acids alone as selection variables; in g/100 g of fatty acids.³Volatile metabolites alone as selection variables; peak area value (arbitrary unit of quantity).⁴Nonvolatile metabolites alone as selection variables; peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetrauteropropionate.⁵All metabolites combined as selection variables.

Figure 1. (A) Observed and predicted CH_4 production (g/d), and (B) residual (i.e., observed – predicted) CH_4 production (g/d), from the regression analyses based on milk fatty acids (g/100 g of fatty acids) alone (1), volatile metabolites alone (2), nonvolatile metabolites alone (3), and all metabolites combined (4). The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the 6 individual experiments described in the Materials and Methods.

intensity. Although the MFA-based prediction models for CH_4 production and CH_4 intensity were equally precise (i.e., both having a r value of 0.76), the MFA-based

prediction model for CH_4 intensity was more accurate (C_b of 0.99 for CH_4 intensity and C_b of 0.88 for CH_4 production). The MFA-based prediction model for CH_4 in-

Figure 2. (A) Observed and predicted CH_4 yield (g/kg of DMI), and (B) residual (i.e., observed – predicted) CH_4 yield (g/kg of DMI), from the regression analyses based on milk fatty acids (g/100 g of fatty acids) alone (1), volatile metabolites alone (2), and all metabolites combined (3). The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the 6 individual experiments described in the Materials and Methods.

Figure 3. (A) Observed and predicted CH₄ intensity [g/kg of fat- and protein-corrected milk (FPCM)], and (B) residual (i.e., observed – predicted) CH₄ intensity (g/kg of FPCM), from the regression analyses based on milk fatty acids (g/100 g of fatty acids) alone (1), volatile metabolites alone (2), nonvolatile metabolites alone (3), and all metabolites combined (4). The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the 6 individual experiments described in the Materials and Methods.

tensity had the ability to describe more of the observed variation in CH₄ emissions compared with MFA-based prediction models for CH₄ production and CH₄ yield. This is evident by the scale shift values, with the MFA-based prediction models for CH₄ production and CH₄ yield having a scale shift that was clearly higher than 1 ($v > 1.67$), whereas the scale shift of the MFA-based prediction for CH₄ intensity was close to 1 ($v = 1.14$).

Volatile Metabolites Alone. The RMSEP of the volatile metabolite-based CH₄ prediction models was 49 g of CH₄/d, 2.3 g of CH₄/kg of DMI, and 2.4 g of CH₄/kg of FPCM. Additionally, the adjusted R² and CCC of the volatile metabolite-based CH₄ prediction models ranged from 0.07 to 0.27, and from 0.07 to 0.29, respectively, with the prediction model for CH₄ yield performing the worst and the prediction model for CH₄ intensity performing the best. The precision of these models (i.e., r) followed the same pattern, which was not the case for the accuracy of these models (i.e., C_b). The C_b value was lowest for the volatile metabolite-based prediction model for CH₄ production (0.42) and highest for the volatile metabolite-based prediction model for CH₄ intensity (0.73). Further, all volatile metabolite-based CH₄ prediction models had a scale shift that was clearly higher than 1 ($v > 2.28$), indicating the inability of volatile metabolites alone to predict the range of observed CH₄ emissions.

Nonvolatile Metabolites Alone. No model was obtained for CH₄ yield with nonvolatile metabolites alone. The significance level for a variable to enter the model was 0.01, whereas the significance level of the

strongest correlation between a nonvolatile metabolite (i.e., UDP-hexose C) and CH₄ yield was 0.035. The RMSEP of the nonvolatile metabolite-based CH₄ prediction models was 45 g of CH₄/d and 2.6 g of CH₄/kg of FPCM. Additionally, the adjusted R² was 0.30 and 0.20, and the CCC was 0.35 and 0.14 for the nonvolatile metabolite-based prediction models for CH₄ production and CH₄ intensity, respectively. Contrary to what was observed for MFA and volatile metabolites alone, the nonvolatile metabolite-based prediction model for CH₄ production performed better than the nonvolatile metabolite-based prediction model for CH₄ intensity. Both nonvolatile metabolite-based CH₄ prediction models had a scale shift that was clearly higher than 1 ($v > 2.26$), indicating the inability of nonvolatile metabolites alone to predict the range of observed CH₄ emissions.

