Between-cow variation in milk fatty acids associated with methane production

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Abstract

We evaluated the between-cow (b-cow) variation and repeatability in omasal and milk fatty acids (FA) related to methane (CH4) emission. The dataset was originated from 9 studies with rumen-cannulated dairy cows conducted using either a switch-back or a Latin square design. Production of CH4 per mole of VFA (Y_CH4VFA) was calculated based on VFA stoichiometry. Experiment, diet within experiment, period within experiment, and cow within experiment were considered as random factors. Empirical models were developed between the variables of interest by univariate and bivariate mixed model regression analysis. The variation associated with diet was higher than the b-cow variation with low repeatability (<0.25) for milk odd- and branch-chain FA (OBCFA). Similarly, for de novo synthesized milk FA, diet variation was ~3-fold greater than the b-cow variation; repeatability for these FA was moderate to high (0.34–0.58). Also, for both cis-9 C18:1 and cis-9 cis-12 cis-15 C18:3 diet variation was more than double the b-cow variation, but repeatability was moderate. Among the de novo milk FA, C4:0 was positively related with stoichiometric Y_CH4VFA, while for OBCFA, anteiso C15:0 and C15:0 were negatively related with it. Notably, when analyzing the relationship between omasal FA and milk FA we observed positive intercept estimates for all the OBCFA, which may indicate endogenous post-ruminal synthesis of these FA, most likely in the mammary gland. For milk iso C13:0, iso C15:0, anteiso C15:0, and C15:0 were positively influenced by omasal proportion of their respective FA and by energy balance. In contrast, the concentration of milk C17:0, iso C18:0, C18:0, cis-11 C18:1, and cis-9 cis-12 cis-15 C18:3 were positively influenced by omasal proportion of their respective FA but negatively related to calculated energy balance. Our findings demonstrate that for most milk FA examined, a larger variation is attributed to diet than b-cow differences with low to moderate repeatability. While some milk FA were positively or negatively related with Y_CH4VFA, there was a pronounced effect of calculated energy balance on these estimates. Additionally, even though OBCFA have been indicated as markers of rumen function, our results suggest that endogenous synthesis of these FA may occur, which therefore, may limit the utilization of milk FA as a proxy for CH4 predictions for cows fed the same diet.
Introduction

Enteric methane (CH$_4$) production is one of the main sources of green-house gas (GHG) emissions from dairy production systems, and enteric CH$_4$ production is among the main targets of GHG mitigation practices for the dairy industry [1]. Therefore, mitigating enteric CH$_4$ emissions is an approach for improving sustainability and profitability of dairy production systems [2]. Direct measurements of CH$_4$ are difficult to perform under regular farm conditions; therefore, the development of prediction equations to estimate CH$_4$ output has gained significance [3–5]. Changes in absorbed fatty acid (FA) composition affected by ruminal metabolism and microbial synthesis of FA can affect milk FA composition [6], and may therefore predict changes in the ruminal fermentation associated with CH$_4$ emissions. It is well established that de novo synthesis in the mammary gland yields short and medium-chain FA (4 to 14 carbons) and a portion of the 16-carbon FA derived from acetate and to a lesser extent BHBA. The remaining 16-carbon and all of the longer-chain FA (greater than 16 carbons) are taken up from the circulating plasma pool originated from absorption from the digestive tract or mobilization from body reserves. Additionally, odd- and branched-chain FA (OBCFA) in milk fat are largely derived from bacteria leaving the rumen [7] and have been suggested as potential biomarkers for rumen function [6]. The potential utilization of milk FA to predict CH$_4$ has been studied from direct in vivo measurements [8, 9] and from meta-analysis approaches [10, 11]. These models have selected several different FA as potential CH$_4$ predictors, which indicates an important influence of other dietary and animal factors influencing these estimates.

Variation in CH$_4$ production has been also attributed to animal factors [3, 4]. Studies conducted in sheep have shown that the variation in ruminal digesta retention time or passage rate is related to CH$_4$ emissions, with high CH$_4$ emitters having a larger rumen volume and digesta pools than low CH$_4$ emitters [12]. Recently, Cabezas-Garcia et al. [13] reported that variables related to animal physiology, such as variation in digesta retention time, can explain most of the between-animal variations in CH$_4$ production. Only small variations were observed in rumen fermentation variables, especially stoichiometric Y$_{CH_4}$VFA, suggesting a minor contribution of the rumen microbiome to CH$_4$ production. Since some studies have indicated that potentially several individual milk FA can be used to predict CH$_4$ emission in lactating dairy cows, the examination of between-animal differences in a data set originating from variations in digestion physiology and different diets is important. Also, because animal variation is likely to be under genetic control, one option to mitigate CH$_4$ emissions that has been suggested is to select for animals that emit less. Heritability of some major milk FA have been previously determined [14, 15]. However, although a large range in the heritability of specific milk FA were reported [14, 15], they did not report heritability and variation in milk FA directly related with rumen function (i.e. OBCFA and trans-FA). Since potentially several individual milk FA can be used to predict CH$_4$ emission in lactating dairy cows, the examination of between-animal differences in a data set originating from variations in digestion physiology and different diets is important. Additionally, integration of data related to rumen function with nutrient outflow and milk output may allow for a better understanding of the variables involved in the observed between-animal and between-diets variation. The objective of our meta-analysis was to evaluate b-cow variation and repeatability in omasal fatty acids and milk fatty acids associated with CH$_4$ emission.

Materials and methods

Data

The dataset was originated from 9 studies [16–25], 29 cows and 33 different diets of rumen cannulated Nordic red dairy cows, conducted using either a Latin square or switch-back
design conducted in the Finland (S1 Table). These studies evaluated a wide range of different dietary condition including different forages strategies, forage conservation method, forage:concentrate levels, and supplementation with different fatty acids. The mean forage-to-concentrate ratio of the diets was 57:43 on a DM basis. The concentrate supplements consisted principally of cereal grains, fibrous by-products from the food industry, and protein supplements, typically canola meal. In some studies diets were supplemented with sunflower oil, rapeseed oil, linseed oil or fish oil. Formic acid-treated grass silage was the main forage source, but red clover silage, extensively fermented grass silage (no additives), fresh chopped grass, and barn-dried hay were used in some studies. The diets were fed ad libitum or at 90 to 95% of ad libitum intake as TMR or fixed amounts of concentrate with forage ad libitum. The complete data set consisted of 135 cow/period observations, which were the experimental unit. A minimum pre-condition for inclusion of a study in the meta-analysis was that feed intake, BW, milk production data, fermentation parameters, omasal FA, and milk FA profile were available.

Individual cow intakes and milk yield were recorded daily throughout the experiment, but only measurements for the last 4–7 d were used for analysis. Samples of milk were collected from each cow over 4 consecutive milkings. Milk samples treated with preservative (bronopol; Valio Ltd., Helsinki, Finland) and were stored at 4°C until analyzed for milk components (MilkoScan 133B analyzer; Foss Electric A/S, Hillerod, Denmark). Unpreserved milk samples were also collected at the same time, stored immediately at −20°C, and composited according to milk yield until analyzed for FA composition. Body weight was measured weekly.

