Meta-analysis of the relationship between milk *trans*-10 C18:1, milk fatty acids <16 C, and milk fat production

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ABSTRACT

The economic value of milk fat and its responsiveness to management strategies provides strong interest in maximizing milk fat production by minimizing occurrence of biohydrogenation-induced milk fat depression (BH-MFD) and maximizing de novo synthesized fatty acids (FA). Tools that allow a timely diagnosis of BH-MFD would improve nutritional management. Specific milk FA or FA categories correlate to milk fat concentration and are of interest for diagnosing the cause of changes in milk fat concentration. The objective of the current study was to characterize the relationship between milk fat concentration and *trans*-10 C18:1, a proxy for BH-MFD, and FA <16 carbons that originate solely from de novo lipogenesis using a meta-analysis approach that used data from the literature and unpublished Penn State experiments. Prior to the meta-analysis, the effect of FA methylation method on milk FA profile was tested to determine potential bias between papers. There was no difference between sodium methoxide, acid, and acid-base methylation methods on *trans*-10 C18:1 concentration, but acid methods resulted in loss of short-chain FA. The relationship between *trans*-10 C18:1 and milk fat percentage was investigated using a 2-component model, where one component described the fraction unresponsive to BH-MFD and the other described a responsive fraction that is exponentially related to *trans*-10 C18:1. The 2 fractions were characterized utilizing a Bayesian hierarchical model accounting for between-study variability. The model was defined by the function $f(x, \theta_1, \theta_2, \theta_3) = \theta_1 + \theta_2 \exp(\theta_3)$, where the unresponsive θ_1 fraction was $2.15 \pm 0.09\%$, the responsive θ_2 fraction was $1.55 \pm 0.08\%$, and the exponential term θ_3 was -0.503 ± 0.07

(posterior mean $\pm$ posterior standard deviation from the Bayesian hierarchical model). A Lin's concordance correlation coefficient of 0.67 suggested good agreement between observations and predictions from the Bayesian hierarchical model, computed only with the model's mean population parameters. There was a linear relationship between milk fat concentration and FA <16 C as a percentage of total FA (intercept = 2.68 ± 0.237 and slope = 0.043 ± 0.011 ; coefficient of determination = 0.31). The relationship between milk FA <16 C and milk fat concentration is weaker than what has been published, likely because multiple factors can reduce de novo FA without reducing milk fat and the broad range of diets present in the literature.

Key words: milk fat depression, biohydrogenation, de novo fatty acid, milk fat

INTRODUCTION

Milk fat is a valuable component of milk and is also the most variable and responsive component to nutrition and management interventions. Biohydrogenation-induced milk fat depression (BH-MFD) is characterized as a decrease in milk fat production with no change in milk or milk protein yield and typically occurs when feeding diets that alter rumen fermentation and increase production of alternate *trans*-10 biohydrogenation intermediates (Bauman and Grinari, 2001). Farmers and nutritionists aim to minimize BH-MFD to maximize fat production. The intrinsic responsiveness of milk fat to nutritional and management interventions and its economic value make tools to diagnose BH-MFD and maximize milk fat production extremely valuable.

Milk fat production is highly variable between herds and cows within a herd due to differences in genetics, season, production level, and other factors. In a recent field study of 79 herds in the upper midwest and northeast regions of the United States, McCarthy et al. (2018) reported milk fat percentage averaged 3.43, but ranged from 2.70 to 4.30; milk fat *trans*-10 C18:1 averaged 1.06% of milk fatty acids (FA), but ranged

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from 0.37 to 4.91; and FA <16 C that are de novo synthesized in the mammary gland averaged 22.5% of FA, but ranged from 17.8 to 26.1. They reported an exponential relationship between milk fat concentration and *trans*-10 C18:1, which is an intermediate of the alternate biohydrogenation pathway associated with BH-MFD. Even though *trans*-10 C18:1 does not directly induce BH-MFD (Lock et al., 2007), it is a good proxy for BH-MFD as it is easier to quantify and is the FA most negatively correlated with milk fat concentration (Kadegowda et al., 2008). The relationship between *trans*-10 C18:1 and milk fat concentration has previously been characterized using a 1-pool exponential or a curvilinear relationship (Bauman and Griinari, 2003; Loores et al., 2005; Lock et al., 2007; Kadegowda et al., 2008; McCarthy et al., 2018), but BH-MFD only results in a maximal milk fat reduction of ~50% biologically challenging the fit of a single pool exponential model.