All Metabolites Combined. When combining the 3 types of milk metabolites, the RMSEP of the CH₄ prediction models was 34 g of CH₄/d, 2.0 g of CH₄/kg of DMI, and 1.5 g of CH₄/kg of FPCM. Additionally, the adjusted R² and CCC of the CH₄ prediction models including all 3 types of metabolites ranged from 0.38 to 0.74, and from 0.44 to 0.80, respectively, with the prediction model for CH₄ yield performing the worst and the prediction model for CH₄ intensity performing the best. A similar pattern was observed for the precision (r), accuracy (C_b), and the scale shift (v) of the CH₄ prediction models combining the 3 types of metabolites. The prediction models using the combination of all 3 types of milk metabolites performed better than the prediction models with volatile metabolites

Table 3. The R² and concordance correlation coefficient (CCC) analysis of the prediction equations and the 10-fold cross-validation results

Model	Overall prediction equation						Cross-validation				
	Adjusted R ²	RMSEP ¹	RMSEP ² (%)	CCC ³	r ⁴	C _b ⁵	v ⁶	μ ⁷	R ² CV	RMSECV ⁸	RMSECV ⁹ (%)
CH ₄ production (g/d)											
MFA ¹⁰	0.51	34	9.6	0.67	0.76	0.88	1.67	-0.019	0.47	41.0	11.5
V ¹¹	0.08	49	13.6	0.13	0.30	0.42	4.56	-0.148	0.09	51.5	14.3
NV ¹²	0.30	45	12.7	0.35	0.48	0.73	2.26	-0.206	0.32	52.6	14.7
All ¹³	0.51	34	9.6	0.67	0.76	0.88	1.67	-0.019	0.48	40.6	11.4
CH ₄ yield (g/kg of DMI)											
MFA	0.38	2.0	8.7	0.44	0.53	0.84	1.83	0.000	0.38	2.1	9.2
V	0.07	2.3	10.1	0.07	0.16	0.46	4.08	0.038	0.08	2.3	10.3
NV	NA ¹⁴	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
All	0.38	2.0	8.7	0.44	0.53	0.84	1.83	0.000	0.39	2.1	9.2
CH ₄ intensity (g/kg of FPCM ¹⁵)											
MFA	0.75	1.7	11.7	0.75	0.76	0.99	1.14	0.052	0.70	2.0	13.7
V	0.27	2.4	16.3	0.29	0.39	0.73	2.28	0.043	0.29	2.4	16.8
NV	0.20	2.6	17.7	0.14	0.21	0.66	2.65	0.054	0.32	2.8	19.3
All	0.74	1.5	10.4	0.80	0.82	0.97	1.24	0.094	0.69	2.0	13.8

¹Root mean squared error of prediction expressed in the same unit as the observed mean.

²Root mean squared error of prediction expressed as a percentage of the observed mean.

³CCC = $r \times C_b$.

⁴Pearson correlation coefficient; a measure of precision.

⁵Bias correction factor; a measure of accuracy.

⁶Scale shift; change in SD between predicted and observed CH₄ emission.

⁷Location shift; if positive under prediction, if negative over prediction.

⁸Root mean squared error of cross-validation expressed in the same unit as the observed mean.

⁹Root mean squared error of cross-validation expressed as a percentage of the observed mean.

¹⁰Milk fatty acids alone as selection variables; in g/100 g of fatty acids.

¹¹Volatile metabolites alone as selection variables; peak area value (arbitrary unit of quantity).

¹²Nonvolatile metabolites alone as selection variables; peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate.

¹³All metabolites combined as selection variables.

¹⁴NA = not applicable, because no model was obtained.

¹⁵Fat- and protein-corrected milk (kg/d) = $[0.337 + 0.116 \times \text{fat (g/100 g of milk)} + 0.06 \times \text{protein (g/100 g of milk)}] \times \text{milk yield (kg/d)}$ (CVB, 2012).

alone and nonvolatile metabolites alone. For both CH₄ production and CH₄ yield, only MFA were selected in the prediction models, resulting in CH₄ prediction models identical to the MFA-based CH₄ prediction models. For CH₄ intensity, all 3 types of milk metabolites were selected in the prediction model: 5 MFA, 1 volatile metabolite, and 1 nonvolatile metabolite.

Cross-Validation. The results of the internal cross-validation of all CH₄ prediction models are also shown in Table 3. Additionally, Supplemental Table S11 (<https://doi.org/10.3168/jds.2017-13334>) shows the MFA, volatile metabolites, and nonvolatile metabolites that were included in the prediction models in the cross-validation, and whether or not these milk metabolites were also part of the best overall prediction models (Table 2). The R²CV and the RMSECV (%) of the MFA-based CH₄ prediction models ranged from 0.38 to 0.70 and from 9.2 to 13.7, respectively. Further, the R²CV and the RMSECV (%) of the volatile metabolite-based CH₄ prediction models ranged from 0.08 to 0.29 and from 10.3 to 16.8, respectively. The R²CV was 0.32 and 0.32, and the RMSECV (%) was 14.7 and 19.3 for the nonvolatile metabolite-based prediction models for CH₄ production and CH₄ intensity, respectively. For the CH₄ prediction models combining all 3 types of milk metabolites, the R²CV and the RMSECV (%) ranged from 0.39 to 0.69 and from 9.2 to 13.8, respectively.