Diet digestibility was determined by total feces collection over 4 to 5 days. Digesta flow measurements were conducted using the omasal sampling technique [26] with a triple-marker system [27] based on Co-EDTA or Cr-EDTA, Yb-acetate, and Cr-mordanted straw or indigestible NDF as markers for liquid, small, and large particles, respectively. Rumen fluid samples (n = 7 or 8) were collected at 1.5 intervals (approximately 500 mL) starting just before morning feeding at 0600 h through rumen cannula using a vacuum pump and flexible tube and analyzed for pH, VFA and ammonia N concentrations [28]. Spot samples (500 mL) of digesta entering the omasal canal were collected 3 times daily at 4-h intervals during 4 consecutive days, to cover a 12-h period that was considered representative of the entire feeding cycle composited, and separated into large particle, small particle, and liquid phases. Each phase was freeze-dried and stored at −20°C, whereas subsamples of each fraction collected for FA analysis were stored at −20°C. Metabolizable energy content of experimental diets was calculated from the concentration of digestible nutrients [0.016 × digestible OM in DM (g/kg); Ministry of Agriculture, Fisheries and Food, 1975] determined by total fecal collection. The energy requirement (MJ/d) for maintenance and milk production was calculated as \[BW (kg^{0.75}) \times 0.515 + ECM yield (kg/d) \times 5.15\]; [29]. Energy balance was calculated as ME intake–ME maintenance–ME production, all expressed as MJ/d.

The VFA ratios acetate/propionate and propionate/butyrate were calculated, and the lipogenic:glucogenic ratio of VFA was determined as (acetate + butyrate)/propionate. Production of CH₄ per mole of VFA (Y,CH₄,VFA) was calculated based on VFA stoichiometry [30] as:

\[Y,CH₄,VFA (mmol/mol of VFA) = 0.5 \times C₂ - 0.25 \times C₃ + 0.5 \times C₄\]

where \(C₂\), \(C₃\), and \(C₄\) are molar proportions (mmol/mol) of acetate, propionate, and butyrate, respectively, in the sum of these VFA.

Total lipid in milk, oil supplements, freeze-dried feed samples and omasal digesta were converted to FA methyl esters (FAME) using standard methods [31, 32]. The FAME were quantified.
using a gas chromatograph equipped with a flame-ionization detector and a CP-Sil 88 column (100 m x 0.25 mm id., 0.2 μm film thickness; Agilent Technologies, Santa Clara, CA).

**Statistical analysis**

All analysis were performed using the MIXED procedure of SAS (SAS version 9.3, SAS Institute Inc., Cary, NC). Variance components of the selected variables was calculated considering random factors of experiment (Exp), diet within experiment [Diet (Exp)], period within experiment [Period (Exp)], and cow within experiment [Cow (Exp)]. Covariance structure was defined in the model using the TYPE = VC (variance components) option in the RANDOM statement. The standard deviation and coefficient of variation for each factor were calculated as the square root of the variance estimate and standard deviation divided by the respective mean value of each factor.

Repeatability values (Rep) for the most relevant variables associated with enteric CH₄ production were calculated as

\[
\text{Rep} = \frac{\sigma^2_{\text{Cow}}}{\sigma^2_{\text{Cow}} + \sigma^2_{\text{Residual}}},
\]

where \(\sigma^2_{\text{Cow}}\) and \(\sigma^2_{\text{Residual}}\) are Cow (Exp) and residual variances, respectively. Repeatability values provide an estimate of the correlation between values from consecutive samples on the same cow, on the same diet, and within the same period of the same experiment. For this study, repeatability was classified as low (<0.25), moderate (0.26–0.50) and high (>0.50).

Empirical models were developed between the variables of interest regarding their biological value by regression analysis within the MIXED procedure of SAS, using the following model:

\[
Y_{ij} = B_0 + B_1 X_{1ij} + b_i + b_1 X_{1ij} + e_{ij},
\]

where \(Y_{ij}\) = the expected value for the dependent variable Y observed at level of j of the independent variable X in study i; \(B_0\) = the overall intercept (fixed effect); \(b_i\) = the random effect of study i on the intercept (i = 1, . . . , 9); \(B_1\) = the regression coefficient of Y on X₁ across all studies (fixed effects), \(X_{1ij}\) = value j of the continuous variable X₁ in study i; \(b_{1i}\) = the random effect of study i on the regression coefficient of Y on X₁ in study i (i = 1, . . . , 9), and \(e_{ij}\) = the residual error.

The models included 2 random statements: a random intercept and slope of X₁ with SUBJECT = Diet (Exp), and a random intercept with SUBJECT = Period (Exp), using the TYPE = VC as the covariance structure for both random statements. The method = ML (maximum likelihood) statement was used in the PROC MIXED model syntax. Only one random independent variable was used to avoid overparameterized models and improve convergence [33].

**Results**

**Data description**

Mean and ranges of nutrient intake, production responses and rumen fermentation parameters are presented in Table 1. Despite the large differences in diet composition, rumen pH and the proportions of major rumen VFA did not vary greatly compared with the proportions of minor VFA. The large variation in intake of total FA is related to several studies in this data set supplemented different sources of FA.

Mean and ranges of proportion of omasal FA are presented in Table 2. As expected, the predominant FA in the omasum was C18:0 with a wide range of it as a proportion of omasal FA.
Also, C16:0, trans-11 C18:1, cis-9 C18:1, and cis-9, cis-12 C18:2 represented the main FA present in the omasum. Variation in the proportion of OBCFA was a similar across all of these FA.

Mean and ranges of milk FA profiles are presented in Table 3. As expected the major FA in milk fat were C16:0, cis-9 C18:1, and C18:0. Although preformed FA were the major FA in milk fat, large variation in the summation of de novo FA, mixed FA and preformed milk FA was also observed. There was a similar variation among the milk OBCFA with iso C17:0 showing the greatest variation. Also, the large variation in milk trans-10 C18:1 and trans-11 C18:1 is related to several studies in this data set which had diets that induced milk fat depression.

### Variance components

In general, the effect of experiment (Exp) was the largest source of variation observed in the data set (not shown). The variance components for rumen fermentation patterns are shown in Table 1.
Table 4. For the molar proportions of acetate and propionate, we observed that the variation related to diet was more than double the b-cow variation, with moderate repeatability, whereas for butyrate and isobutyrate the diet and b-cow variance components were similar. Diet and b-cow variance components were similar for both rumen pH and total VFA, and they were more repeatable than molar proportions of individual VFA. The b-cow variation for Y_CH was only 0.010, and repeatability was low.

