Milk metabolome relates enteric methane emission to milk synthesis and energy metabolism pathways

E. C. Antunes-Fernandes,*† S. van Gastelen,*‡ J. Dijkstra,‡ K. A. Hettinga,†¹ and J. Vervoort§

*Top Institute Food and Nutrition, PO Box 557, 6700 AN Wageningen, the Netherlands

†Food Quality and Design Group, Wageningen University, PO Box 17, 6700 AH Wageningen, the Netherlands

‡Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH Wageningen, the Netherlands

§Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA, Wageningen, the Netherlands

ABSTRACT

Methane (CH₄) emission of dairy cows contributes significantly to the carbon footprint of the dairy chain; therefore, a better understanding of CH₄ formation is urgently needed. The present study explored the milk metabolome by gas chromatography-mass spectrometry (milk volatile metabolites) and nuclear magnetic resonance (milk nonvolatile metabolites) to better understand the biological pathways involved in CH₄ emission in dairy cattle. Data were used from a randomized block design experiment with 32 multiparous Holstein-Friesian cows and 4 diets. All diets had a roughage:concentrate ratio of 80:20 (dry matter basis) and the roughage was grass silage (GS), corn silage (CS), or a mixture of both (67% GS, 33% CS; 33% GS, 67% CS). Methane emission was measured in climate respiration chambers and expressed as CH₄ yield (per unit of dry matter intake) and CH₄ intensity (per unit of fat- and protein-corrected milk; FPCM). No volatile or nonvolatile metabolite was positively related to CH₄ yield, and acetone (measured as a volatile and as a nonvolatile metabolite) was negatively related to CH₄ yield. The volatile metabolites 1-heptanol-decanol, 3-nonanone, ethanol, and tetrahydrofuran were positively related to CH₄ intensity. None of the volatile metabolites was negatively related to CH₄ intensity. The nonvolatile metabolites acetoacetate, creatinine, ethanol, formate, methylmalonate, and *N*-acetylsugar A were positively related to CH₄ intensity, and uridine diphosphate (UDP)-hexose B and citrate were negatively related to CH₄ intensity. Several volatile and nonvolatile metabolites that were correlated with CH₄ intensity also were correlated with FPCM and not significantly related to CH₄ intensity anymore when FPCM was included as covariate. This suggests that changes in these milk metabolites may be related to

changes in milk yield or metabolic processes involved in milk synthesis. The UDP-hexose B was correlated with FPCM, whereas citrate was not. Both metabolites were still related to CH₄ intensity when FPCM was included as covariate. The UDP-hexose B is an intermediate of lactose metabolism, and citrate is an important intermediate of Krebs cycle-related energy processes. Therefore, the negative correlation of UDP-hexose B and citrate with CH₄ intensity may reflect a decrease in metabolic activity in the mammary gland. Our results suggest that an integrative approach including milk yield and composition, and dietary and animal traits will help to explain the biological metabolism of dairy cows in relation to methane CH₄ emission.

Key words: dairy cow, milk metabolome, enteric methane emission, energy metabolism

INTRODUCTION

Enteric methane (CH₄) production in ruminants mainly occurs in the rumen and is a natural byproduct of microbial feed fermentation and degradation, an essential process to provide nutrients to the animal. An increase of DMI results in a higher CH₄ production because more substrate is available for rumen microbiota to degrade, but diet characteristics, including the type of carbohydrates and fat content, can also have a large effect on CH₄ production (Kirchgeßner et al., 1995). Due to the large contribution (approximately 52%) of CH₄ emission to the total greenhouse gas (GHG) emissions of the dairy sector (Gerber et al., 2013), mitigation strategies have been widely investigated (Hristov et al., 2013). Dietary changes to influence CH₄ emission are among the most direct CH₄ mitigation strategies (Knapp et al., 2014). Their importance increases because they are also candidates for implementation at dairy farms. According to Dijkstra et al. (2011), evaluating dietary mitigation strategies should be based on CH₄ production relative to feed intake because it avoids confounding effects of DMI on total CH₄ production (CH₄ produced per animal). However, uncertainties in measuring DMI at farm level makes an accurate rela-

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¹Corresponding author: kasper.hettinga@wur.nl

tion of CH₄ to DMI difficult in practice (Bannink et al., 2011). Others have related CH₄ mitigation strategies to their effect on the product (milk) of a dairy farm (Knapp et al., 2014).

To assess GHG emissions by the dairy chain, it is also possible to relate CH₄ production per unit of milk [usually expressed per unit of ECM or per unit of fat- and protein-corrected milk (**FPCM**)]. Higher production levels related to nutritional and nonnutritional management strategies may reduce CH₄ emissions per unit of milk (FAO, 2010). Emissions per unit of animal product reflect the accuracy of management practices on the composite of feed intake, GHG emission, and animal productivity (FAO, 2010). Therefore, evaluating CH₄ production in relation to feed intake and in relation to milk production are complementary.

Many studies have focused on the effect of CH₄ mitigation strategies on milk composition, but mainly on the macro constituents level (Mohammed et al., 2011; Hart et al., 2015). Less attention has been paid to individual metabolites of milk, with the exception of milk fatty acids (**MFA**; Odongo et al., 2007; Chilliard et al., 2009). This focus on MFA is because of the relation between MFA and ruminal activity with respect to microbial metabolism and type of VFA formed (Vlaeminck and Fievez, 2005). Changes in feeding can result in clear changes in MFA, which are partly related to how feed is degraded in the rumen (Halmemies-Beauchet-Filleau et al., 2014). Although MFA may predict CH₄ emission accurately within a limited range of dietary variation (e.g., variation in lipid source only; Chilliard et al., 2009), MFA cannot accurately predict the differences in CH₄ emission on a wider range of diets (van Lingen et al., 2014; Williams et al., 2014).

Milk volatile metabolite and nonvolatile metabolite profiles can be used to monitor animal health, feeding regimens, and metabolism in dairy cows. Based on different feeding regimens, indole and skatole present in the volatile fraction of milk were pointed out as indicative of the feeding regimen of dairy cows (Toso et al., 2002; Croissant et al., 2007). Further, Hettinga et al. (2008) used the milk volatile metabolite profile to detect and differentiate mastitis caused by different pathogens. Also, Klein et al. (2012) indicated the ratio of the nonvolatiles glycerophosphocholine and choline as possible predictor for developing ketosis in dairy cows and Lu et al. (2013), showed that phosphate sugars can be related to energy balance of the cow, due to a different organization of the epithelial membrane in relation to energy balance. These authors also showed that determining milk components using different techniques simultaneously can be useful for a more integrated understanding of the metabolism of cows (Klein et al., 2010; Lu et al., 2013).