DISCUSSION

Relationship Between Individual Milk Metabolites and CH₄ Emission

Milk Fatty Acids. Van Gastelen and Dijkstra (2016) reviewed studies that investigated the predictive power of MFA composition for CH₄ emission. In line with this review, in the present study several SMCFA were positively related to CH₄ emission. In general, these SMCFA remained related to CH₄ intensity, or the relationship appeared or strengthened, upon including FPCM as covariate. These positive relationships are the result of the de novo synthesis of these MFA in the mammary gland mainly from acetate and butyrate produced in the rumen (Bauman and Griinari, 2003), which are both positively related to CH₄ emission (Ellis et al., 2008). The main exception is C4:0. The observed negative relationship between C4:0 and CH₄ emission in the present study was also observed by Dijkstra et al. (2011) and van Lingen et al. (2014). The significant negative relationship between C4:0 and CH₄ intensity disappeared ($P > 0.10$) upon including FPCM as covariate, which may indicate a dilution effect with C4:0 increasing with decreasing FPCM.

The *iso* OBCFA were often positively related to CH₄ emission in the present study and remained positively related to CH₄ intensity upon including FPCM as covariate, which is in agreement with *iso* OBCFA being generally more abundant in fibrolytic bacteria (Vlaeminck et al., 2006). The *anteiso* OBCFA are generally more abundant in amylolytic bacteria and thus expected to be negatively relatively to CH₄ emission (Vlaeminck et al., 2006). This was observed for *anteiso* C17:0 and CH₄ production, but *anteiso* C15:0 was positively related to CH₄ intensity. The latter MFA remained positively related to CH₄ intensity upon including FPCM as covariate. A high level of ruminal propionate is related to low CH₄ production, and propionate is a substrate for de novo synthesis of C15:0 and C17:0. Hence, both C15:0 and C17:0 are hypothesized to be negatively related to CH₄ emissions (Vlaeminck and Fievez, 2005). In the present study, only C17:0 was negatively related to CH₄ production, whereas C15:0 was positively related to all units of CH₄ emission and C17:0 was positively related to CH₄ yield. The reason behind these positive relationships is not completely clear to us, but they are in agreement with other studies, such as Chilliard et al. (2009), Dijkstra et al. (2011), and van Lingen et al. (2014), although inconsistent with the findings of Rico et al. (2016). Additionally, it is unclear why C15:0 and C17:0 are differently related to CH₄ emission, despite their similar synthesis pathways.

The negative relationships found in the present study between C18:1, C18:2, and C18:3 isomers in milk and CH₄ emissions are in general agreement with others (van Lingen et al., 2014; Rico et al., 2016), and can be explained by dietary UFA and their biohydrogenation products (van Gastelen and Dijkstra, 2016). The relationship between CH₄ intensity and several C18:1 isomers as well as C18:2 *cis*-9, *trans*-11 strengthened or appeared after correcting for FPCM yield. This suggests that the relationship between these MFA and FPCM can hamper the direct relation between MFA and CH₄ intensity. The relationships found in the present study between CH₄ emissions and long-chain fatty acids, which derive from absorption from the digestive tract and body fat mobilization (Bauman and Griinari, 2003), have been reported before (i.e., Chilliard et al., 2009; Rico et al., 2016; van Gastelen et al., 2017). The individual relationships found in the present study between CH₄ emission and the long-chain fatty acids with more than 20 carbons were generally unaffected when including FPCM as a covariate. Additionally, these long-chain fatty acids were also included in the CH₄ prediction models. This together suggests that these MFA are important in terms of CH₄ prediction.

Volatile Metabolites. In contrast to Antunes-Fernandes et al. (2016), in the present study, many volatile

metabolites in milk were related to CH₄ emission and the relationships found between the volatile metabolites and CH₄ intensity were not only the result of the relationship between the volatile metabolites in milk and FPCM. The lack of relationships found by Antunes-Fernandes might be the result of the limited variation in dietary treatments used, which was the exchange of fiber-rich grass silage with starch-rich corn silage. As shown by Hettinga et al. (2008), the volatile composition of milk was affected by supplementing diets with specific byproducts (including onions and cabbage) but was not affected by variation in the starch to fiber content of the diet, even though the latter manipulation has an influence on ruminal fermentation and CH₄ emission (Hassanat et al., 2013; van Gastelen et al., 2015). The present study involved a wide variety of dietary treatments, including different qualities of forage, which are known to effect the volatile composition of milk (Thomson et al., 2005), ruminal fermentation, and CH₄ emission (Warner et al., 2016).