The variance components for the proportion of omasal FA are presented in Table 5. For the OBCFA including iso C13:0, anteiso C13:0, iso C15:0, iso C16:0, iso C17:0, anteiso C17:0, C17:0 and iso C18:0 the variation associated with diet was greater than the between-cow variation with low repeatability. For anteiso C15:0 and C15:0 the variation associated with diet was also greater than the between-cow variation with moderate repeatability. Although the variation associated with diet for C16:0 was more than double the between-cow variation, this FA had the highest repeatability. C18:0, cis-9 C18:1 and cis-11 C18:1 had low repeatability, and the variation associated with diet was greater than the between-cow variation. Similarly, for trans-10 C18:1, trans-11 C18:1, cis-9, cis-12 C18:2 and cis-9 cis-12 cis-15 C18:3 diet variation was greater than between-cow variation with low repeatability, whereas cis-9 trans-11 C18:2 had diet and between-cow variance components similar and high repeatability.

The variance components for milk FA are presented in Table 6. Milk FA can be classified in three groups because they are derived from 2 sources: de novo synthesis in the mammary gland (≤ 16 carbon FA) and originating from extraction from plasma (> 16 carbon FA). Mixed source FA (16-carbon milk FA) can be originate from both pools. Interestingly, for the summation of milk FA by source (de novo, mixed and preformed), the diet variation was greater than the b-cow variation, but these groups of FA had moderate to high repeatability. For de novo milk FA C4:0, C6:0 and C8:0 the diet variation was approximately 3-fold greater than the b-cow variation; repeatability for these FA was moderate to high. For OBCFA...
including iso C13:0, anteiso C13:0, iso C16:0, iso C17:0, the diet variation was 2 to 3-fold the b-cow variation with low repeatability. Although diet variation was greater than b-cow variation for iso C15:0, C15:0, anteiso C15:0, anteiso C17:0, C17:0, and iso C18:0, these FA had moderate repeatability. Similarly, diet variation for C16:0 was more than double the b-cow variation, though this FA had the highest repeatability. C18:0, and cis-11 C18:1 had moderate repeatability, and the variation related to diet was greater than the b-cow variation. For both preformed milk FA (cis-9 C18:1 and cis-9, cis-12, cis-15 C18:3) diet variation was greater than the b-cow variation, but repeatability was moderate. For trans-10 C18:1, trans-11 C18:1 and cis-9 trans-11 C18:2 diet variation was greater than b-cow variation with low repeatability.

Empirical models–simple regressions

Relationships between milk FA and stoichiometric Y\_CH\_4 VFA are presented in Fig 1 and S2 Table. The C4:0 and C6:0 ($P < 0.05$) were positively related with stoichiometric Y\_CH\_4 VFA. For the OBCFA, anteiso C15:0 ($P < 0.01$) and C15:0 ($P < 0.01$) were negatively associated with stoichiometric CH\_4 VFA. Additionally, milk trans-11 C18:1 ($P = 0.02$), cis-11 C18:1 ($P < 0.01$), cis-9, trans-11 C18:2 ($P = 0.01$), and cis-9, cis-12, cis-15 C18:3 were negatively related with stoichiometric CH\_4 VFA. There was no relationship between the summation of
milk FA by source (de novo, mixed and preformed) and stoichiometric CH\textsubscript{4}VFA. We evaluated the relationship between milk OBCFA and rumen VFA (S3 Table). There was no relationship ($P > 0.05$) between both anteiso C13:0 and iso C13:0 milk FA and concentration of rumen VFA (propionate, valerate, isovalerate, and BCVFA). Milk C15:0 was positively associated with rumen propionate ($P < 0.01$) and valerate ($P = 0.01$). Milk anteiso C15:0 was positively associated with rumen propionate ($P = 0.05$), while milk iso C15:0 was negatively associated with isovalerate ($P = 0.01$) and BCVFA ($P = 0.02$). Milk anteiso C17:0 was positively associated with isovalerate ($P < 0.01$) and BCVFA ($P = 0.01$), while C17:0 was negatively related to with BCVFA ($P = 0.05$).

### Table 4. Variance component estimates for methane estimate and rumen fermentation parameters in dairy cows.

| Variable                  | Estimate | SE    | Z value\(^1\) | SD\(^2\) | CV\(^3\) | Rep\(^4\) |
|---------------------------|----------|-------|---------------|----------|----------|-----------|
| CH\textsubscript{4}VFA, mmol/mol | Diet (Exp) | 110   | 38.9          | <0.01    | 10.5     | 0.03      | 0.22      |
|                           | Cow (Exp)  | 23.6  | 14.54         | 0.05     | 4.86     | 0.01      |
|                           | Residual   | 84.7  | 14.96         | <0.01    | 9.20     | 0.03      |
| TVFA, mmol                | Diet (Exp) | 24.0  | 9.91          | <0.01    | 4.90     | 0.05      | 0.46      |
|                           | Cow (Exp)  | 30.9  | 11.03         | <0.01    | 5.56     | 0.05      |
|                           | Residual   | 36.9  | 7.35          | <0.01    | 6.07     | 0.06      |
| Acetate, mmol/mol         | Diet (Exp) | 416   | 130.0         | <0.01    | 20.4     | 0.03      | 0.37      |
|                           | Cow (Exp)  | 56.6  | 23.27         | <0.01    | 7.52     | 0.01      |
|                           | Residual   | 97.8  | 19.43         | <0.01    | 9.89     | 0.02      |
| Propionate, mmol/mol      | Diet (Exp) | 151   | 46.2          | <0.01    | 12.3     | 0.07      | 0.28      |
|                           | Cow (Exp)  | 40.7  | 20.44         | 0.02     | 6.38     | 0.03      |
|                           | Residual   | 103   | 23.1          | <0.01    | 10.2     | 0.05      |
| Butyrate, mmol/mol        | Diet (Exp) | 30.9  | 19.33         | 0.05     | 5.56     | 0.05      | 0.10      |
|                           | Cow (Exp)  | 15.6  | 16.20         | 0.17     | 3.95     | 0.03      |
|                           | Residual   | 147   | 23.2          | <0.01    | 12.1     | 0.10      |
| Isobutyrate, mmol/mol     | Diet (Exp) | 0.19  | 0.125         | 0.07     | 0.43     | 0.05      | 0.02      |
|                           | Cow (Exp)  | 0.02  | 0.085         | 0.42     | 0.13     | 0.02      |
|                           | Residual   | 0.91  | 0.184         | <0.01    | 0.96     | 0.12      |
| Isovalerate, mmol/mol     | Diet (Exp) | 2.73  | 1.002         | <0.01    | 1.65     | 0.13      | 0.35      |
|                           | Cow (Exp)  | 1.46  | 0.586         | <0.01    | 1.21     | 0.10      |
|                           | Residual   | 2.76  | 0.541         | <0.01    | 1.66     | 0.13      |
| Ratio\(^5\)              | Diet (Exp) | 0.10  | 0.039         | <0.01    | 0.32     | 0.08      | 0.08      |
|                           | Cow (Exp)  | 0.01  | 0.014         | 0.23     | 0.10     | 0.03      |
|                           | Residual   | 0.12  | 0.020         | <0.01    | 0.34     | 0.09      |
| BCVFA\(^6\)              | Diet (Exp) | 3.09  | 1.276         | <0.01    | 1.76     | 0.09      | 0.23      |
|                           | Cow (Exp)  | 1.51  | 0.792         | 0.03     | 1.23     | 0.06      |
|                           | Residual   | 5.16  | 1.024         | <0.01    | 2.27     | 0.11      |
| pH                       | Diet (Exp) | 0.02  | 0.007         | <0.01    | 0.14     | 0.02      | 0.55      |
|                           | Cow (Exp)  | 0.02  | 0.006         | <0.01    | 0.14     | 0.02      |
|                           | Residual   | 0.02  | 0.003         | <0.01    | 0.12     | 0.02      |

\(^1\)Probability of Z-value.