Many fields of research analyze the same bio-matrix with different methods and integrate the resulting information to better monitor, predict, and interpret biological processes. Although milk volatile metabolite and nonvolatile metabolite profiles have been used to monitor digestion and metabolism in dairy cows, to the best of our knowledge these profiles have not been related to CH₄ emission. The present study explores the milk metabolome by GC-MS (GC-MS metabolomics; milk volatile metabolites) and proton nuclear magnetic resonance (¹**H-NMR** metabolomics; milk nonvolatile metabolites) to better understand the biological pathways involved in CH₄ emission.

MATERIALS AND METHODS

Experimental Design

Data from a completely randomized block design experiment were used with a total of 32 multiparous lactating Holstein-Friesian cows fed 4 diets that differed in grass silage (**GS**) and corn silage (**CS**) content. The experiment was fully described by van Gastelen et al. (2015). The experiment was conducted in 2012 in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands).

The 4 diets had a roughage:concentrate ratio of 80:20 based on DM content. The composition of the concentrate was similar for all diets, whereas the roughage consisted of 100% GS, 67% GS and 33% CS, 33% GS and 67% CS, and 100% CS (ingredient as percentage of the total amount of roughage in the diet, all DM basis). Feed intake was restricted (95% of ad libitum DMI) to avoid confounding effects of DMI on CH₄ production. After an adaptation period of 12 d, on d 13, cows were housed in climate respiration chambers (**CRC**) for a 5-d period. Cows were milked and fed twice daily. Production of CH₄ was determined in 10 min intervals during 3 full 24-h periods in the CRC. The details of the CRC used in this experiment are extensively described by van Gastelen et al. (2015).

Milk Yield and Composition

Milk yield was recorded during each milking, and a milk sample (10 mL) was collected for analyses of fat, protein, and lactose content by mid-infrared spectroscopy by Qlip (Zutphen, the Netherlands). In addition, a representative milk sample (5 g/kg of milk production) was obtained at each milking from each cow. The first milk sample was collected on d 13 in the afternoon and the last milk sample was collected on d 17 in the morning, whereas cows were housed in the

CRC. Sodium azide (0.05% wt/wt) was added to the pooled samples of the first 4 milkings, followed by the same procedure for milking 5 to 8 in a separate bottle. Both bottles were stored at 5°C. After the last milking, these 2 sub-samples (milkings 1 to 4 and 5 to 8) were pooled, and stored in 10-mL aliquots at -40°C for milk composition analyses.

Analytical Procedures

Volatile Metabolites. To determine the volatile metabolite profile, GC-MS metabolomics was performed based on the method described by Hettinga et al. (2008) and Settachaimongkon et al. (2014). Milk samples were thawed overnight in a refrigerator (7°C). A 5-mL milk sample was preheated in 10-mL vials sealed with silicon/Teflon septa and magnetic caps for 1 min at 60°C. Volatile metabolites were extracted from the headspace for 5 min with a 75- μ m PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA) using the Triplus autosampler (CTC Analytics Ag, Zwingen, Switzerland). The volatile metabolites were thermally desorbed from the fiber by heating it in a Best PTV injector (Thermo-Finnigan, San Jose, CA) with an empty liner for 5 min at 250°C. The fiber was subsequently cleaned for 10 min at 290°C. Vials without milk (only air) were used as blank samples.

Gas chromatography separation of volatile metabolites was performed on a Trace GC/MS (Thermo-Fisher Scientific, Waltham, MA). Volatiles were separated on a polar Stabilwax column of 30 m length, 0.32 mm, and 1- μ m film thickness (Restek, Breda, the Netherlands). Oven temperature was kept at 40°C for 3 min, after which it was increased to 220°C at 15°C/min, with 1 min holding at 220°C. Helium at a flow rate of 1.5 mL/min was used as a carrier gas. Mass spectrometry analysis was performed in electron impact mode (70 eV) in the range of 33 to 250 m/z , with 2 scans/s; the mass range of m/z 33 to 250 was used. The ion source was kept at 225°C.

The resulting chromatograms were analyzed using the AMDIS software (NIST, Gaithersburg, MD); data were deconvoluted to obtain pure mass spectra for improved peak identification. Identification of volatile metabolites was based on AMDIS software referred to NIST/EPA/NIH database (<http://www.nist.gov/srd/nist1a.cfm>) and matching mass spectra and retention time with an in-house library based on previous milk analyses (Hettinga et al., 2009). Fragment patterns were not specific enough to identify the chain length of 3 alkanes and these were labeled as alkane A, B, and C. Peak integration was subsequently performed using the XCalibur software package (Thermo-Scientific, Austin, TX). Peak area of the milk samples was corrected for

the peak area of the blank samples, resulting in a peak area in arbitrary units that was used for statistical analyses.

Nonvolatile Metabolites. To determine the non-volatile metabolite profile of the milk samples, ¹H-NMR metabolomics was performed. The procedure is described in detail by Lu et al. (2013). In short, milk samples were ultra-centrifuged to isolate milk serum. One-dimensional nuclear Overhauser enhancement spectroscopy (1-D-NOESY) spectra were obtained for all milk serum samples, using a nuclear magnetic resonance Bruker spectrometer Avance III with a 600 MHz/54 mm UltraShielded Plus magnet equipped with a CryoPlatform cryogenic cooling system, a BCU-05 cooling unit, an ATM automatic tuning and matching unit (Bruker, Rheinstetten, Germany). To assign milk serum nonoverlapping metabolite resonances, comparisons were made with published literature (Klein et al., 2010, 2012), the Human Metabolome Database version 2.0 online library (<http://www.hmdb.ca>), as well as internal standards. The peak area of each assignment is relative to the calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate, resulting in a relative peak area in arbitrary units that was used for statistical analyses.

Statistical Analysis

One cow was excluded from the experiment, because of large feed residuals while housed in the CRC, which resulted in much lower DMI compared with the last 4 d in the tie-stall and the other cows in that block (van Gastelen et al., 2015). In addition, the results of GC-MS metabolomics of another cow could not be used because these results were considered outliers (i.e., 9 from 25 volatile metabolites were not found in the milk of this cow, and acetone was 10 times more present in the milk of this cow compared with the milk of other cows). Last, the results of ¹H-NMR metabolomics of a third cow could not be used because the sample showed repeatedly a high background interference, which impaired peak integration. Therefore, relations between metabolites measured with both techniques were based on 29 samples, whereas all remaining data analyses of volatile and nonvolatile metabolites, CH₄ emission, and production traits were based on 30 samples.

Data on DMI, milk production, milk composition, and MFA are described by van Gastelen et al. (2015). The summary statistics of the volatile metabolites and nonvolatile metabolites are presented in Supplemental Tables S1 and S2, respectively (<http://dx.doi.org/10.3168/jds.2015-10248>). All statistical analyses were done using SPSS version 21 (SPSS Inc., Chicago, IL).