Milk FA in the dairy cow can be categorized according to their biological origin. Fatty acids <16 C originate entirely from de novo synthesis in the mammary gland, FA >16 C originate from uptake of preformed FA from plasma, and 16 C FA originate from both sources. Recent advances in Fourier-transform infrared spectroscopy analysis allow rapid prediction of these categories in milk and has driven interest in the relationship between milk FA profile and milk concentration (Barbano et al., 2014). A positive linear relationship between FA <16 C (de novo FA) and milk fat percentage has been reported, suggesting that a lower concentration of de novo FA is associated with lower milk fat (Barbano et al., 2017). Fatty acids <16 C have also been suggested as a metric for herd health, management, nutrition and lactation performance (Woolpert et al., 2016, 2017). However, these relationships have been mostly developed using contemporary rations in the northeastern USA.

The objective of this study was to characterize the relationship between milk fat concentration and the concentration of *trans*-10 C18:1 and milk FA <16 C across a broad range of diets and conditions. First, a comparison of methylation methods was conducted to support integration of data across the literature. Data from previous experiments from the literature and our research group at Penn State were then used in a meta-analytical approach. We hypothesized that the relationship between milk fat production and milk fat *trans*-10 in dairy cows could be explained using a 2-component model that accounts for the fraction of milk fat synthesis that is not responsive to BH-MFD. Further, we hypothesized that the concentration of FA <16C in milk fat would be linearly related to milk fat concentration, but the relationship would be weaker than previously reported in publications that used data sets with less diversity in diets and management.

MATERIALS AND METHODS

Database Development

A database was developed of experiments reporting milk yield, milk composition, and milk FA profile using modern capillary column procedures from Holstein cows after the transition period. The first 35 publications were compiled by Hinrichsen et al. (2006). Additional papers were identified by reviewing titles in the table of contents of the *Journal of Dairy Science*, keyword searches in Pubmed and *Journal of Dairy Science*, and targeted author search of investigators active in the investigation of milk fat production in dairy cows. Experiments that induced MFD by *trans*-10, *cis*-12 CLA treatments were excluded from the database. The final database included 223 publications and 3 unpublished Penn State experiments with a total of 1,103 treatment means. Hurtaud and Peyraud (2007) was excluded because it reported an extremely high *trans*-10 C18:1 of 11.3% of milk FA if feeding 2 kg/d of camelina seed meal (240 g/d of polyunsaturated FA). Roy et al. (2006) was excluded because it reported a *trans*-10 C18:1 value of 18.6% feeding an extremely low forage diet that was 20.6% NDF and 49.9% starch (a total of 12 treatment means were excluded). Odongo et al. (2007) was excluded because it reported milk FA <16 C at 40.6% of FA if feeding myristic acid (not commercially available) at 5% of the diet, which invalidates the assumption that FA <16 C are only from mammary de novo synthesis (C14:0 increased from 12.8 vs. 30.6%; a total of 2 treatment means were excluded). Furthermore, studentized residuals outside of ± 3.5 and Cook's distance >1 were considered outliers. A complete reference list of all studies in the database and a summary description of unpublished Penn State experiments is provided as Supplemental File S1 and Supplemental Table S1, respectively (<https://doi.org/10.3168/jds.2019-18129>).

Dry matter was recorded as kilograms per day, milk fat as a percentage of milk, milk fat yield as kilograms per day, and milk FA as a percentage of total milk FA. Fatty acids <16 C were calculated as the summation of C6:0, C8:0, C10:0, C12:0, C14:0, and *cis*-9 C14:1. Butyric acid (C4:0) was omitted because of high analytical variation and it originates from both the normal FA synthase dependent lipogenesis pathway and a second pathway that is equivalent of a reversal of β -oxidation (Palmquist, 2009).

Comparison of Fatty Acid Methylation Methods

Multiple protocols were used across experiments in the database for ester derivative formation before FA

profile analysis by GC. Protocols based on methyl ester formation were categorized into 3 broad categories according to the methylation reagent used: sodium methoxide (**NaM**) methylation, acid methylation (e.g., sodium trifluoride or methanolic HCl), and acid-base methylation (e.g., NaM followed by methanolic HCl). Eighty percent of all studies were classified into these categories, with the most common method being NaM-based methylation (58% of all studies). Studies not included in these categories used transesterification protocols based on butyl or isopropyl ester formation or were literature and metanalytic reviews. A method bias was experimentally tested to determine bias especially concerning possible isomerization of *trans*-10 C18:1. Twelve milk fat samples with a known range of *trans*-10 C18:1 concentrations were selected from past studies that investigated BH-induced MFD and were methylated by base methylation with sodium methoxide (Chouinard et al., 1999), acid methylation with methanolic HCl (Sukhija and Palmquist, 1988), and dual acid-base methylation with sodium methoxide followed by methanolic HCl (Jenkins, 2010). Samples were analyzed with a GC equipped with a flame ionization detector as described by Baldin et al. (2018).