\(^2\)Calculated as the square root of the variance component estimate.

\(^3\)Calculated as SD divided by the respective mean value of the variable.

\(^4\)Rep = $\sigma^2$ Cow/($\sigma^2$ Cow + $\sigma^2$ Residual).

\(^5\)Ratio = (Acetate + Butyrate) / (Propionate + Valerate).

\(^6\)BCVFA = Isovalerate + Isobutyrate.

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Table 5. Variance component estimates of omasal fatty acids (FA) in dairy cows.

| Variable | Estimate | SE  | Z value | SD² | CV³ | Rep⁴ |
|----------|----------|-----|---------|-----|-----|------|
| Selected FA, g 100g/FA |          |     |         |     |     |      |
| C13:0 iso | Diet (Exp) | 0.0001 | 0.00004 | <0.01 | 0.01 | 0.30 | 0.04 |
|          | Cow (Exp) | 0.00002 | 0.000007 | 0.37 | 0.002 | 0.04 |
|          | Residual | 0.0001 | 0.00001 | <0.01 | 0.008 | 0.21 |
| C13:0 anteiso | Diet (Exp) | 0.0016 | 0.00081 | 0.02 | 0.04 | 1.68 | 0.17 |
|          | Cow (Exp) | 0.0006 | 0.00052 | 0.11 | 0.02 | 1.02 |
|          | Residual | 0.003 | 0.0006 | <0.01 | 0.05 | 2.24 |
| C15:0 iso | Diet (Exp) | 0.006 | 0.0020 | <0.01 | 0.08 | 0.28 | 0.05 |
|          | Cow (Exp) | 0.0002 | 0.00031 | 0.30 | 0.01 | 0.05 |
|          | Residual | 0.003 | 0.0005 | <0.01 | 0.05 | 0.19 |
| C15:0 anteiso | Diet (Exp) | 0.39 | 0.151 | <0.01 | 0.63 | 1.23 | 0.30 |
|          | Cow (Exp) | 0.18 | 0.092 | 0.02 | 0.42 | 0.83 |
|          | Residual | 0.41 | 0.082 | <0.01 | 0.64 | 1.25 |
| C15:0 | Diet (Exp) | 0.72 | 0.241 | <0.01 | 0.85 | 1.08 | 0.31 |
|          | Cow (Exp) | 0.17 | 0.083 | 0.02 | 0.42 | 0.53 |
|          | Residual | 0.40 | 0.084 | <0.01 | 0.63 | 0.80 |
| C16:0 iso | Diet (Exp) | 0.003 | 0.0011 | <0.01 | 0.06 | 0.29 | 0.02 |
|          | Cow (Exp) | 0.0001 | 0.00034 | 0.41 | 0.009 | 0.04 |
|          | Residual | 0.003 | 0.0006 | <0.01 | 0.06 | 0.29 |
| C16:0 | Diet (Exp) | 11.9 | 3.60 | <0.01 | 3.45 | 0.30 | 0.53 |
|          | Cow (Exp) | 2.46 | 0.841 | <0.01 | 1.57 | 0.14 |
|          | Residual | 2.14 | 0.433 | <0.01 | 1.46 | 0.13 |
| C17:0 iso | Diet (Exp) | 0.003 | 0.0010 | <0.01 | 0.06 | 0.24 | 0.02 |
|          | Cow (Exp) | 0.0002 | 0.000108 | 0.44 | 0.004 | 0.02 |
|          | Residual | 0.001 | 0.0002 | <0.01 | 0.03 | 0.14 |
| C17:0 anteiso | Diet (Exp) | 0.003 | 0.0009 | <0.01 | 0.05 | 0.27 | 0.10 |
|          | Cow (Exp) | 0.0002 | 0.00021 | 0.21 | 0.01 | 0.07 |
|          | Residual | 0.002 | 0.0003 | <0.01 | 0.04 | 0.20 |
| C17:0 | Diet (Exp) | 0.01 | 0.003 | <0.01 | 0.10 | 0.21 | 0.21 |
|          | Cow (Exp) | 0.0002 | 0.00014 | 0.05 | 0.02 | 0.03 |
|          | Residual | 0.0009 | 0.00015 | <0.01 | 0.03 | 0.06 |
| C18:0 iso | Diet (Exp) | 0.0002 | 0.00007 | <0.01 | 0.01 | 0.26 | 0.05 |
|          | Cow (Exp) | 0.00005 | 0.000010 | 0.32 | 0.002 | 0.04 |
|          | Residual | 0.0001 | 0.00002 | <0.01 | 0.009 | 0.16 |
| C18:0 | Diet (Exp) | 83.5 | 25.11 | <0.01 | 9.14 | 0.18 | 0.06 |
|          | Cow (Exp) | 0.95 | 1.452 | 0.26 | 0.98 | 0.02 |
|          | Residual | 15.4 | 2.50 | <0.01 | 3.93 | 0.08 |
| C18:1, trans-10 | Diet (Exp) | 10.6 | 3.15 | <0.01 | 3.26 | 1.71 | 0.15 |
|          | Cow (Exp) | 0.29 | 0.201 | 0.08 | 0.54 | 0.28 |
|          | Residual | 1.67 | 0.321 | <0.01 | 1.29 | 0.68 |
| C18:1, trans-11 | Diet (Exp) | 5.68 | 1.752 | <0.01 | 2.38 | 0.46 | 0.13 |
|          | Cow (Exp) | 0.24 | 0.193 | 0.10 | 0.49 | 0.10 |
|          | Residual | 1.64 | 0.272 | <0.01 | 1.28 | 0.25 |
| C18:1, cis-9 | Diet (Exp) | 0.91 | 0.283 | <0.01 | 0.95 | 0.31 | 0.14 |
|          | Cow (Exp) | 0.04 | 0.031 | 0.10 | 0.21 | 0.07 |
|          | Residual | 0.26 | 0.052 | <0.01 | 0.51 | 0.17 |

(Continued)
The relationship between concentrations and flows of omasal OBCFA on milk FA are presented in Figs 2 and 3. We observed positive relationship between concentration of omasal OBCFA and the concentration of milk OBCFA ($P < 0.01$), as well as positive intercepts for all of the OBCFA evaluated ($P < 0.01$). Similarly, for most OBCFA, we observed positive relationships between omasal flow of OBCFA and yield of milk OBCFA ($P < 0.01$) with exception for milk isoC17:0 that was not affected by isoC17:0 omasal flow ($P = 0.13$). Regarding the intercept values, for all milk OBCFA, we observed positive intercepts ($P < 0.01$).