The relations between individual volatile metabolites or nonvolatile metabolites, and CH₄ intensity (g/kg of FPCM) or CH₄ yield (g/kg of DMI) were established by linear regression with CH₄ intensity and CH₄ yield as dependent variables and milk volatile or nonvolatile metabolites as independent variables. All coefficients were calculated over all diets. To evaluate the influence of FPCM on the established relations between individual volatile metabolites or nonvolatile metabolites and CH₄ intensity, FPCM was included as a covariate in the linear regressions. To evaluate the influence of diet on the established relations between individual volatile metabolites or nonvolatile metabolites, and CH₄ yield and intensity, dietary treatment was included as a covariate in the linear regressions. Pearson correlation coefficients between milk (non)volatile metabolites and milk and animal traits were determined, with a 2-tailed test for significance ($P < 0.05$). Multivariate analysis of the data was done by principal component analysis in R version 3.2.3. (R Core Team, 2013).

RESULTS AND DISCUSSION

Relation Between Individual Metabolites and CH₄ Intensity or Yield

Volatile Metabolites. In the present study, a total of 25 volatile metabolites were identified. These included ketones, aldehydes, alcohols, hydrocarbons, sulfur compounds, esters, and terpenes. The relations between each volatile metabolite and CH₄ intensity (g/kg of FPCM) and CH₄ yield (g/kg of DMI) are shown in Tables 1 and 2, respectively.

The volatile metabolites 1-heptanol-decanol, 3-nonanone, ethanol, and tetrahydrofuran were positively related ($P < 0.044$) to CH₄ intensity, and none of the volatile metabolites were negatively related to CH₄ intensity. None of the volatile metabolites were positively related to CH₄ yield, whereas acetone was negatively related ($P = 0.043$) to CH₄ yield.

The relations between each volatile metabolite and CH₄ intensity including FPCM as a covariate are shown in Table 1. Including FPCM as a covariate in the regression model resulted in no relationship between the volatile metabolites and CH₄ intensity (Table 1). This suggests that the previous relations were due to a relation between the volatile metabolites and FPCM.

Nonvolatile Metabolites. In the present study, 30 resonances could be assigned either to a compound or to a member of a class of compounds (Supplemental Table S3; <http://dx.doi.org/10.3168/jds.2015-10248>). The relations between each nonvolatile metabolite and CH₄ intensity or CH₄ yield are shown in Tables 3 and 4, respectively. The nonvolatile metabolites, acetoacetate,

creatinine, ethanol, formate, methylmalonate, and *N*-acetylsugar A were positively related ($P < 0.030$) to CH₄ intensity. Citrate and uridine diphosphate (UDP)-hexose B were negatively related ($P < 0.026$) to CH₄ intensity. None of the nonvolatile metabolites were positively related to CH₄ yield, whereas acetone was the only nonvolatile metabolite negatively related ($P = 0.046$) to CH₄ yield.

The relations between each nonvolatile metabolite and CH₄ intensity, including FPCM as a covariate, are shown in Table 3. When including FPCM as a covariate in the regression model, none of the nonvolatile metabolites were positively related to CH₄ intensity (Table 3). This suggests that the relation between CH₄ intensity and acetoacetate, creatinine, ethanol, formate, methylmalonate, and *N*-acetylsugar A were due to the relation between these nonvolatiles metabolites and FPCM. Citrate and UDP-hexose B remained negatively related ($P < 0.026$) to CH₄ intensity upon including FPCM as a covariate in the regression model. The significant relationship with CH₄ intensity in the presence of FPCM as a covariate suggests that the metabolic pathways in which these metabolites are involved relate to CH₄ production in dairy cows, independent of FPCM yield.

Our results suggest that relating milk metabolites to CH₄ intensity without including FPCM as a covariate may identify milk metabolites that are also or exclusively related to FPCM rather than CH₄ emission. This dependency seems to be true for both volatile and nonvolatile metabolites, and indicates that an increase in milk yield may reduce the concentration of some milk constituents. Bovenhuis et al. (2015) reported similar findings showing the genetic polymorphism in the *DGAT1* gene related to a higher milk yield and reduced milk protein content. However, changes in milk yield do not account for all the variation in milk composition. Hristov et al. (2015) reported that without changes in milk yield, milk of cows with a lower CH₄ production (g/d) due to feeding of 3-nitrooxypropanol had a higher content of de novo synthesized fatty acids. Due to the relation between rumen VFA and de novo synthesized fatty acids, the results of Hristov et al. (2015) suggest that milk composition also depends on blood-derived compounds and mammary gland metabolic activity (Bauman and Grinari, 2003; Jenkins and McGuire, 2006). Taken together, these 2 processes contribute to changes in milk composition, and in our data set it was difficult to distinguish between a dilution effect due to higher milk yield or a higher metabolic activity related to higher milk yield. Therefore, throughout the discussion we will consider them both.

Comparison of Both Techniques. Milk metabolites can be present in solution and be measured by ¹H-NMR. Upon heating, some milk metabolites can

Table 1. Linear regression between methane intensity (g/kg of fat- and protein-corrected milk; FPCM¹) and milk volatile metabolites (peak area²) and between methane intensity (g/kg of FPCM) and milk volatile metabolites, including FPCM as a covariate³