Statistical Analysis

The method comparison add-in of JMP Pro 13.2.2 (SAS Institute Inc., Cary, NC) was used to conduct a paired *t*-test to compare methylation methods using NaM-based methylation as the reference method. Because there was a large loss of short-chain FA with the acid and acid-base methylation, the short-chain FA were calculated relative to total FA <16 C to reduce bias during the method comparison. A paired *t*-test was done with the matched pairs platform to analyze the mean difference of each methylation procedure to the reference. A distribution analysis that included mean, median, skewness, standard deviation, and the 10th and 90th percentile was conducted for milk yield, milk fat percentage, milk fat *trans*-10 C18:1, and milk FA <16 C as a percentage of total FA for each of the analysis using JMP Pro 13.2.1.

A Bayesian hierarchical modeling approach was taken to characterize the relationship between *trans*-10 C18:1 and milk fat percentage to allow the specification of a nonlinear model and account for between-study variability. Bayesian hierarchical models are particularly suited for meta-analyses because these models allow individual studies to have their specific set of parameters. These study-specific parameters are constrained to follow a common distribution centered at the vector mean population parameters that is common to all studies.

The mean population parameters can be seen, in the frequentist sense, as representation of fixed effects, and the deviations from these, for each set of study-specific parameters, as the random effects that are allowed to enter the model nonlinearly. The estimated population parameters determine the model that can be used by future model users to understand and predict the relationship between milk fat percentage and *trans*-10 C18:1 as % of milk FA at the population for which the studies are a sample. A total of 117 papers reported 579 treatment means for *trans*-10 C18:1 and milk fat percentage. The model was specified in 3 hierarchies. The first hierarchy specified the model parameters and variances as follows:

$$y_{ij} \sim N[f(x_{ij}, \theta_i), s_{ij}\sigma^2], \quad [1]$$

where y_{ij} is the milk fat percentage for the j th mean ($j = 1, \dots, n_i$) in the i th study ($i = 1, \dots, m$), x_{ij} is *trans*-10 C18:1 as % of milk FA, θ_i is a vector of parameters specific to the i th study, f is the function describing the relationship between y and x , s_{ij} is the scale of the variance set as the centered truncated standard error of the mean (**SEM**) from the original studies to account for heterogeneous error's variances, and σ^2 the variance. Standard errors from studies that reported standard error of the difference or root mean square error were converted to SEM as described by Roman-Garcia et al. (2016). Studies that did not report SEM or information needed to calculate SEM for milk fat percentage were not included in the analysis. The SEM were truncated at the 5th percentile by eliminating the SEM higher and lower than the top and bottom 5th percentile and centered with the division of SEM by the average SEM. A percentile approach was taken rather than truncating to half of the average SEM because the latter resulted in an excessive number of SEM being truncated (53.5% from mixed models and 15.2% from fixed effect models would have been truncated). Truncation and standardization were conducted separately for studies that used fixed and mixed models (184 and 809 treatment means, respectively).

The function f used to model the response is described as

$$f(x_{ij}, \theta_i) = \theta_{1,i} + \theta_{2,i} \exp(-x_{ij} \theta_{3,i}), \quad [2]$$

where $\theta_i = (\theta_{1,i} \ \theta_{2,i} \ \theta_{3,i})^T$ with $\theta_{1,i}$ as a constant term representing the unresponsive fraction, $\theta_{2,i}$ the initial responsive fraction associated with an exponential rate (i.e., at $x_{ij} = 0$), and $\theta_{3,i}$ the exponential rate. The second hierarchy defines a model for between-study variability where $\theta_i = \beta + \mathbf{DLb}_i$. Specifically, β is a vector

of mean population parameters, $\mathbf{D}$ is a diagonal matrix with the coefficient scales, $\mathbf{L}$ is a lower triangular matrix with the Cholesky factors of the correlation matrix, and $\mathbf{b}_i$ is a vector following a normal distribution centered at zero: $\mathbf{b}_i \sim N(\mathbf{0}_3, \mathbf{I}_3)$. In this notation, $\mathbf{0}_3$ is a 3×1 vector of zeros and $\mathbf{I}_3$ is a 3×3 identity matrix.