In the present study, acetone was negatively related to both CH₄ production and CH₄ intensity. Acetone is a ketone body that can be used to identify cows with negative energy balance and subclinical ketosis (Andersson and Emanuelson, 1985). Gravert et al. (1991) reported that the quality of grass or maize silage was negatively related to milk acetone, and it has been suggested that, in general, silage feeding or the content of butyric acid in silage may affect milk acetone (Andersson and Emanuelson, 1985; Andersson and Lundström, 1985). This suggests that milk acetone can be affected by the same dietary factors that can affect CH₄ emissions, explaining the negative relationship found in the present study.

Kalač (2011) reported that the occurrence of both acids and alcohols in silage result in the formation of various ethyl esters. Limited information is available on the transfer efficiency of both acids and esters from silage to milk, but the dietary treatments could have affected the relationships found in the present study. However, esters can also be formed within the mammary gland, from esterification of short-chain alcohols and FFA (Toso et al., 2002), and they can indicate bacterial action (Hettinga et al., 2009). In the present study, both volatile esters (ethyl acetate and ethyl butanoate) tended to be or were positively related to CH₄ intensity, but including FPCM as a covariate resulted in the disappearance or weakening of these relationships. This suggests that the positive relationships with CH₄ intensity were due to a relationship between FPCM and both volatile esters.

In the present study, 1-pentanol was negatively related to CH₄ production and CH₄ intensity. According to Moio et al. (1993), primary alcohols are formed by

reduction of their respective aldehyde. Consequently, 1-pentanol is formed by the reduction of pentanal, which was not identified in the present study. Based on Villeneuve et al. (2013), it seems likely that the content of 1-pentanol in milk reflects the content of its respective aldehyde, pentanal. Straight-chain aldehydes, such as pentanal and hexanal, can derive from lipid degradation (Moio et al., 1993). Dietary lipids are negatively associated with CH₄ emissions (Grainger and Beauchemin, 2011), potentially explaining the negative relationships found between CH₄ emission and both 1-pentanol and hexanal in the present study. Further, dimethyl sulfone was negatively related to CH₄ intensity. In the rumen, dimethyl sulfide is derived from the catabolism of sulfur AA, particularly methionine (Taylor and Kiene, 1989). Dimethyl sulfide is subsequently oxidized to dimethyl sulfone, which can be transferred to milk (Villeneuve et al., 2013). This suggests that the relationship between CH₄ intensity and dimethyl sulfone could be the result of the dietary protein content, although the effect of dietary protein content on CH₄ emissions is variable in the literature (Ellis et al., 2009; Reynolds et al., 2010).

We also identified 4 volatile FFA in the present study, all positively related to CH₄ yield and CH₄ intensity, but not with CH₄ production. The concentration of FFA in milk is generally low and can be the result of incomplete esterification in the mammary gland before lipid secretion (Marsili, 2003) or spontaneous lipolysis (Chazal et al., 1987). The latter study also reported higher FFA concentrations in milk from cows fully fed on good quality grass silage compared with cows fully fed on good quality hay. Additionally, Chazal and Chilliard (1986) observed that milk from cows fed poor quality grass silage had higher FFA levels than milk from cows supplemented with corn silage. Moreover, Thomson et al. (2005) reported that FFA concentrations in milk were highest in summer when the quality of the pasture declines. This together suggests that the positive relation between volatile FFA in milk and both CH₄ yield and CH₄ intensity might be the results of dietary composition and quality, which affect both the composition of volatile metabolites in milk and enteric CH₄ production.

Nonvolatile Metabolites. Similar to Antunes-Fernandes et al. (2016), nonvolatile metabolites were generally better correlated with CH₄ intensity than with CH₄ yield. Antunes-Fernandes et al. (2016) reported that the positive relationship between CH₄ intensity and the nonvolatile metabolites acetoacetate, creatinine, ethanol, formate, methylmalonate, and *N*-acetylsugar A were due to the relationship between these nonvolatile metabolites and FPCM. This was also observed for creatinine and ethanol in the present study, as well as for the metabolites betaine, citrate, *N*-acetylsugar B,