**Empirical models–multiple regressions**

We evaluated whether calculated ME balance and proportion of omasal FA would affect milk FA profile (Table 7). The concentration of several milk FA was affected by energy balance. Some milk OBCFA (isoC13:0, isoC15:0, anteiso C15:0, and C15:0) were positively associated with the omasal proportion of their respective FA (all $P < 0.01$) and by energy balance ($P < 0.01$). In contrast, the concentration of milk C17:0, isoC18:0, C18:0, and cis-11 C18:1 were positively influenced by omasal proportion of their respective FA ($P < 0.01$) but negatively associated with energy balance ($P < 0.05$). For milk cis-9 C18:1, there was no effect of omasal cis-9 C18:1 ($P = 0.69$), but it was inversely related with energy balance ($P < 0.01$). We observed minor effects of DMI associated with rumen VFA on milk OBCFA (S4 Table).

**Discussion**

Lately, several studies have focused on developing reliable and low-cost measures of ruminant enteric CH$_4$ emissions on an individual-animal basis. Determining the variability among cows offers the potential for genetic selection of animals that have lower CH$_4$ emissions, which is an attractive mitigation strategy because genetic improvements are cumulative and permanent [34]. Milk FA are a promising CH$_4$ proxy because of the direct link to microbial digestion in the rumen and energy balance [35]. Additionally, breeding for reduced CH$_4$ production has been proposed and therefore indicators of CH$_4$ production based on milk FA are of particular interest [9, 36]. A large range in the heritability of CH$_4$ production ($h^2$: 0.12 to 0.44) estimated

### Table 5. (Continued)

| Variable                  | Estimate (Diet (Exp)) | SE   | Z value | SD   | CV   | Rep  |
|---------------------------|-----------------------|------|---------|------|------|------|
| C18:1, cis-11             |                       |      |         |      |      |      |
| Diet (Exp)                | 0.05                  | 0.023| <0.01   | 0.23 | 0.32 | 0.09 |
| Cow (Exp)                 | 0.001                 | 0.0021| 0.20   | 0.04 | 0.05 |      |
| Residual                  | 0.02                  | 0.003| <0.01   | 0.12 | 0.18 |      |
| C18:2, cis-9 cis-12       |                       |      |         |      |      |      |
| Diet (Exp)                | 0.48                  | 0.153| <0.01   | 0.69 | 0.30 | 0.12 |
| Cow (Exp)                 | 0.02                  | 0.022| 0.13   | 0.14 | 0.06 |      |
| Residual                  | 0.14                  | 0.031| <0.01   | 0.38 | 0.16 |      |
| C18:2, cis-9 trans-11     |                       |      |         |      |      |      |
| Diet (Exp)                | 0.02                  | 0.007| <0.01   | 0.13 | 0.20 | 0.53 |
| Cow (Exp)                 | 0.02                  | 0.008| <0.01   | 0.16 | 0.24 |      |
| Residual                  | 0.02                  | 0.004| <0.01   | 0.15 | 0.22 |      |
| C18:3, cis-9 cis-12 cis-15|                       |      |         |      |      |      |
| Diet (Exp)                | 0.14                  | 0.041| <0.01   | 0.37 | 0.35 | 0.20 |
| Cow (Exp)                 | 0.005                 | 0.0030| 0.07   | 0.07 | 0.06 |      |
| Residual                  | 0.02                  | 0.004| <0.01   | 0.14 | 0.13 |      |

1Probability of Z-value.
2Calculated as the square root of the variance component estimate.
3Calculated as SD divided by the respective mean value of the variable.
4Rep = σ$^2$ Cow/(σ$^2$ Cow + σ$^2$ Residual).

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Table 6. Variance component estimates of milk fatty acids (FA) in dairy cows.

| Variable | Estimate | SE  | Z value | SD² | CV³ | Rep⁴ |
|----------|----------|-----|---------|-----|-----|------|
| Selected FA, g 100g/FA |          |     |         |     |     |      |
| C4:0 Diet (Exp) | 0.18     | 0.054 | <0.01   | 0.42 | 0.13 | 0.51  |
| Cow (Exp) | 0.04     | 0.014 | <0.01   | 0.20 | 0.06 |       |
| Residual | 0.04     | 0.007 | <0.01   | 0.20 | 0.06 |       |
| C6:0 Diet (Exp) | 0.11     | 0.033 | <0.01   | 0.33 | 0.18 | 0.48  |
| Cow (Exp) | 0.01     | 0.005 | <0.01   | 0.12 | 0.06 |       |
| Residual | 0.02     | 0.003 | <0.01   | 0.12 | 0.07 |       |
| C8:0 Diet (Exp) | 0.06     | 0.018 | <0.01   | 0.24 | 0.22 | 0.58  |
| Cow (Exp) | 0.01     | 0.004 | <0.01   | 0.11 | 0.10 |       |
| Residual | 0.01     | 0.002 | <0.01   | 0.10 | 0.09 |       |
| C13:0 iso⁵ Diet (Exp) | 2.00     | 0.600 | <0.01   | 0.04 | 1.51 | 0.18  |
| Cow (Exp) | 0.30     | 0.210 | 0.07    | 0.02 | 0.66 |       |
| Residual | 1.00     | 0.200 | <0.01   | 0.04 | 1.41 |       |
| C13:0 anteiso Diet (Exp) | 0.03     | 0.010 | 0.01    | 0.16 | 14.2 | 0.15  |
| Cow (Exp) | 0.01     | 0.005 | 0.09    | 0.08 | 7.23 |       |
| Residual | 0.04     | 0.007 | <0.01   | 0.19 | 17.1 |       |
| C15:0 iso⁵ Diet (Exp) | 1.00     | 0.400 | <0.01   | 0.04 | 0.17 | 0.40  |
| Cow (Exp) | 0.30     | 0.130 | 0.01    | 0.02 | 0.08 |       |
| Residual | 0.50     | 0.100 | <0.01   | 0.02 | 0.10 |       |
| C15:0 anteiso⁵ Diet (Exp) | 3.00     | 0.800 | <0.01   | 0.05 | 0.12 | 0.57  |
| Cow (Exp) | 2.00     | 0.700 | <0.01   | 0.05 | 0.11 |       |
| Residual | 2.00     | 0.300 | <0.01   | 0.04 | 0.09 |       |
| C15:0⁵ Diet (Exp) | 0.02     | 0.006 | <0.01   | 0.13 | 0.13 | 0.27  |
| Cow (Exp) | 0.005    | 0.0023 | 0.02   | 0.07 | 0.07 |       |
| Residual | 0.01     | 0.002 | <0.01   | 0.11 | 0.11 |       |
| C16:0 iso⁵ Diet (Exp) | 0.01     | 0.004 | 0.03    | 0.09 | 0.38 | 0.02  |
| Cow (Exp) | 0.001    | 0.0023 | 0.40   | 0.02 | 0.11 |       |
| Residual | 0.02     | 0.005 | <0.01   | 0.15 | 0.67 |       |
| C16:0 Diet (Exp) | 13.9     | 3.60  | <0.01   | 3.45 | 0.13 | 0.58  |
| Cow (Exp) | 2.26     | 0.835 | <0.01   | 1.57 | 0.06 |       |
| Residual | 2.14     | 0.429 | <0.01   | 1.46 | 0.05 |       |
| C17:0 iso⁵ Diet (Exp) | 0.50     | 0.210 | 0.01    | 0.02 | 0.13 | 0.17  |
| Cow (Exp) | 0.20     | 0.130 | 0.08    | 0.01 | 0.08 |       |
| Residual | 0.90     | 0.190 | <0.01   | 0.03 | 0.18 |       |
| C17:0 anteiso⁵ Diet (Exp) | 1.00     | 0.400 | 0.01    | 0.03 | 0.12 | 0.27  |
| Cow (Exp) | 0.60     | 0.300 | 0.02    | 0.03 | 0.09 |       |
| Residual | 2.00     | 0.300 | <0.01   | 0.04 | 0.15 |       |
| C17:0⁵ Diet (Exp) | 10.00    | 2.000 | <0.01   | 0.07 | 0.15 | 0.47  |
| Cow (Exp) | 0.60     | 0.210 | <0.01   | 0.02 | 0.05 |       |
| Residual | 0.70     | 0.110 | <0.01   | 0.03 | 0.05 |       |
| C18:0 iso⁵ Diet (Exp) | 0.10     | 0.020 | <0.01   | 0.01 | 0.15 | 0.43  |
| Cow (Exp) | 0.03     | 0.013 | <0.01   | 0.01 | 0.11 |       |
| Residual | 0.05     | 0.009 | <0.01   | 0.01 | 0.12 |       |
| C18:0 Diet (Exp) | 4.99     | 1.514 | <0.01   | 2.23 | 0.21 | 0.27  |
| Cow (Exp) | 0.39     | 0.192 | 0.02    | 0.63 | 0.06 |       |
| Residual | 1.06     | 0.210 | <0.01   | 1.03 | 0.10 |       |