$$\theta_i = \beta + \mathbf{D} \mathbf{L} \mathbf{b}_i$$

$$\begin{bmatrix} \theta_{1,i} \\ \theta_{2,i} \\ \theta_{3,i} \end{bmatrix} = \begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix} + \begin{bmatrix} \tau_1 & 0 & 0 \\ 0 & \tau_2 & 0 \\ 0 & 0 & \tau_3 \end{bmatrix} \begin{bmatrix} l_{11} & 0 & 0 \\ l_{21} & l_{22} & 0 \\ l_{31} & l_{32} & l_{33} \end{bmatrix} \begin{bmatrix} b_{1,i} \\ b_{2,i} \\ b_{3,i} \end{bmatrix} \quad [3]$$

The third-level hierarchy specifies weakly informative priors used for all parameters. In particular, τ_1 , τ_2 , τ_3 , and σ were assigned half-Cauchy priors with a small scale (i.e., 2.5) setting weakly informative priors (Stan Development Team, 2016a). The Cholesky factors of the correlation matrix were assigned weakly informative priors using LKJCholesky distribution with shape parameter equal to 2 (Stan Development Team, 2016a). Briefly, this distribution is a parameterization of the LKJ correlation matrix density (Lewandowski et al., 2009) in terms of its Cholesky factors (Stan Development Team, 2016a). All models were fitted in Stan using the Hamiltonian Monte Carlo No-U-Turn Sampler (Carpenter et al., 2017) through the rstan package of R version 3.6.1 (Stan Development Team, 2016b). Two chains with over-dispersed initial values were specified for each parameter, chain mixing, autocorrelation, posterior densities, and the Gelman-Rubin diagnostics (Gelman and Rubin, 1992) were used to visually assess chain convergence and determine the required burn-in period. To summarize the Bayesian statistical method, a hierarchical approach was used to define a statistical model that directly accounts for the sources of variation within and between studies in our database and allows the relationship between y and x to be modeled by a nonlinear functional form. For discussion purposes, the relationship between *trans*-10 C18:1 and milk fat percentage without the random effect of experiment was characterized in JMP 13.2.2 in the nonlinear module with the built in exponential 3P model.

The relationship between milk de novo synthesized FA and milk fat percentage was characterized with a linear mixed effects model that contained random intercepts and slopes describing the effects of between-study variability (St-Pierre, 2001). An analysis that used a total of 427 treatment means from 78 papers that used NaM-based methylation was conducted first. The analysis was also done for each individual de novo

FA. Data were analyzed with PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC) with the following model:

$$y_{ij} = \beta_0 + \beta_1 X_{ij} + S_i + b_i X_{ij} + \epsilon_{ij}, \quad [4]$$

where y_{ij} is the milk fat percentage for the j th mean ($j = 1, \dots, n_i$) in the i th study ($i = 1, \dots, m$), β_0 is the overall intercept, β_1 is the slope associated with de novo FA, X_{ij} is the de novo FA, S_i is the random effect of study, b_i is the random slope associated with the i th study, and ϵ_{ij} is the random error component. Weights were used in PROC MIXED to account for heterogeneous error variances. Weighting factors were calculated as the reciprocal of the truncated SEM as described above.

To assess robustness and allow use of the larger database, a second analysis of the relationship between de novo FA and milk fat concentration was conducted using all experiments reporting FA <16 C and included the fixed effect of method as described by St-Pierre (2001). Each of the FA <16 C were first corrected to account for the loss of short-chain FA associated with derivatization protocols that include a prolonged incubation period at elevated temperatures or post-derivatization extraction procedure. The corrected values of C8:0, C10:0, C12:0, C14:0, and *cis*-9 C14:1 were summed together.

Milk fat percentage plotted in figures was adjusted to account the random effect of study as described by St-Pierre (2001). Briefly, the resulting linear model was used to calculate predicted milk fat percentage and residuals for each prediction were calculated and subsequently added to predicted milk fat percentage to obtain adjusted milk fat percentage that accounts for the random effects. In the case of the model that included the fixed effect of method, if heterogeneous slopes were detected (significant interaction between method and predictor) the line for each method was used to predict milk fat percentage. The coefficient of determination for the models were calculated by regressing the adjusted milk fat percentage values by the predictor variable in JMP Pro 13.2.1 using the weighting option.