and sugar A. This suggests that these metabolites have no clear relationships with the ruminal CH₄ emission metabolism, but rather are related to changes in milk yield or metabolic processes involved in milk synthesis. In contrast to Antunes-Fernandes et al. (2016), the 3 nonvolatile metabolites acetoacetate, methylmalonate, and *N*-acetylsugar A remained related to CH₄ intensity upon including FPCM as a covariate. Acetoacetate is a ketone body and can be positively related to fiber-rich diets and subsequently ketogenic VFA (van Kneegsel et al., 2007), explaining the positive relationship found between acetoacetate and CH₄ intensity. The concentration of methylmalonate in milk has been associated with dietary composition (Bauman and Griinari, 2001). When high grain or low forage diets are fed, the ruminal production of vitamin B₁₂ decreases, whereas the production of propionate increases. This results in the accumulation of methylmalonate in the liver and subsequently, by transport via the circulatory system, in elevated methylmalonate supply to the mammary gland and increased concentration in milk (Bauman and Griinari, 2001). High grain or low forage diets are also associated with decreased CH₄ emissions, and hence a negative relationship between methylmalonate and CH₄ emission would be expected. In the present study, however, methylmalonate was positively related to CH₄ intensity. The latter may be explained by the absence of a negative relationship between ruminal propionate and CH₄ emissions in some of the studies of which the data were used for the present analysis (e.g., van Gastelen et al., 2015; Hatew et al., 2016).

N-Acetylsugars are intermediates of biological pathways that occur in cell cytosol (Lu et al., 2013). *N*-acetylsugars C, D, and E were significantly and positively related to CH₄ intensity only when including FPCM as a covariate. The results of the present study suggest that *N*-acetylsugars are related to the ruminal CH₄ production pathway. According to Lu et al. (2013), a higher concentration of *N*-acetylsugars could indicate leakage of cellular components to milk or higher permeability of the cell membrane in the epithelial cells in the mammary gland. Both can subsequently be associated with the differences in epithelial cell membrane stability. Tian et al. (2016) found lower concentrations of *N*-acetylsugars in milk of cows experiencing heat stress, and Antunes-Fernandes et al. (2016) found some *N*-acetylsugars tending to be negatively related to CH₄ yield. However, it is unclear how differences in dietary composition could have changed the epithelial cell membrane stability in the mammary gland and how this relates to CH₄ emissions, warranting more research.

Antunes-Fernandes et al. (2016) showed that citrate and UDP-hexose B were both negatively related to CH₄ intensity, potentially reflecting decreased meta-

bolic activity in the mammary gland with increased CH₄ intensity as citrate is an intermediate of the Krebs cycle (Bremer and Davis, 1974) and UDP-hexose B is an intermediate of lactose metabolism (Cant et al., 2002). In the present study, citrate was also negatively related to CH₄ intensity. However, this relationship disappeared upon inclusion of FPCM as a covariate. This suggests that changes in milk citrate may be related to the energy metabolism of the mammary gland, as previously suggested by Faulkner and Peaker (1982), which is not necessarily related to changes in CH₄ intensity. Furthermore, UDP-hexose B was not related to CH₄ intensity in the present study, which is in disagreement with Antunes-Fernandes et al. (2016). The other UDP-hexoses were negatively related to CH₄ production (i.e., UDP-hexose D), CH₄ yield (i.e., UDP-hexose C), and CH₄ intensity (i.e., UDP-hexose D upon including FPCM as a covariate). The negative relationship with CH₄ production is surprising, because UDP-hexoses are intermediates of lactose synthesis (Cant et al., 2002), and milk yield is controlled by the synthesis of lactose. Increased milk yield is often associated with increased feed intake and it is consistently reported in literature that increased feed intake is positively associated with CH₄ production (Hristov et al., 2013). Subsequently, a positive relation between UDP-hexoses and CH₄ production would be expected. The negative relationship between UDP-hexoses and CH₄ yield can probably be explained by increased feed intake and the related decreased ruminal retention time of starch, which may result in increased postruminal digestion and subsequently glucose absorption (Rius et al., 2010) and conversion to UDP-hexoses in the mammary gland.

Acetate was positively related to CH₄ emission, and remained related to CH₄ intensity upon including FPCM as a covariate. This is in agreement with ruminal acetate production being positively associated with CH₄ emission (Ellis et al., 2008). Furthermore, Krause and Oetzel (2005) demonstrated that ruminal succinate is usually only found at very low levels in the rumen, but that the concentration increased during SARA. In general, diets with a high starch content and a low fiber content are associated with decreased ruminal pH and CH₄ emission (Beauchemin et al., 2008). Hence, a negative relationship between ruminal succinate and CH₄ emission would be expected. However, we found a positive relationship between succinate in milk and CH₄ emission. This suggests that succinate in milk does probably not directly reflect ruminal succinate levels.