(Continued)
using milk FA has been reported [15], even though the $R^2$ of the equations were not much different (0.63 to 0.73). In the present study, repeatability and b-cow variation estimated by variance components were used to identify suitable animal variables of rumen fermentation, omasal FA and milk FA related to b-cow differences in estimated CH$_4$ emissions. Despite the limited number of observations in our analysis due to our selection criteria focused on the integration of data on fermentation parameters, omasal FA and milk FA, we established important relationships involving animal factors, digestion, omasal flow and milk output related to CH$_4$.

A limitation in our study is that we did not have a direct measurement of CH$_4$, but rather we used the approach proposed by Wolin [30] to calculate Y$_{CH_4}$VFA. This method could be criticized because it assumes that all fermented substrates have a formula C$_6$H$_{12}$O$_6$, while some carbohydrates deviate from this general formula. Although this consideration is important, these carbohydrates usually comprise only a small part of ruminant diets [30].

### Table 6. (Continued)

| Variable                          | Estimate | SE     | Z value$^1$ | SD$^2$ | CV$^3$ | Rep$^4$ |
|-----------------------------------|----------|--------|-------------|--------|--------|---------|
| C18:1, trans-10                   | Diet (Exp) | 3.38   | 0.999       | <0.01  | 1.84   | 1.81    | 0.14    |
|                                   | Cow (Exp) | 0.08   | 0.057       | 0.08   | 0.28   | 0.28    |
|                                   | Residual | 0.48   | 0.093       | <0.01  | 0.69   | 0.68    |
| C18:1, trans-11                   | Diet (Exp) | 1.50   | 0.449       | <0.01  | 1.22   | 0.64    | 0.10    |
|                                   | Cow (Exp) | 0.03   | 0.026       | 0.15   | 0.17   | 0.09    |
|                                   | Residual | 0.23   | 0.045       | <0.01  | 0.48   | 0.25    |
| C18:1, cis-9                      | Diet (Exp) | 11.4   | 3.56        | <0.01  | 3.44   | 0.34    | 0.43    |
|                                   | Cow (Exp) | 3.01   | 1.131       | <0.01  | 1.74   | 0.09    |
|                                   | Residual | 3.95   | 0.656       | <0.01  | 1.99   | 0.11    |
| C18:1, cis-11                     | Diet (Exp) | 0.03   | 0.008       | <0.01  | 0.16   | 0.27    | 0.30    |
|                                   | Cow (Exp) | 0.004  | 0.0018      | 0.02   | 0.06   | 0.10    |
|                                   | Residual | 0.01   | 0.002       | <0.01  | 0.09   | 0.15    |
| C18:2, cis-9 trans-11             | Diet (Exp) | 0.20   | 0.063       | <0.01  | 0.45   | 0.57    | 0.17    |
|                                   | Cow (Exp) | 0.01   | 0.006       | 0.12   | 0.08   | 0.11    |
|                                   | Residual | 0.05   | 0.009       | <0.01  | 0.23   | 0.30    |
| C18:3, cis-9 cis-12 cis-15$^5$     | Diet (Exp) | 10.0   | 4.00        | <0.01  | 1.22   | 0.24    | 0.46    |
|                                   | Cow (Exp) | 2.0    | 0.60        | <0.01  | 0.04   | 0.08    |
|                                   | Residual | 2.0    | 0.40        | <0.01  | 0.05   | 0.09    |
| <16-carbon                        | Diet (Exp) | 15.2   | 4.63        | <0.01  | 3.90   | 0.15    | 0.60    |
|                                   | Cow (Exp) | 3.76   | 1.266       | <0.01  | 1.94   | 0.07    |
|                                   | Residual | 2.53   | 0.434       | <0.01  | 1.59   | 0.06    |
| 16-carbon                         | Diet (Exp) | 11.5   | 3.49        | <0.01  | 3.39   | 0.11    | 0.52    |
|                                   | Cow (Exp) | 2.25   | 0.780       | <0.01  | 1.50   | 0.05    |
|                                   | Residual | 2.10   | 0.435       | <0.01  | 1.45   | 0.05    |
| >16-carbon                        | Diet (Exp) | 49.3   | 14.78       | <0.01  | 7.02   | 0.16    | 0.38    |
|                                   | Cow (Exp) | 3.70   | 1.550       | 0.01   | 1.92   | 0.04    |
|                                   | Residual | 6.16   | 1.051       | <0.01  | 2.48   | 0.06    |

$^1$Probability of Z-value.
$^2$Calculated as the square root of the variance component estimate.
$^3$Calculated as SD divided by the respective mean value of the variable.
$^4$Rep = $\sigma^2$ Cow/($\sigma^2$ Cow + $\sigma^2$ Residual).
$^5$These FA are reported in mg/100 g FA.