RESULTS AND DISCUSSION

Comparison of Methylation Procedures

Twelve milk fat samples representing low, mid, and high concentrations of *trans*-10 C18:1 were selected from past experiments and methylated with the 3 most prominent methylations procedures in the database to assess methylation method bias (Table 1). No evidence

was found to suggest that these methylation procedures biases concentration of *trans*-10 C18:1 ($P = 0.69$, acid vs. NaM; $P = 0.67$, acid-base vs. NaM). Therefore, all *trans*-10 C18:1 means available were used to evaluate the relationship between *trans*-10 C18:1 and milk fat percentage. However, there was an effect of the acid and acid-base methylation procedures on concentration of FA <16 C, as these protocols require prolonged incubation periods at elevated temperatures and post-methylation extraction procedures with an aqueous layer. Acid methylation tended to decrease FA <16 C by 1.20% (0.29 percentage units) compared with NaM ($P = 0.06$) and acid-base methylation decreased FA <16C by 26% (6.6 percentage units) compared with NaM ($P < 0.001$). Vapor pressure and normal boiling points, 2 chemical properties directly related to volatility, have an inverse relationship with carbon chain length in FA methyl ester increasing susceptibility of shorter chain fatty acid methyl ester to volatilization (Yuan et al., 2005). The acid methylation procedure resulted in a 12% loss of C4:0 compared with NaM ($P < 0.001$) and acid-base methylation resulted in a complete loss of C4:0 and C6:0 ($P < 0.001$ for both). Our results agree with Ungerfeld et al. (2019), which found an effect of laboratory, defined by the authors as location and research group that conducted the research, and attributed it to the diverse protocols used to analyze milk FA profile. Therefore, the analysis of the relationship was done in 2 ways: (1) using only NaM methylation and (2) using all available treatment means with the fixed effect of method in the model to account for method effects.

Description of the Database

The intraexperimental relationship of milk *trans*-10 C18:1 and FA <16 C with milk fat percentage supports the need to include the random effect of experiment in our statistical models (Figure 1). Including the effect

of experiment in our models accounts for differences in technical accuracy to quantify the C18:1 isomer region in a GC-FID chromatogram within experiments and non-nutritional factors (e.g., genetics, season, substrate supply) that affect milk fat percentage between experiments (Figure 1). Descriptive statistics indicated a range from no to severe BH-MFD in the database, which is important to assessing the relationships with BH-MFD (Table 2). For the *trans*-10 C18:1 analysis, milk yield averaged 33.8 kg/d ($n = 571$ treatment means) and the 10th and 90th percentile were 21.0 and 44.1 kg/d, respectively. Milk fat percentage averaged 3.46% ($n = 579$ treatment means) and the 10th and 90th percentile were 2.67 and 4.18%, respectively. *Trans*-10 C18:1 averaged 1.39% of milk FA ($n = 579$ treatment means) and the 10th and 90th percentile were 0.32 and 3.67% of milk FA. Fatty acids <16 C had a mean of 20.0% of milk FA ($n = 457$ treatment means) with a 10th percentile of 15.0% of milk FA and a 90th percentile of 23.6% of milk FA.

For the de novo FA analysis with only NaM-based methylation studies, milk yield averaged 34.3 kg/d ($n = 423$ treatment means) and the 10th and 90th percentile were 23.6 and 44.3 kg/d, respectively. Milk fat percentage averaged 3.50% ($n = 427$ treatment means) and the 10th and 90th percentile were 2.67 and 4.18%, respectively. FA <16 C had a mean of 20.4% of milk FA ($n = 427$ treatment means) with a 10th percentile of 15.1% of milk FA and a 90th percentile of 24.3% of milk FA. For the analysis with all available treatment means, milk yield averaged 32.7 kg/d ($n = 693$ treatment means) and the 10th and 90th percentile were 21.9 and 43.6 kg/d, respectively. Milk fat percentage averaged 3.56% ($n = 697$ treatment means) and the 10th and 90th percentile were 2.69 and 4.36%, respectively. After correcting for methylation procedure, FA <16 C had a mean of 19.5% of milk FA ($n = 697$ treatment means) with a 10th percentile of 14.3% of milk FA and a 90th percentile of 23.8% of milk FA.