The decrease of acetylcarnitine in milk with increasing CH₄ production and CH₄ intensity (acetylcarnitine became negative related to CH₄ intensity upon including FPCM as a covariate) is in agreement with the results of the SMCFA in milk. It has been shown that

acetylcarnitine reflects the inhibition of de novo fatty acid synthesis from acetate in the mammary gland (Erflle et al., 1970). De novo fatty acid synthesis in the mammary gland is inhibited by specific *trans* UFA, which are formed during ruminal biohydrogenation of dietary UFA (Bauman and Griinari, 2003). Certain dietary strategies (including low-fiber diets and high-concentrate diets) alter the rumen environment, lowering the ruminal pH and subsequently increasing the *trans* UFA being formed from ruminal biohydrogenation. Both dietary UFA and lower ruminal pH are also associated with reduction in CH₄ emission, explaining the negative relationship between acetylcarnitine and CH₄ emission.

Hippurate was positively related to CH₄ production and CH₄ intensity in the present study. Boudonck et al. (2009) showed that the concentration of hippurate was lower in milk of cows receiving organic diets (mainly forage-based) than in milk of cows receiving conventional diets (mainly concentrate-based). This suggests that increased forage content in the diet, which is accompanied by increased dietary fiber, increases hippurate content in milk. This might explain the relationship found in the present study, because dietary forage content, as well as dietary fiber content, is positively associated with CH₄ emission (Beauchemin et al., 2008).

Overall, these results indicate that, next to MFA, both volatile and nonvolatile metabolites in milk are often related to CH₄ emissions. These relationships are most likely the result of changes in dietary composition that affect not only enteric CH₄ production, but also the profile of volatile and nonvolatile metabolites in milk. This illustrates that these milk metabolites might provide useful information increasing our understanding of CH₄ emission of dairy cows.

Prediction Models for CH₄ Emission

Milk Fatty Acids. The prediction potential of MFA varies between studies. The adjusted R² values for the MFA-based prediction models for both CH₄ production and CH₄ yield from the present study are lower than the ones reported by other studies (Chilliard et al., 2009; Dijkstra et al., 2011; Rico et al., 2016). In contrast, the adjusted R² values for the MFA-based prediction models for CH₄ intensity of the present study are higher than those reported in literature (i.e., van Lingen et al., 2014). The scale shift results of the present study indicate that MFA are able to describe more of the observed variation in CH₄ intensity than of the observed variation in CH₄ production and CH₄ yield. It is known that MFA are related to ruminal CH₄ production pathways (Ellis et al., 2008; Chilliard et al., 2009), but it appears that the MFA can predict CH₄

intensity better than CH₄ production and CH₄ yield. According to Dehareng et al. (2012), this might be due to CH₄ intensity taking milk yield into account, which is directly associated with enteric CH₄ production by cows and reflected by the MFA profile because of possible dilution effects. The results of the present study indeed show that some of the MFA are related to CH₄ intensity due to their relationship with FPCM (e.g., C4:0). However, this is not the case for all MFA that are important for the prediction of CH₄ intensity (such as *iso* C15:0).

Volatile Metabolites. The CH₄ prediction potential of volatile metabolites alone appears low and is considerably less promising compared with MFA. Although the prediction potential of the volatile metabolites for CH₄ production in this study is higher than van Gastelen et al. (2017) in which no model could be obtained for CH₄ production, the adjusted R² and CCC values of the volatile metabolite-based prediction models for CH₄ yield and CH₄ intensity are lower than the ones reported by van Gastelen et al. (2017).

Similar to van Gastelen et al. (2017), the potential of volatile metabolites to predict CH₄ intensity in the present study was greater than their potential to predict CH₄ production and CH₄ yield, which is evidenced by the higher adjusted R² and CCC values and lower scale shift. However, even for CH₄ intensity, the large scale shift (>2.28) shows that the variation in predicted CH₄ emissions was considerably smaller than that in observed CH₄ emission. This is illustrated in Figures 1, 2, and 3 and indicates the inability of volatile metabolites alone to predict the range of observed CH₄ emissions.

Based on 1 experiment with 4 dietary treatments, van Gastelen et al. (2017) concluded that volatile metabolites alone hold potential to predict CH₄ intensity, which might be the result of the relationship between volatile metabolites in milk and FPCM yield (Antunes-Fernandes et al., 2016). This is not supported by the results of the present study. First, the prediction potential of volatile metabolites for CH₄ intensity is low. Second, most volatile metabolites remained significantly related to CH₄ intensity upon including FPCM as a covariate. We therefore propose that, with a wide range of forage-based diets and a variety of quantity and quality of grass, grass silage, and corn silage, volatile metabolites in milk hold little potential to predict CH₄ emissions, despite the relationships found between individual volatile metabolites and CH₄ emissions.