Volatile metabolite (peak area)	Linear regression between methane intensity and milk volatile metabolites ⁴				Linear regression between methane intensity and milk volatile metabolites, including FPCM as a covariate ⁵						
	Slope	SE	Slope <i>P</i>	R ²	Slope (volatile)	SE	Slope <i>P</i> (volatile)	Slope (FPCM)	SE	Slope <i>P</i> (FPCM)	R ²
1-Heptanol	-2.47×10^{-7}	1.000×10^{-6}	0.855	0.001	1.79×10^{-7}	1.07×10^{-6}	0.868	-0.366	0.089	<0.001	0.388
1-Heptanol-decanol	3.35×10^{-6}	2.000×10^{-6}	0.044	0.137	1.22×10^{-6}	1.48×10^{-6}	0.417	-0.334	0.096	-0.002	0.403
1-Pentanol	4.42×10^{-7}	3.755×10^{-7}	0.249	0.047	2.16×10^{-7}	3.09×10^{-7}	0.490	-0.355	0.089	<0.001	0.398
2-Butanone	4.49×10^{-8}	5.527×10^{-8}	0.424	0.023	3.02×10^{-8}	4.43×10^{-8}	0.501	-0.362	0.088	<0.001	0.398
2-Heptanone	1.60×10^{-7}	1.271×10^{-7}	0.218	0.054	2.51×10^{-8}	1.04×10^{-7}	0.384	-0.354	0.089	<0.001	0.405
3-Nonanone	2.47×10^{-6}	6.561×10^{-7}	0.001	0.336	1.28×10^{-6}	8.16×10^{-7}	0.129	-0.250	0.113	0.035	0.439
Acetone	4.55×10^{-10}	3.928×10^{-9}	0.909	0.001	2.03×10^{-9}	3.16×10^{-6}	0.527	-0.377	0.090	<0.001	0.397
Alkane A	9.76×10^{-7}	1.000×10^{-6}	0.493	0.017	1.12×10^{-6}	1.10×10^{-6}	0.321	-0.369	0.087	<0.001	0.410
Alkane B	2.11×10^{-6}	2.000×10^{-6}	0.309	0.037	1.25×10^{-7}	1.73×10^{-6}	0.943	-0.365	0.093	0.001	0.388
Alkane C	-1.95×10^{-7}	3.831×10^{-7}	0.614	0.009	5.38×10^{-8}	3.10×10^{-7}	0.986	-0.366	0.090	<0.001	0.388
Benzene-alkane	2.47×10^{-6}	2.000×10^{-6}	0.184	0.062	-5.4×10^{-7}	1.68×10^{-6}	0.751	-0.382	0.100	0.001	0.390
Benzene-compound	6.16×10^{-7}	6.454×10^{-7}	0.348	0.031	7.34×10^{-7}	5.04×10^{-7}	0.157	-0.373	0.086	<0.001	0.432
Butanoic acid	6.28×10^{-9}	8.263×10^{-9}	0.454	0.020	4.94×10^{-9}	6.59×10^{-9}	0.460	-0.363	0.088	<0.001	0.400
Cyclohexane	1.10×10^{-5}	7.000×10^{-6}	0.139	0.076	-7.53×10^{-7}	6.77×10^{-6}	0.912	-0.372	0.100	0.001	0.388
Dimethyl-sulfone	-3.26×10^{-7}	3.190×10^{-7}	0.316	0.036	-1.69×10^{-7}	2.60×10^{-7}	0.520	-0.358	0.089	<0.001	0.397
Ethanol	5.71×10^{-7}	2.166×10^{-7}	0.013	0.199	3.20×10^{-7}	1.98×10^{-7}	0.118	-0.312	0.091	0.002	0.442
Ethyl ester-acetic acid	1.95×10^{-7}	2.269×10^{-7}	0.398	0.026	6.75×10^{-8}	1.86×10^{-6}	0.719	-0.361	0.090	<0.001	0.391
Hexanal	-2.13×10^{-9}	2.763×10^{-8}	0.939	<0.001	1.21×10^{-8}	2.22×10^{-8}	0.589	-0.374	0.089	<0.001	0.394
Hexanoic acid	6.96×10^{-9}	5.109×10^{-9}	0.184	0.062	5.34×10^{-9}	4.10×10^{-9}	0.204	-0.356	0.086	<0.001	0.424
Hydrogen-azide	5.22×10^{-8}	2.18×10^{-8}	0.170	0.170	2.51×10^{-8}	2.01×10^{-8}	0.222	-0.321	0.094	0.002	0.421
Ketone A	1.36×10^{-6}	8.571×10^{-7}	0.124	0.082	9.42×10^{-7}	6.98×10^{-7}	0.187	-0.349	0.087	<0.001	0.427
Limonene	-6.09×10^{-7}	1.400×10^{-5}	0.966	<0.001	8.60×10^{-6}	1.13×10^{-5}	0.454	-0.380	0.089	<0.001	0.400
Octanoic acid	1.09×10^{-8}	6.681×10^{-9}	0.113	0.087	7.84×10^{-9}	5.42×10^{-9}	0.160	-0.349	0.086	<0.001	0.431
Tetrahydrofuran	3.22×10^{-8}	1.283×10^{-8}	0.018	0.184	9.14×10^{-9}	1.35×10^{-8}	0.503	-0.327	0.106	0.005	0.398

¹Fat- and protein-corrected milk (kg/d) = $[0.337 + 0.116 \times \text{fat (g/100 g of milk)} + 0.06 \times \text{protein (g/100 g of milk)}] \times \text{milk yield (kg/d)}$.

²Numbers are peak area values (arbitrary units).

³Milk volatile metabolites are ordered alphabetically, n = 30.

⁴Parameters were extracted from the equation: CH₄ intensity = a + b × volatile metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the volatile metabolite, and e is the error.

⁵Parameters were extracted from the equation: CH₄ intensity = a + b × volatile metabolite + c × FPCM + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the volatile metabolite, c is the slope of the regression line associated with FPCM, and e is the error.

be volatilized and measured by GC-MS. Ethanol and acetone were the only compounds detected both by ^1H -NMR and GC-MS. A positive correlation was found between the (relative) areas of ethanol ($P = 0.031$, $R^2 = 0.401$; $n = 29$) and acetone ($P < 0.001$, $R^2 = 0.684$; $n = 29$) measured by both methods. This is an important prerequisite for the combined analysis of milk metabolome.

In our data set, both volatile and nonvolatile metabolites are generally better correlated with CH_4 intensity than with CH_4 yield. Further, nonvolatile metabolites are also better related to CH_4 intensity than volatile metabolites, and the relationship of 2 nonvolatile metabolites (UDP-hexose B and citrate) remained significant after including FPCM as a covariate in the regression model. The weaker relation between volatile metabolites and CH_4 intensity might be due to the direct transfer of volatile compounds from diet to milk or the interaction between volatile metabolites and rumen metabolism (Urbach, 1990; Désage et al., 1996; Toso et al., 2002) that are not related to the ruminal pathways leading to CH_4 production.

Multivariate Analysis. Principal component analysis was conducted to identify general differences in volatile and nonvolatile metabolite profiles. The extraction of 2 components explained only 46.2% of the variation of volatile metabolites and 45.0% of the variation of nonvolatile metabolites (Supplemental Figure

S1A and S1B, respectively; <http://dx.doi.org/10.3168/jds.2015-10248>). Further, no clear correlation was found between the groups of variables and the factors.