Table 1. Summary statistics of transesterification method comparison for select milk fatty acids (FA)¹

FA ²	1-step vs. NaM				2-step vs. NaM			
	1-step	NaM	SEM	P-value	2-step	NaM	SEM	P-value
C4:0	16.3	18.5	0.09	<0.001	0	18.8	0.97	<0.001
C6:0	9.2	9.1	0.02	<0.001	0	9.2	0.26	<0.001
C8:0	5.1	5.0	0.01	<0.001	1.7	5.1	0.13	<0.001
<i>trans</i> -10 C18:1	2.04	2.05	0.01	0.69	1.94	1.93	0.02	0.67
FA <16 C	24.65	24.94	0.14	0.06	18.57	25.16	0.25	<0.001

¹Sodium methoxide (NaM) methylation was used as the reference method to compare acid methylation with methanolic HCl (1-step) and dual acid-base methylation with NaM followed by methanolic HCl (2-step). Means were compared by a paired *t*-test.

²Individual de novo synthesized FA were calculated relative to total de novo fatty acid content to avoid the bias of loss of fatty acid.

Table 2. Summary statistics of data used in each analysis of relationship between fatty acids (FA) and milk fat concentration

Variable	N ¹	Mean	Median	Skewness ²	SD	Percentile	
						10th	90th
<i>trans</i> -10 C18:1 data set							
Milk yield, kg/d	571	33.8	34.4	−0.40	8.53	21	44.11
Milk fat, %	579	3.46	3.49	0.23	0.60	2.67	4.18
<i>trans</i> -10 C18:1, % FA	579	1.39	0.7	2.08	1.52	0.33	3.67
de novo, % FA	457	20.0	20.5	0.17	3.45	14.99	23.57
De novo NaM data set ³							
Milk yield, kg/d	423	34.3	33.7	−0.07	7.70	23.60	44.3
Milk fat, %	427	3.50	3.57	−0.34	0.55	2.71	4.14
de novo, % FA ⁴	427	20.4	20.9	−0.01	3.68	15.07	24.33
<i>trans</i> -10 C18:1, % FA	326	1.50	0.71	1.86	1.61	0.37	4.15
De novo all-method data set							
Milk yield, kg/d	693	32.7	32.3	−0.05	8.12	21.90	43.60
Milk fat, %	697	3.56	3.60	−0.05	0.64	2.69	4.36
Corrected de novo, % FA ⁵	697	19.5	19.9	−0.02	3.92	14.25	23.82
<i>trans</i> -10 C18:1, % FA	479	1.51	0.70	3.24	1.87	0.32	4.10

¹n = number of treatment means used on each analysis. Total number of treatment means in the database was 1,103 from 227 papers and 3 unpublished Penn State experiments.

²Skewness is a measure of asymmetry of a distribution. Normally distributed data have a skewness of zero. Negative or positive skewness indicate distributions that are skewed to the left or right, respectively.

³Only papers that use sodium methoxide (NaM)-based methylation and reported all FA in summation were included.

⁴Summation of C6:0, C8:0, C10:0, C12:0, C14:0, and *cis*-9 C14:1.

⁵Summation of C8:0, C10:0, C12:0, C14:0 and *cis*-9 C14:1 corrected for the loss of C4:0 and C6:0 associated with analytical methods that include prolonged incubation periods at high temperature. All papers that reported the FA included in the calculation of corrected de novo FA included independent of analytical method.

Relationship of *Trans*-10,C18:1 and Milk Fat Percent

The BH theory of MFD proposes that during altered ruminal fermentation there is a shift in rumen biohydrogenation pathways that results in the production of specific *trans* isomers that inhibit milk fat production (Bauman and Griinari, 2003). *Trans*-10,*cis*-12 CLA has been identified as a bioactive FA that inhibits milk fat

during BH-MFD (Baumgard et al., 2000). There is an established relationship between *trans*-10,*cis*-12 CLA and *trans*-10 C18:1 as *trans*-10,*cis*-12 CLA is a transient intermediate during ruminal biohydrogenation (Bauman and Griinari, 2003). Milk FA profile is rapidly modified within 7 d during induction and recovery of BH-MFD, allowing timely use of milk fatty acid profile as a diagnostic tool (Rico and Harvatine, 2013). The