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deviations from the C₆H₁₂O₆ formula could influence variance component of Diet (Exp) but not that of Cow (Exp), which is the major interest of this study. Additionally, the stoichiometric relationships between VFA production and production of H₂ (substrate for hydrogenotrophic methanogens) suggest that CH₄ emissions are positively associated with the acetate:propionate ratio in ruminal fluid; however, the relationship between CH₄ emission and both VFA and pH are variable in the literature and not as straightforward as expected from theory [35]. In our study, the b-cow coefficient of variation (CV) for Y CH₄ VFA was only 0.01, while the variation in the variance components for diet was 3 times greater than the b-cow variation. Similar to our results, b-cow CV of 0.01, and 0.104 for predicted Y CH₄ VFA, and total CH₄ production were previously reported [13]. Between-animal CV in CH₄ production reported in the literature differ, reflecting differences in feed intake and methodology.

Rumen VFA pattern can be expected to be related to CH₄ production due to changes in H₂ balance, such that high acetate and butyrate production enhance CH₄ production, whereas high propionate production is associated with low CH₄ emissions [37]. In the present study, b-cow variation in rumen VFA pattern was small (CV ranged from 0.01 to 0.05). Similarly, a previous study reported for sheep a CV for CH₄ production of 0.098 [38], whereas the CV for molar proportions of acetate, propionate, and butyrate was 0.011, 0.047, and 0.036, respectively. Greater b-cow variation and repeatability for traits such as digestibility, passage, and efficiency of microbial cell synthesis has been previously reported [13] indicating that
between-animal variation in CH$_4$ may be more closely related to these characteristics than the composition of the rumen microbiome.

The milk fat in ruminants contain greater proportions of saturated FA compared with dietary intake because of extensive biohydrogenation of unsaturated FA in the rumen [39]. During the biohydrogenation of FA, several trans FA intermediates are formed under different dietary conditions [40], and therefore they may also be indicators of changes in rumen function. In our study, for both omasal and milk trans FA (trans-10 C18:1 and trans-11 C18:1) diet variation was 5-fold greater than b-cow variation indicating that rumen conditions influencing the synthesis of these FA was more strongly associated with differences in diets than with differences between the cows. Variance components and repeatability of trans-10 C18:1 and trans-11 C18:1 were similar between omasal flow and milk indicating that these FA are more related with ruminal changes than post-ruminal metabolism. Also, milk trans-11 C18:1 and cis-11 C18:1 were similar between omasal flow and milk indicating that these FA are more related with ruminal changes than post-ruminal metabolism. Also, milk trans-11 C18:1 and cis-11 C18:1 were negative related with stoichiometric Y$_{\text{CH}_4}\text{VFA}$. Similarly, a previous study [9] indicated a negative correlation between concentration of cis-9, cis-12, cis-15 C18:3 in milk fat and CH$_4$ production. Additionally, milk trans-11 C18:1 and cis-11 C18:1 were negative correlated with CH$_4$ production [10]. Negative association of some unsaturated FA (i.e. cis-9 C18:1) with CH$_4$ production are expected, especially during negative energy balance where intake and CH$_4$ production are low compared with cows in positive energy balance. Also, a negative association of unsaturated FA
and CH$_4$ is expected since during biohydrogenation some H$_2$ are used by rumen bacteria. In addition, increased unsaturated FA in milk may indicate dietary unsaturated fat supplementation and thus decreased intake of fermentable carbohydrates, and or reduce ruminal fermentation of organic matter, and thereby CH$_4$ production. Furthermore, several trans and cis FA occurring in milk fat are biohydrogenation intermediates of both cis-9, cis-12 C18:2 and cis-9, cis-12, cis-15 C18:3 [39]. Rumen conditions with low fiber and high concentrate diets may induce changes in the extent of biohydrogenation and formation of biohydrogenation intermediates [40]. With reduced rumen pH, the predominant biohydrogenation pathway of cis-9, cis-12 C18:2 may shift to the trans-10 pathway [40]. Therefore, these observations explain the negative correlation obtained between the concentration of some milk trans FA and CH$_4$ production, whereas diet factors are more strongly related to the differences in these FA than to between-animal differences.

OBCFA are suggested to reflect rumen function including ruminal fermentation pattern, duodenal flow of microbial protein and acidosis [6]. Overall for OBCFA omasal flow the variation associated with diet was considerably greater than the between-cow variation with low repeatability. Furthermore, in our study, we observed weak associations between rumen VFA profile and milk iso and anteiso OBCFA. Similarly, a previous study observed that rumen and milk OBCFA responses were minimal following infusion of large amounts of VFA (acetate, propionate and isovalerate) and suggested that shifts in ruminal OBCFA are primarily affected by diet factors.
by altered populations of different rumen microbial strains driven by dietary composition as opposed to altered VFA available in the extracellular space for FA synthesis [41]. In the rumen, de novo FA in bacteria are synthesized by two types of FA synthetases: straight-chain and branched-chain FA synthetase [42]. Linear odd-chain FA are formed when propionyl-CoA, instead of acetyl-CoA, is used as a primer [42]. In our study, we observed that milk C15:0 was positively associated with rumen propionate and valerate, which agrees with previous findings suggesting that C15:0 and C17:0 are formed through elongation of propionate or valerate [6]. Additionally, when we considered DMI in the equations, we also observed a positive effect of propionate and DMI on milk C15:0, while for C17:0 a positive relationship with valerate and DMI was detected. Similar to our results, [43] reported milk concentrations of C15:0 and the sum of C17:0 and \textit{cis} \textit{-9} C17:1 to be positively related to propionate concentration in the rumen. Since it is expected that propionate production is negatively related to \textit{CH}_4 production, this suggests a negative relationship between the concentration of these OBCFA in milk and \textit{CH}_4 production. In the present study, we did not observe an association between the proportion of omasal C15:0 and C17:0 and Y \textit{CH}_4 VFA, while milk C15:0 was negatively related to \textit{CH}_4 VFA. Similarly, the results from previous studies have been equivocal and reporting negative correlations between milk C15:0 and C17:0 and \textit{CH}_4 production [44] or no significant relationships between these FA with \textit{CH}_4 yield [10].