Changes in CH_4 Intensity May Be Related to the 1-Carbon Metabolism and Energy Metabolism Pathways

Formate is positively related to CH_4 intensity; however, when FPCM is included in the regression model the relation is no longer significant. This may be explained by the negative correlation between formate and FPCM ($P = 0.024$, data not shown). The relation between formate and CH_4 intensity may therefore be explained by the milk synthesis processes, more specifically the 1-C metabolism in postabsorption pathways. One-carbon donors, including formate, are important in eukaryotic 1-C metabolism as they connect parallel mitochondrial and cytosolic pathways. Activated 1-C compounds, such as formate, are produced by the mitochondria, after which an enzymatic cascade in the cytoplasm will allow formate to be further incorporated in 1-C metabolism (Appling, 1991; Christensen and MacKenzie, 2006). The 1-C metabolism is a housekeeping process involving diverse mechanisms such as biosynthesis of lipids and proteins as well as methylation reactions (Christensen and MacKenzie, 2006). These processes are entwined with milk synthesis (Bian et al., 2015) as

Table 2. Linear regression between methane yield (g/kg of DMI) and milk volatile metabolites (peak area)¹

Volatile metabolite ² (peak area)	Intercept	SE	Slope	SE	Slope <i>P</i>	<i>R</i> ²
1-Heptanol	24.8	0.59	-1.72×10^{-6}	1.000×10^{-6}	0.184	0.062
1-Heptanol-decanol	23.4	1.33	9.52×10^{-7}	2.000×10^{-6}	0.569	0.012
1-Pentanol	23.8	0.98	1.18×10^{-7}	3.734×10^{-7}	0.755	0.004
2-Butanone	23.9	0.57	2.33×10^{-8}	5.418×10^{-8}	0.671	0.007
2-Heptanone	24.1	0.49	5.11×10^{-9}	1.270×10^{-7}	0.968	<0.001
3-Nonanone	24.3	1.43	-8.54×10^{-8}	7.828×10^{-7}	0.914	<0.001
Acetone	25.1	0.54	-7.53×10^{-9}	3.545×10^{-9}	0.043	0.139
Alkane A	23.9	0.93	3.54×10^{-7}	1.000×10^{-6}	0.799	0.002
Alkane B	24.4	1.32	-4.64×10^{-7}	2.000×10^{-6}	0.819	0.002
Alkane C	24.6	0.61	-3.53×10^{-7}	3.682×10^{-7}	0.346	0.032
Benzene-alkane	24.4	1.31	-4.02×10^{-7}	2.000×10^{-6}	0.827	0.002
Benzene-compound	23.9	0.43	5.53×10^{-7}	6.290×10^{-7}	0.387	0.027
Butanoic acid	24.0	0.47	3.61×10^{-9}	8.088×10^{-9}	0.659	0.007
Cyclohexane	24.7	1.16	-4.0×10^{-6}	7.000×10^{-6}	0.587	0.011
Dimethyl-sulfone	23.3	0.71	3.98×10^{-7}	3.067×10^{-7}	0.205	0.057
Ethanol	23.2	1.04	2.13×10^{-7}	2.318×10^{-7}	0.366	0.029
Ethyl ester-acetic acid	25.3	1.12	-2.44×10^{-7}	2.187×10^{-7}	0.273	0.043
Hexanal	24.6	0.50	-3.53×10^{-8}	2.602×10^{-8}	0.186	0.062
Hexanoic acid	23.9	0.42	4.08×10^{-9}	5.071×10^{-9}	0.428	0.023
Hydrogen-azide	23.4	1.18	1.41×10^{-8}	2.310×10^{-8}	0.548	0.013
Ketone A	23.9	0.48	6.62×10^{-7}	8.608×10^{-7}	0.449	0.021
Limonene	23.5	1.23	6.85×10^{-6}	1.400×10^{-5}	0.620	0.009
Octanoic acid	23.9	0.43	5.25×10^{-9}	6.727×10^{-9}	0.442	0.021
Tetrahydrofuran	23.8	0.70	6.98×10^{-9}	1.374×10^{-8}	0.615	0.009

¹Numbers are peak area values (arbitrary units). Milk volatile metabolites are ordered alphabetically, $n = 30$.

²Parameters were extracted from the equation: CH_4 yield = $a + b \times \text{volatile metabolite} + e$, where a is the intercept of the regression line, b is the slope of the regression line associated with the metabolite, and e is the error.

Table 3. Linear regression between methane intensity (g/kg of fat- and protein-corrected milk; FPCM¹) and milk nonvolatile metabolites (relative area²) and between methane intensity (g/kg of FPCM) and milk nonvolatile metabolites, including FPCM as a covariate¹