Figure 1. Relationship between milk fat concentration and milk fatty acid (FA) profile. (A) Intrastudy relationship between *trans*-10 C18:1 and milk fat percentage (126 experiments with 613 means). (B) Intrastudy relationship between de novo FA (Σ C6:0, C8:0, C10:0, C10:1, C12:0, C14:0, C14:1) and milk fat percentage (42 experiments with 455 means). Treatment means within a study are connected by a line and each color represents an individual experiment.

concentration of *trans*-10 C18:1 is much higher than *trans*-10,*cis*-12 CLA in milk increasing quantification accuracy and diagnostic range (0.3–9% of FA vs. non-detectable to 0.1% of FA, respectively), thus it provides more opportunity as a diagnostic tool. A relationship between *trans*-10 C18:1 and milk fat percentage has been reported, but previous approaches have used a single pool exponential model (e.g., Bauman et al., 2006). Biohydrogenation-induced MFD results in up to ~50% maximal decrease in milk fat (Bauman and Griinari, 2001; Baumgard et al., 2001; Harvatine et al., 2009) and a simple exponential model fails to account for the portion of milk fat that is unresponsive to BH-MFD.

The 2-component model used in the current work to describe the relationship between *trans*-10 C18:1 and milk fat percentage models the fractions of milk fat that are responsive and unresponsive to BH-MFD. The

analysis included all the studies that reported *trans*-10 C18:1 because no bias was detected for analytical methods. The unresponsive fraction was 2.51 ± 0.09 , the initial responsive fraction was 1.55 ± 0.08 , and the exponential rate was -0.503 ± 0.07 (posterior means $\pm$ posterior SD; Figure 2). Kadegowda et al. (2008) conducted a break-point analysis of published literature and reported a maximal reduction to 2.58% milk fat when *trans*-10 C18:1 reached 2.52% of FA ($n = 63$), which is a similar value to the unresponsive fraction characterized with the 2-pool model. A Lin's concordance correlation coefficient of 0.67 suggested good agreement between observations and predictions from the Bayesian hierarchical model, computed only with the mean population parameters of the model. In short, the Lin's concordance correlation coefficient measures agreement between 2 variables, with 1 representing perfect agreement and 0 no agreement.

Figure 2. Characterization of the relationship between milk fat percentage, *trans*-10 C18:1, and de novo milk fatty acids (FA; C6:0 to <C16) as a percentage of milk FA. (A) Relationship between *trans*-10 C18:1 and milk fat in the entire data set ($n = 579$ from 117 experiments). (B) Relationship between *trans*-10 C18:1 and milk fat in studies conducted at Penn State showing the individual points and the same fitted model as in panel A ($n = 205$ from 18 experiments). (C) Relationship between de novo synthesized FA (C6:0 to <C16) and study adjusted milk fat concentration of all sodium methoxide methylation-based papers ($n = 427$ from 78 papers). (D) Relationship between de novo synthesized FA (C6:0 to <C16) and study adjusted milk fat concentration in studies from Penn State ($n = 205$ from 18 experiments). The Lin's concordance correlation coefficient (CCC) between observations and predictions or the coefficient of determination calculated by regressing the adjusted milk fat percentage values with their respective Σ de novo values and model root mean square error (RMSE) are shown.

It is important to note that the fraction of milk fat responsive to BH-MFD is smaller than the maximal response reported with purified infusion of *trans*-10,*cis*-12 CLA, which can decrease milk fat to 1.6 percentage units (Baumgard et al., 2001). Interestingly, De Veth et al. (2004) found a better fit between CLA and the decrease in milk fat compared with control with up to a 50% decrease observed. Estimating milk fat *trans*-10,*cis*-12 CLA concentration using the equation reported by Bauman and Grinari (2003), the maximal value of *trans*-10 C18:1 in the current database (7.98% of FA) would predict a 0.12% *trans*-10,*cis*-12 CLA of milk FA ($\text{trans-10, cis-12 CLA} = 0.013(\text{trans-10 C18:1}) + 0.011$). This would correspond to a predicted 27% decrease in milk fat yield using the equations of De Veth et al. (2004) ($\text{Change in milk fat yield} = -49.51 + 51.71e^{(-1.2971 \times \text{trans-10, cis-12 CLA})}$). Similarly, the fraction of milk fat percentage responsive to BH-MFD is estimated to be 1.31% milk fat, which is 34% of total milk fat. For the purpose of discussion, when fitting the nonlinear model with the random effect of study results in an unresponsive fraction of 2.58 ± 0.06 , responsive fraction of 1.71 ± 0.06 (32% of milk fat), and exponential of -0.711 ± 0.08 (parameter $\pm$ SD). Overall the models with and without the random effect of experiment similarly characterize the fraction of milk fat responsive and unresponsive fraction to BH-MFD. According to the current data set, the maximal decrease in milk fat during BH-MFD is much smaller than observed with infusions of *trans*-10,*cis*-12 CLA, possibly due to lower maximal ruminal production of bioactive isomers during BH-MFD.