Importantly, when we evaluated the effect of omasal OBCFA (g/100 g of FA) on their respective milk OBCFA (g/100 g of FA) and the omasal flow of OBCFA (g/d) on their respective yield of milk OBCFA (g/d), we detected positive intercepts, which may indicate endogenous synthesis or elongation in the mammary gland. Similar to our results, a previous study reported greater secretion of C15:0, C17:0, and iso C17:0 in milk fat than could be accounted for by intestinal absorption [45]. In the mammary gland, endogenous chain elongation using
propionyl-CoA as precursor [46] explains the occurrence of certain odd-chain FA (i.e. C5:0, C7:0, C9:0 and C11:0) in milk and it may also increase the amount of other odd-chain FA transferred from the duodenum (C13:0, C15:0 and C17:0) into milk. Also, milk secretion of iso C17:0 and anteiso C17:0 in excess of duodenal flows of those FA has been also observed indicating synthesis in tissues [47]. Limited synthesis of the iso 17:0 has also been reported [7], and methodological issues due to coelution of cis-9 C16:1 with anteiso C17:0 [48] are also possible factors that affect these differences between omasal flow and milk FA secretion. In addition to mammary gland, other tissues have also been shown to have the ability to synthesize OBCFA from propionate [49] and, therefore, OBCFA that are present in milk in greater amounts than their respective duodenal flow could partially be a result of the synthesis in the mammary gland and increasing amounts of OBCFA mobilized from other tissues. Repeatability was lower for iso C13:0, iso C15:0, and iso C17:0 omasal flow compared to milk output. Additionally, we observed that milk iso C13:0 and iso C15:0 were positively associated to rumen propionate concentration and feed intake, which in turn suggest that potentially other factors can affect the output of these OBCFA in milk. Therefore, the endogenous synthesis of OBCFA, elongation of some OBCFA into their longer chain equivalents, and synthesis in other tissues may limit their use as biomarkers of rumen function and CH$_4$ proxy.

Additionally, we observed that energy balance is an important factor influencing milk FA profile. For milk OBCFA, iso C13:0, iso C15:0, anteiso C15:0, and C15:0 were positively influenced by omasal proportion of their respective FA and by energy balance. Similar to our results, Craninx et al. [50] reported that OBCFA with chain lengths of 14 or 15 carbon atoms showed an increasing pattern as lactation period progressed and cows entered in positive energy balance, whereas OBCFA with chain lengths of 17 carbon atoms showed the opposite pattern of response. In dairy cows, cis-9 C18:1, C18:0 and C16:0 are the main FA present in adipose tissue [51]. During early lactation, mobilization of body reserves of fat increases the circulation of these FA and their uptake by the mammary gland. Therefore, the decrease in the concentration of these OBCFA in milk fat may be a dilution effect since other long-chain FA will increase during periods of negative energy balance. Therefore, some of the inconsistency when predicting CH$_4$ using concentration of milk FA can be explained by energy balance and lactation stage, both being factors that can influence the relationship between milk FA and CH$_4$ emission.

The short- and medium-chain FA (4 to 14 carbons) and a portion of the 16-carbon FA are derived from de novo synthesis from acetate and to a lesser extent BHBA [40]. Therefore, since acetate and butyrate production in the rumen is associated with H$_2$ production, some de novo milk FA may be a proxy for CH$_4$. In contrast to our expectation, we found weak relationships between most de novo milk FA and Y_CH$_4$VFA, with only the concentration of milk C4:0 and a tendency for C6:0 being positively associated with Y_CH$_4$VFA. A positive correlation between de novo FA and CH$_4$ (g/d) has been reported [9], while others reported that C12:0, and C14:0 were positively associated with CH$_4$ (g/d) [52]. Also, for de novo milk FA C4:0, C6:0 and C8:0 and for C16:0 the diet variation was 3-fold higher than the b-cow variation, but repeatability for these FA was high. Also, a previous study reported that the concentration of C16:0 in milk fat was moderately positively related to CH$_4$ yield (g/kg of DMI), and concentrations of C6:0, C8:0, and C10:0 in milk fat tended to be weakly positively related to CH$_4$ yield [10]. Although diet is still the major factor impacting the variance components of de novo FA, these FA seem more promising as proxies for CH$_4$ parameters as heritability for short and medium chain FA are greater than those for mixed and unsaturated milk FA [53]. However, selecting animals for low C4:0 to C12:0 milk FA may result in lower CH$_4$, but also may reduce milk fat content and yield due to the correlation between milk de novo FA concentration and these traits.
Although we estimated CH\textsubscript{4} using VFA stoichiometry rather than directly measuring CH\textsubscript{4} our b-cow estimates are in line with previous reports. Additionally, we generated b-cow estimates for trans-FAs and OBCFA that have been related with rumen function, but our analysis of omasal flow of FA and animal factors suggest that factors, such as feed intake and energy balance, should be considered because they are likely associated to post-ruminal changes in the appearance of these FA into milk fat. Additionally, a recent study used the equations from the meta-analysis of van Lingen et al. \cite{10} to quantify the CH\textsubscript{4} emissions traits predicted by selected milk FA and to assess their main sources of variation \cite{54}. They reported wide variability in estimated CH\textsubscript{4} emissions traits among different farms within dairy system (TMR fed vs. hay + concentrate) indicating that factors related with feeding management and other animal management practices are likely related to CH\textsubscript{4} emissions \cite{54}. Feeding management factors (i.e. feeding frequency, bunk space, etc.) influence feed intake, slug-feeding, rumen pH and animal behavior \cite{55}, which in turn may affect CH\textsubscript{4} emissions. Although we did not characterize and have available data in our data set regarding feed management, we cannot rule out the possibility that factors that influence feed behavior may impact CH\textsubscript{4} estimates.

**Conclusion**

Our findings demonstrate that for most omasal and milk FA examined, a larger variation can be attributed to dietary factors than b-cow differences with low to moderate repeatability. Even though we observed that some milk FA were positively or negatively associated with Y\textsubscript{,CH\textsubscript{4}VFA}, other factors such as energy balance had a pronounced effect on these estimates. Therefore, this may preclude the utilization of milk FA as a proxy for CH\textsubscript{4} predictions. Based on our dataset, between-animal variation in milk FA profile was small, which may suggest that caution should be exercised when using milk FA to select low-emitting animals in breeding programs. Because of the greater between-diet variability compared with between-animal variation for most milk FA, they may be used as a proxy for detecting differences between diets and farms; however, these differences can also be predicted by empirical models.

**Supporting information**

S1 Table. Data sources and characteristics of included studies. (DOCX)

S2 Table. Influence of milk fatty acid (FA) on stoichiometry methane (CH\textsubscript{4} VFA) estimated by univariate mixed model regression analysis (CH\textsubscript{4} VFA = A + BX\textsubscript{1}) in dairy cows. (DOCX)

S3 Table. Influence of rumen VFA on the concentration of milk odd- and branched-chain fatty acids (OBCFA) (g 100 g/ FA), estimated by univariate mixed model regression analysis (OBCFA = A + BX\textsubscript{1}) in dairy cows. (DOCX)

S4 Table. Influence of rumen VFA, and DMI on milk odd- and branched-chain fatty acids (OBCFA), estimated by bivariate mixed model regression analysis (OBCFA = A + BX\textsubscript{1} + BX\textsubscript{2}) in dairy cows. (DOCX)

S1 Data. (XLSX)
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Conceptualization: K. J. Shingfield, P. Huhtanen.
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