Nonvolatile metabolite (relative peak area)	Linear regression between methane intensity and milk nonvolatile metabolites ³				Linear regression between methane intensity and milk nonvolatile metabolites, including FPCM as a covariate ⁴						
	Slope	SE	Slope <i>P</i>	R ²	Slope (nonvolatile)	SE	Slope <i>P</i> (nonvolatile)	Slope (FPCM)	SE	Slope <i>P</i> (FPCM)	R ²
Acetate	36.09	40.550	0.381	0.028	12.34	28.51	0.666	-0.438	0.079	<0.001	0.547
Acetoacetate	193.99	72.200	0.012	0.205	75.30	59.55	0.217	-0.399	0.083	<0.001	0.569
Acetone	-33.41	23.999	0.175	0.065	-17.41	17.00	0.315	-0.429	0.078	<0.001	0.561
Acetylcarnitine	-6.00	26.234	0.847	0.001	-6.77	18.01	0.710	-0.443	0.078	<0.001	0.546
Betaine	-1.33	6.295	0.834	0.002	-2.90	4.31	0.506	-0.446	0.078	<0.001	0.551
Butyrate	3.59	2.049	0.091	0.099	1.27	1.53	0.416	-0.424	0.081	<0.001	0.555
BHB	42.80	35.945	0.244	0.048	14.59	25.72	0.575	-0.434	0.079	<0.001	0.549
Carnitine	-2.84	16.426	0.864	0.001	-14.38	11.14	0.208	-0.460	0.077	<0.001	0.570
Choline	2.25	1.397	0.119	0.085	0.48	1.06	0.656	-0.431	0.082	<0.001	0.547
Citrate	-2.04	0.866	0.026	0.166	-1.43	0.603	0.025	-0.412	0.072	<0.001	0.622
Creatine	-3.11	6.388	0.631	0.008	-2.88	2.10	0.516	-0.442	0.077	<0.001	0.551
Creatinine	130.24	45.098	0.007	0.229	58.60	36.98	0.125	-0.390	0.082	<0.001	0.583
Ethanol	328.82	88.707	0.001	0.329	167.66	115.62	0.159	-0.367	0.092	<0.001	0.577
Formate	225.08	98.756	0.030	0.156	63.71	80.17	0.434	-0.415	0.085	<0.001	0.554
Galactose-1-phosphate	-0.31	130.124	0.998	<0.001	42.23	89.45	0.641	-0.446	0.078	<0.001	0.547
Glycerophosphocholine	2.10	1.371	0.137	0.077	1.242	0.965	0.209	-0.427	0.077	<0.001	0.570
Hippurate	59.46	32.558	0.079	0.106	43.00	22.41	0.066	-0.425	0.074	<0.001	0.598
Lactate	6.73	13.128	0.612	0.009	5.83	9.01	0.523	-0.442	0.077	<0.001	0.551
Lactose	-0.72	0.387	0.074	0.11	-0.485	0.270	0.084	-0.422	0.075	<0.001	0.592
Malonate	-6.60	39.219	0.868	0.001	7.254	27.07	0.791	-0.445	0.078	<0.001	0.545
Methylmalonate	101.03	33.512	0.005	0.245	34.28	29.93	0.262	-0.392	0.088	<0.001	0.565
<i>N</i> -Acetylsugar A	43.12	16.054	0.012	0.205	5.31	14.94	0.724	-0.424	0.094	<0.001	0.546
<i>N</i> -Acetylsugar B	0.95	2.868	0.743	0.004	-3.75	2.00	0.072	-0.498	0.079	<0.001	0.596
<i>N</i> -Acetylsugar C	1.31	3.717	0.728	0.004	4.01	2.49	0.119	-0.464	0.076	<0.001	0.584
<i>N</i> -Acetylsugar D	2.36	10.318	0.821	0.002	11.23	6.93	0.117	-0.469	0.076	<0.001	0.584
<i>N</i> -Acetylsugar E	24.76	28.472	0.392	0.026	32.81	18.87	0.093	-0.452	0.074	<0.001	0.590
Orotate	3.89	25.592	0.880	0.001	-4.55	17.65	0.799	-0.455	0.078	<0.001	0.545
Oxaloacetate	86.94	94.549	0.366	0.029	53.89	65.46	0.418	-0.437	0.077	<0.001	0.555
Oxoglutarate	29.74	32.466	0.367	0.029	-9.77	23.69	0.683	-0.453	0.082	<0.001	0.547
Phosphocreatine	1.67	32.153	0.959	<0.001	28.48	21.92	0.205	-0.463	0.077	<0.001	0.571
Phosphorylcholine	-2.38	4.010	0.558	0.012	3.21	2.88	0.274	-0.473	0.081	<0.001	0.564
Proline	-37.24	19.839	0.071	0.112	3.24	16.53	0.846	-0.451	0.089	<0.001	0.544
Pyruvate	4.67	28.595	0.871	0.001	17.83	19.51	0.369	-0.451	0.077	<0.001	0.557
Succinate	55.40	37.836	0.154	0.071	-20.62	27.49	0.460	-0.429	0.079	<0.001	0.553
Sugar A (derivative phosphate)	4.69	55.663	0.933	<0.001	-5.65	38.32	0.884	-0.443	0.078	<0.001	0.544
Sugar B (derivative phosphate)	-13.96	31.110	0.657	0.007	33.85	22.05	0.136	-0.487	0.080	<0.001	0.580
Sugar C (derivative phosphate)	-12.93	22.771	0.575	0.011	19.06	16.31	0.253	-0.474	0.081	<0.001	0.566
Uridine diphosphate (UDP)-hexose A	-282.90	303.410	0.359	0.03	-12.78	217.50	0.954	-0.442	0.080	<0.001	0.544
UDP-hexose B	-347.39	88.662	0.001	0.354	-185.00	78.55	0.026	-0.353	0.081	<0.001	0.621
UDP-hexose C	144.61	76.051	0.068	0.114	87.48	54.05	0.116	-0.419	0.076	<0.001	0.584
UDP-hexose D	-169.83	210.770	0.427	0.023	-17.70	149.15	0.906	-0.441	0.079	<0.001	0.544

¹Milk nonvolatile metabolites are ordered alphabetically, $n = 30$. FPCM (kg/d) = $[0.337 + 0.116 \times \text{fat (g/100 g of milk)} + 0.06 \times \text{protein (g/100 g of milk)}] \times \text{milk yield (kg/d)}$.²Numbers are relative peak area values in relation to the peak area of internal standard (arbitrary units).³Parameters were extracted from the equation: $\text{CH}_4 \text{ intensity} = a + b \times \text{nonvolatile metabolite} + e$, where a is the intercept of the regression line, b is the slope of the regression line associated with the metabolite, and e is the error.⁴Parameters were extracted from the equation: $\text{CH}_4 \text{ intensity} = a + b \times \text{metabolite} + c \times \text{FPCM} + e$, where a is the intercept of the regression line, b is the slope of the regression line associated with the metabolite, c is the slope of the regression line associated with FPCM, and e is the error.

Table 4. Linear regression between methane yield (g/kg of DMI) and milk nonvolatile metabolites (relative area)^{1,2}

Nonvolatile metabolite ³ (relative peak area)	Intercept	SE	Slope	SE	Slope <i>P</i>	R ²
Acetate	25.6	1.75	−32.91	36.288	0.372	0.029
Acetoacetate	25.5	2.07	−50.55	71.864	0.488	0.017
Acetone	25.6	0.79	−43.20	20.664	0.046	0.135
Acetylcarnitine	25.1	1.99	−12.38	23.388	0.601	0.010
Betaine	27.0	1.70	−9.32	5.358	0.093	0.098
Butyrate	24.0	0.56	0.35	1.931	0.858	0.001
BHB	26.2	1.85	−37.62	32.213	0.253	0.046
Carnitine	29.2	2.67	−26.95	13.806	0.061	0.120
Choline	23.1	1.15	1.11	1.290	0.397	0.026
Citrate	25.9	2.28	−0.70	0.838	0.412	0.024
Creatine	25.7	2.28	−4.10	5.691	0.477	0.018
Creatinine	23.1	2.97	15.41	45.908	0.740	0.004
Ethanol	25.4	2.17	−59.55	96.318	0.541	0.013
Formate	25.9	1.77	−99.20	94.431	0.302	0.038
Galactose-1-phosphate	24.7	0.95	−83.17	115.441	0.477	0.018
Glycerophosphocholine	23.8	1.07	0.32	1.277	0.804	0.002
Hippurate	22.3	1.57	34.50	30.141	0.262	0.045
Lactate	25.1	0.99	−13.12	11.546	0.265	0.044
Lactose	30.2	7.66	−0.29	0.363	0.430	0.022
Malonate	25.8	1.81	−33.90	34.543	0.335	0.033
Methylmalonate	24.3	1.34	−6.30	34.512	0.856	0.001
N-Acetylsugar A	28.7	2.54	−27.76	15.241	0.079	0.106
N-Acetylsugar B	27.0	1.51	−4.75	2.411	0.059	0.122
N-Acetylsugar C	23.7	0.92	1.33	3.326	0.693	0.006
N-Acetylsugar D	23.9	0.99	2.00	9.239	0.830	0.002
N-Acetylsugar E	24.1	1.24	−0.79	25.834	0.976	<0.001
Orotate	23.8	1.29	5.15	22.903	0.824	0.002
Oxaloacetate	25.0	1.95	−41.34	85.567	0.633	0.008
Oxoglutarate	24.5	1.61	−8.39	29.458	0.778	0.003
Phosphocreatine	23.0	1.15	27.73	28.309	0.336	0.033
Phosphorylcholine	23.5	0.66	3.83	3.539	0.288	0.040
Proline	25.0	1.64	−10.39	18.745	0.584	0.011
Pyruvate	25.7	2.27	−18.05	25.387	0.483	0.018
Succinate	24.0	1.92	3.18	35.144	0.929	<0.001
Sugar A (derivative phosphate)	25.6	1.33	−56.85	48.672	0.253	0.046
Sugar B (derivative phosphate)	23.5	1.08	15.27	27.805	0.587	0.011
Sugar C (derivative phosphate)	23.6	0.67	17.01	20.251	0.408	0.025
Uridine diphosphate (UDP)-hexose A	24.5	0.88	−150.60	274.370	0.587	0.011
UDP-hexose B	25.2	1.31	−88.20	97.360	0.373	0.028
UDP-hexose C	25.3	2.21	−39.70	71.965	0.586	0.011
UDP-hexose D	24.4	1.15	−54.77	190.607	0.776	0.003