Nonvolatile Metabolites. The adjusted R² and CCC values reported in this study for nonvolatile metabolite-based CH₄ prediction models were considerably lower than those reported by van Gastelen et al. (2017). Our results suggest that nonvolatile metabolites

in milk have low CH₄ prediction potential. For CH₄ yield no model could be derived at all, which was to be expected given the low number of relationships (i.e., significant and tendency) between individual nonvolatile metabolites and CH₄ yield. The variation in the predicted CH₄ emissions was considerably smaller than that in observed CH₄ emissions, as evidenced by the large scale shift (>2.26). This is visualized in Figures 1 and 3, and suggests that nonvolatile metabolites lack the ability to predict the range of CH₄ emissions observed.

Van Gastelen et al. (2017) concluded, based on 1 experiment with 4 dietary treatments, that nonvolatile metabolites hold potential to predict CH₄ intensity, which could largely be explained by the relationship between the nonvolatile metabolites in milk and FPCM yield as observed by Antunes-Fernandes et al. (2016). This is not supported by the results of the present study. We therefore propose that, with a wide range of forage-based diets with a variety of quantity and quality of grass, grass silage, and corn silage, nonvolatile metabolites in milk hold little potential to predict CH₄ emissions despite the significant relationships found between individual nonvolatile metabolites and CH₄ emissions.

All Metabolites Combined. No single proxy accurately predicts CH₄ emission, and combinations of 2 or more proxies are likely to be a better solution to predict CH₄ emission (reviewed by Negussie et al., 2017). In comparison with MFA alone, combining the 3 types of milk metabolites did not improve the potential to predict CH₄ production and CH₄ yield. The prediction models were actually identical to the ones obtained when selecting MFA alone. Also in the 10-fold cross-validation, volatile and nonvolatile metabolites were rarely included in the prediction models (Supplemental Table S11; <https://doi.org/10.3168/jds.2017-13334>). These results clearly show that combining MFA with both volatile and nonvolatile metabolites has no added value in terms of CH₄ production and CH₄ yield prediction potential relative to MFA alone.

Similarly, for CH₄ intensity, combining MFA with volatile and nonvolatile metabolites hardly improved prediction potential. Five MFA were included in the CH₄ intensity prediction model, of which 4 were identical to the MFA included in the MFA-based prediction model for CH₄ intensity. Despite the inclusion of one volatile metabolite (2-heptanone) and one nonvolatile metabolite (acetylcarnitine) in the combined model, the adjusted R² was marginally lower (0.74) than that of the MFA-based prediction model for CH₄ intensity (0.75). The CCC value of the prediction model for CH₄ intensity combining the 3 metabolites was slightly higher than the one reported for the MFA-based CH₄

intensity prediction model (0.80 and 0.75, respectively), which was mainly the result of increased precision (*r*).

In terms of the 10-fold cross-validation, MFA alone and the combination of the 3 types of milk metabolites performed equally well, having similar R²CV and RMSECV values. When considering the metabolites that were included in the prediction models of the 10-fold cross-validation (Supplemental Table S11; <https://doi.org/10.3168/jds.2017-13334>), it appears that the CH₄ prediction models combining the 3 types of milk metabolites are less robust than the MFA-based CH₄ prediction models, especially for CH₄ intensity. Variation in the metabolites that were included in the cross-validation was smaller for the MFA alone model (12 metabolites) than for the 3 types of metabolites combined (22 metabolites). Moreover, the MFA included in the best overall MFA-based prediction model for CH₄ intensity were included at least 2 times in the 10-fold cross-validation. Contrary, 2 of the metabolites in the best overall prediction model for CH₄ intensity combining the 3 types of milk metabolites were included only once (i.e., acetylcarnitine) or not at all (i.e., C24:0) in the cross-validation. The cross-validation results motivated us to develop a second best overall prediction model for CH₄ intensity, but without acetylcarnitine and C24:0. This resulted in a CH₄ intensity prediction model with an adjusted R² of 0.72, a RMSEP of 1.6 g of CH₄/kg of FPCM, a CCC of 0.78, and a scale shift of 1.23. This illustrates that the prediction potential for CH₄ intensity hardly differed when excluding acetylcarnitine and C24:0, which suggests that the prediction model for CH₄ intensity combining the 3 types of metabolites is not robust.