¹Milk nonvolatile metabolites are ordered alphabetically, n = 30.²Numbers are relative peak area values in relation to the peak area of internal standard (arbitrary units).³Parameters were extracted from the equation: CH₄ yield = a + b × metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the metabolite, and e is the error.

in healthy cows, milk metabolites may be secreted into milk via transcellular routes (McManaman and Neville, 2003). Therefore, a negative relation between formate and FPCM might reflect a change in postabsorption 1-C metabolism, possibly in the mammary gland.

Acetoacetate, together with volatile 3-nonane, are ketone bodies that are positively correlated with CH₄ intensity (Tables 3 and 1, respectively). The presence of ketones bodies in milk is often used to monitor changes in energy metabolism of dairy cows (Enjalbert et al., 2001). A higher amount of ketone bodies in blood plasma and subsequently in milk occurs when there is a surplus of acetyl-CoA for the tricarboxylic acid (TCA)

cycle (Enjalbert et al., 2001; Wellen and Thompson, 2012). In general, diets relatively rich in fiber that promote production of the ketogenic VFA, acetic acid, and butyric acid in the rumen may give rise to higher levels of ketone bodies in blood than glucogenic diets (diets relatively rich in rumen bypass starch delivering glucose in the small intestine or rich in rapidly fermentable carbohydrates that promote production of propionic acid in the rumen; van Knegsel et al., 2007). Diet composition is known to be related to CH₄ production in the rumen, with fiber generally resulting in higher CH₄ production per unit substrate degraded in comparison with starch (Ellis et al., 2008; Hristov et al., 2013).

Hence, a positive relationship between ketone bodies in milk and CH₄ intensity might be explained from the ketogenic or glucogenic nutrient supply to the rumen and its effect on rumen fermentation. However, higher concentrations of these ketone bodies in milk were not significantly related to CH₄ yield, indicating that such differences in ketogenic or lipogenic supply to the rumen and effects on rumen fermentation do not have a role in the observed relationships with CH₄ intensity. The correlation between acetoacetate, 3-nonanone, and CH₄ intensity disappears when FPCM is included as a covariate in the regression model. In fact, acetoacetate and 3-nonanone are negatively related ($P = 0.022$, $P < 0.001$, respectively, data not shown) to FPCM. This may indicate a dilution effect of certain milk metabolites due to a higher milk yield.

The TCA cycle in the mitochondria is of paramount significance to the metabolic efficiency of the cell and therefore the metabolism of the cow. In the mitochondria, ATP is produced from acetyl-CoA originating from glucose, fatty acids, lactate, pyruvate, and AA (Hardie and Carling, 1997). The production and utilization of ATP is therefore a flexible situation in which molecules are interconverted depending on the needs of the cell. In this situation, molecules with a high-energy phosphate, such as phosphocreatine, have been widely studied. Together with its precursor creatine, they form an important pool of energy in the cell, which can be used by the mitochondria. A consequence of this metabolism is the formation of creatinine, which is often monitored for energy status of tissues (Wyss and Kaddurah-Daouk, 2000). In our data set, a positive correlation is present between creatinine and CH₄ intensity ($P = 0.007$, Table 4) and a negative correlation between creatinine and milk yield expressed as FPCM ($P = 0.026$, data not shown). Further, the positive correlation between creatinine and CH₄ intensity disappears when FPCM is included as a covariate in the regression model. Therefore, in our data set, changes in creatinine concentration in milk seem related to changes in milk yield and therefore milk synthesis. This may indicate a change in the metabolic activity fueled by the mitochondria.

As discussed in this section, a negative relation was found between milk formate, acetoacetate, 3-nonanone, creatinine, and milk yield, expressed as FPCM. This supports the idea of a possible dilution effect on milk metabolites. Further, when FPCM is included as a covariate in the regression models, the above mentioned metabolites are no longer related to CH₄ intensity. An increase in CH₄ intensity resulting from a lower milk yield may therefore be associated with a lower metabolic rate, explaining the changes in milk metabolites related to milk synthesis and energy metabolism.

Changes in CH₄ Intensity Are Related to Lactose Synthesis

In our data set, CH₄ intensity is negatively related to UDP-hexose B ($P = 0.001$, Table 3). This negative relation may be due to the fact that UDP-sugars, including UDP-hexoses, are intermediates in lactose synthesis (Cant et al., 2002). For the production of lactose, glucose is transported from the bloodstream into the cytosol of epithelial cells, where part of it is converted into UDP-sugars. These are precursors of galactose that together with glucose will form lactose (Cant et al., 2002). This may explain the positive correlation between UDP-hexose B and lactose yield (g/d; Figure 1A). Milk yield is mainly controlled by the synthesis of lactose due to the contribution of this disaccharide to the osmolality of milk (Linzell and Peaker, 1971), and as discussed previously, an increase in milk yield (as FPCM) per animal reduces CH₄ intensity. Although in our data set UDP-hexose B is positively related ($P = 0.008$, data not shown) to FPCM, even when including FPCM in the regression model the UDP-hexose B is still significantly related to CH₄ intensity. This suggests that the lower amount of UDP-hexose B in milk might be related to the involvement of glucose in different metabolic pathways in the mammary gland. The large majority of glucose that is taken up by the epithelial cells is used in the biosynthesis of lactose (Cant et al., 2002). Glucose is also involved in NADPH production, which is paramount in the de novo MFA synthesis, and is required to synthesize glycerol, forming the backbone of triglycerides (Dijkstra et al., 1996). Thus, the negative relation between UDP-hexose B and CH₄ intensity may be explained by a decrease in metabolic rate in the mammary gland due to a lower milk yield.

In our data set, milk citrate is negatively related to CH₄ intensity ($P = 0.026$, Table 3) and is not related to FPCM ($P = 0.347$, data not shown). Therefore, when including FPCM in the regression model, citrate is still negatively related to CH₄ intensity ($P = 0.025$, Table 3). Milk citrate is regarded as a marker for the energy metabolism in the mammary gland because the mammary epithelium is impermeable to citrate (Linzell et al., 1976; Faulkner and Peaker, 1982). In our study, a decrease in milk citrate and associated increase in CH₄ intensity (Table 3) could indicate a disruption in the TCA cycle in the mammary gland because citrate is a regulatory compound of the acetyl-CoA metabolism in the mitochondria (Bremer and Davis, 1974). Further, by providing NADPH, the citrate metabolism and the TCA cycle may be indirectly involved in de novo synthesis of MFA in the mammary gland, inducing a negative correlation between citrate and de novo synthesized MFA (Faulkner and Peaker, 1982). In our

Figure 1. Scatter plots of (A) uridine diphosphate (UDP)-hexose B and lactose yield (g/d), and (B) CH_4 intensity (g/kg of fat- and protein-corrected milk; FPCM) and BW (kg). r indicates the Pearson correlation coefficient, $n = 30$.

data set, C12:0 and C14:0 were the only de novo synthesized MFA negatively related to milk citrate (data not shown). This may be due to the contribution of NADPH to the elongation step during the de novo MFA synthesis. In each elongation step, rumen-derived acetate and NADPH are needed and longer de novo MFA require more elongation steps and therefore more NADPH; C6:0 requires 2 elongation steps in contrast to the 5 elongation steps needed for C12:0 (Garnsworthy et al., 2006).

Together with NADPH, each elongation step in the de novo synthesis of MFA requires rumen-derived acetate, which mainly originates from fermentation of complex carbohydrates in the rumen. Decreased rates of MFA synthesis in the mammary gland induced by dietary changes have been shown to increase citrate levels of milk (Garnsworthy et al., 2006). Increasing the level of rapidly fermentable carbohydrates by increasing concentrate proportion of the diet, or increasing dietary lipid content, are generally associated with reduced de novo MFA synthesis, and such diets in general are also

associated with reduced CH_4 production (Ellis et al., 2008). This may further explain the observed negative relationship between citrate and CH_4 intensity in the present study.

Milk Acetone Relates to CH_4 Yield

Expressing CH_4 emission relative to feed intake avoids confounding effects of DMI on total CH_4 production (Dijkstra et al. (2011)). In our data set, acetone measured both by ^1H -NMR and GC-MS was the only metabolite negatively related to CH_4 yield. Acetone is a ketone body that may be positively or negatively related to milk production. In general, milk acetone levels of energy deficient cows increase rapidly and may indicate subclinical ketosis (Miettinen, 1994), and subclinical ketosis may negatively affect milk production. Milk acetone is also influenced by parity and lactation stage, and a rise in milk production was accompanied by a rise in milk acetone levels (Heuer et al., 2001). In our study, CH_4 yield and acetone were not related to milk production (expressed as FPCM; $P < 0.675$, data not shown), and therefore the mechanisms explaining the relation between CH_4 yield and acetone are not totally clear. In general, levels of ketone bodies including acetone are not just related to energy balance of the cow, but also to glucose levels (Andersson, 1988). As discussed in a previous section, glucogenic diets are generally associated with reduced CH_4 production. Therefore, we assessed the effect of diet on the established relations between individual volatile metabolites or nonvolatile metabolites, and CH_4 emission, by including dietary treatment as a covariate in the linear regressions. The majority of relations were not significantly changed (data not shown). Only hydrogen-azide became significantly related to CH_4 intensity ($P = 0.043$) and sugar A (derivative phosphate) became significantly related to CH_4 yield, ($P = 0.031$). More specifically, including treatment as a covariate in the regression model did not significantly change the relation between acetone (nonvolatile) and CH_4 yield ($P = 0.044$), but acetone (volatile) was no longer significantly related to CH_4 yield ($P = 0.229$). Our results suggest that although acetone may help to understand changes in the physiology of the dairy cow associated with CH_4 yield, it may also be influenced by the diet.

Body Weight Relates to CH_4 Intensity, But Not to CH_4 Yield

In the present study, BW of cows was positively related to CH_4 intensity ($P = 0.012$, $r = 0.444$; Figure 1B), but was not related to CH_4 yield ($P = 0.671$, $r = 0.079$). The importance of BW in relation to CH_4

production has been previously acknowledge by other authors who have included BW in regression equations to predict CH₄ production (Kirchgebner et al., 1995; Moraes et al., 2014). In a meta-analysis using a Bayesian model selection procedure, Moraes et al. (2014) identified BW as a key explanatory variable in predicting CH₄ emissions, in addition to the key dietary variables energy intake, dietary fiber, and lipid proportions. The association between BW and CH₄ emission might be explained by the relation between BW and rumen capacity (Demment and Van Soest, 1985). A higher BW is proportional to a larger rumen capacity. When feed intake is kept constant, a higher rumen capacity results in a lower passage rate (Demment and Van Soest, 1985), resulting in a higher CH₄ production (Moraes et al., 2014).

Because feed intake, either as DMI or gross energy intake, are confounding factors of BW and enteric CH₄ production (Ellis et al., 2008), cows in the present experiment had a restricted feed intake, and no correlation between BW and CH₄ yield ($P = 0.671$, $r = 0.079$) or BW and FPCM ($P = 0.232$, $r = -0.221$) was observed. A higher BW requires more feed to be used for maintenance purposes, thus having less feed available for milk production, which is expected to increase CH₄ intensity. However, the absence of relation between BW and FPCM supports the idea that rumen size and passage rate may explain the positive relation between BW and CH₄ intensity.

CONCLUSIONS

The results of the present study suggest that milk is a suitable matrix to better understand the biological pathways involved in CH₄ emission. In general, milk nonvolatile metabolites have a more pronounced relationship with CH₄ emission compared with milk volatile metabolites, especially when referring to CH₄ intensity (g/kg of FPCM) rather than CH₄ yield (g/kg of DMI). However, relationships between several metabolites and CH₄ intensity are partly dependent on milk production (as FPCM). The relations between milk UDP-hexose B and citrate with CH₄ intensity (g/kg of FPCM) remained significant when FPCM was included as a covariate in the regression models. The UDP-hexose B is an intermediate metabolite of lactose metabolism, whereas citrate is an important intermediate of Krebs cycle-related energy metabolic processes. This suggests that CH₄ intensity may be related to lactose synthesis and energy metabolism in the mammary gland. The negative relation between milk acetone and CH₄ yield may be related to glucogenic nutrient supply, and implies that acetone is important to monitor CH₄ emission related to feed intake. We observed a positive

relationship between BW and CH₄ intensity, which may be related to differences in rumen capacity and rumen passage rate. Our results suggest that an integrative approach including milk production and composition, dietary and animal traits will help to explain the biological metabolism of dairy cows in relation to CH₄ emission.

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