Overall, our results indicate that combining MFA with milk volatile metabolites and nonvolatile metabolites does not improve the CH₄ prediction potential relative to MFA alone. This is in agreement with van Gastelen et al. (2017) for CH₄ production and CH₄ yield. However, van Gastelen et al. (2017) reported a considerable improvement in the prediction potential when combining MFA with volatile and nonvolatile metabolites. The difference between the present study and van Gastelen et al. (2017) for CH₄ intensity might be explained by the differences in the data set used by both studies. Although both studies only involved forage-based diets, the present study involved more observations (123 vs. 29, respectively) and a larger variation in dietary treatments, CH₄ emissions, DMI, and FPCM.

Our analyses suggest that the relationship between CH₄ emission and both volatile and nonvolatile metabolites is largely driven by dietary composition and rumen fermentation. When investigating this relationship within a single experiment, with a small range of dietary treatments, these individual relationships en-

sure sufficient prediction potential, as evidenced by van Gastelen et al. (2017) for CH₄ intensity. However, as illustrated in the present study, upon combining data of multiple experiments that represent a wide range of dietary treatments, the diet driven individual relationships between CH₄ emission and both volatile and nonvolatile metabolites are still present, but the prediction potential decreases considerably. This suggests that the wider range of dietary treatments hampers the CH₄ prediction potential of volatile and nonvolatile metabolites. In contrast to volatile and nonvolatile metabolites, MFA are more directly related to the ruminal CH₄ pathways (rumen microbial origin) and retain their prediction potential despite a wider range of dietary treatments, suggesting that MFA profile represents a more robust indicator for CH₄ emission of dairy cows.

It is important to note though, that this study was based upon 6 experiments with forage-based diets only (forage varied between 70 and 85 g/100 g of diet DM). Hence, the diets represent only a relative narrow range of forage-to-concentrate ratios. Additionally, milk production of the cows did not exceed 35 kg/d, and all cows were restricted in their feed intake to ensure similar feed intake between treatments, thus avoiding confounding effects of DMI on CH₄ production. The relationships between CH₄ emission and the 3 types of milk metabolites might be different, when more experiments would be included, involving more individual observations, and with more variety in dietary composition, feed intake, and milk yield.

Potential limitations aside, based on the results of the present study, it can be concluded that MFA have the greatest potential to predict CH₄ emission of dairy cows compared with milk volatile and nonvolatile metabolites. Negussie et al. (2017) assessed several existing potential proxies for CH₄ emissions of dairy cows, including proxies related to (1) feed intake and feeding behavior, (2) rumen function, metabolites, and microbiome, (3) milk production and composition, (4) hindgut and feces, and (5) measurements at the level of the whole animal (e.g., BCS, BW, and lactation stage). The authors of that review indicated that the accuracy of MFA to estimate CH₄ emission was moderate to high, which is considerably higher than that of rumen-related variables, major milk components such as fat, lactose, and protein, and most variables of the whole animal, but similarly accurate as BW, digestibility, and milk yield (Negussie et al., 2017). Only feed intake (both alone and in combination with dietary composition) and milk infrared spectroscopy scored higher in terms of accuracy to estimate CH₄ emission (Negussie et al., 2017). Because it is still a major challenge to measure feed intake in practice and because the current number

of studies relating milk infrared spectroscopy with CH₄ emission is limited, MFA remain an interesting proxy for CH₄ emission of dairy cows.

CONCLUSIONS

Changes in concentrations of individual milk metabolites (i.e., MFA, volatile metabolites, and nonvolatile metabolites) can be related to the ruminal CH₄ production pathway. These relationships are largely diet-driven (i.e., diet composition, intake, and passage affect both ruminal CH₄ production and the milk metabolites). Some of the relationships between individual milk metabolites and CH₄ intensity, however, were partly dependent on milk production (FPCM). Furthermore, the CH₄ prediction potential of both volatile metabolites alone and nonvolatile metabolites alone is low, independent of the unit of CH₄ emission. The CH₄ prediction potential of MFA alone depended greatly on the unit in which CH₄ emission was expressed. The potential was lowest for CH₄ yield, intermediate for CH₄ production, and highest for CH₄ intensity. This study also demonstrates that, relative to MFA alone, CH₄ prediction potential does not increase when combining MFA with volatile and nonvolatile metabolites, in particular for CH₄ production and CH₄ yield. Volatile and nonvolatile metabolites in milk contain information that may increase our understanding of enteric CH₄ production of dairy cows, but it is not worthwhile to determine the volatile and nonvolatile metabolites in milk to estimate CH₄ emission of dairy cows. Milk fatty acids have moderate potential to predict CH₄ emission of dairy cattle fed forage-based diets, and the models can aid in the effort to understand and mitigate CH₄ emissions of dairy cows.

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