There was a large variation in milk fat percentage at low levels of *trans*-10 C18:1. Treatment means with *trans*-10 C18:1 below 0.5% of milk FA averaged 3.83% milk fat with a standard deviation of 0.56 and ranged from 1.83 to 5.93%. This was expected as milk fat percentage is multifactorial trait affected by genetics (Grisart et al., 2002; Winter et al., 2002), season (Salfer et al., 2019), level of dietary fat (Palmquist, 2009), fat supplement inclusion (Palmquist and Jenkins, 2017), and acetate supply (Urrutia et al., 2019), among other factors. Furthermore, the effect of these factors on the variation in milk fat is apparent when treatment means from Penn State experiments are plotted alone (Figure 2B). The Penn State experiments were conducted under similar management, diet, and climate conditions and had less variation at low levels of *trans*-10 C18:1. Penn State treatments means with *trans*-10 C18:1 below 0.5% of milk FA averaged 3.79% milk fat with a SD of 0.20 and ranged from 3.33 to 4.25%. Future work may focus on explaining the variation of milk fat percentage in the absence of apparent BH-MFD.

Relationship Between Fatty Acids <16 C and Milk Fat Percent

De novo FA are made through lipogenesis in the mammary gland. Mammary blood flow provides negligible amounts of FA with a chain length below 16 C under normal dietary conditions, thus it is assumed that those FA originate only from de novo origin. The main carbon substrates for de novo lipogenesis in the mammary gland are acetate and BHB, 2 main products of ruminal fermentation (Palmquist et al., 1969). Because of their intrinsic relationship with rumen function and the advent of technology that allows rapid and cost-effective prediction of milk FA categories by Fourier-transform infrared spectroscopy, there has been a recent interest in the relationship of milk FA <16 C with production traits such as milk fat concentration. Specifically, de novo FA concentration as a percentage of milk and milk FA has been shown to be a positive linear predictor of bulk tank milk fat and protein percentage on dairy farm in the northeastern United States (Barbano et al., 2014, 2017; Woolpert et al., 2016, 2017). It is important to note that the concentration of FA <16 C as a percentage of milk and milk fat concentration are not independent, as FA <16 C is an intrinsic component of total milk fat and thus a relationship by regression is expected. However, specific management practices that affect cow behavior and rumen conditions (i.e., feeding frequency, bunk space, physically effective fiber, and ether extract) have been related to milk FA <16 C as a percentage of total FA and farms categorized as high de novo farms had higher milk fat and protein concentrations (Woolpert et al., 2017). Dietary, management, and analytical factors have also been associated with individual de novo FA and the average chain length of de novo FA (Ungerfeld et al., 2019).

The current approach to characterize the relationship between FA <16 C and milk fat percentage used a mixed model approach to introduce random study effects on the intercept and slope, as suggested by St-Pierre (2001). The analysis was performed in 2 ways because of the analytical bias of esterification protocols reported above. First, only treatment means from studies that use NaM-based methylation were used (approximately 58% of all studies). There was a linear relationship between milk fat percent and FA <16 C [intercept = 2.68 ± 0.237 and slope = 0.043 ± 0.011 ; partial R^2 of 0.31 (R^2 does not include variation explained by random effect of experiment)], verifying that de novo FA are a positive linear predictor of milk fat percentage (Figure 2). It is important to note that the relationship in our analysis is weaker than previously reported; for example, Barbano et al. (2017) reported

an R^2 of 0.81 when regressing milk fat percentage and de novo FA as a percentage of milk. This is also weaker than the relationship of some individual de novo FA and milk fat percentage in the current database discussed below (Figure 3). It is important to note that when the random effect of experiment is not considered, FA <16 C are still linear predictors of milk fat percentage, but model parameters and R^2 are different (intercept = 2.03, slope = 0.072, R^2 = 0.24). This supports the value of modeling the random effect of experiment and is in agreement with the variation between studies discussed by Ungerfeld et al. (2019).

A second analysis was conducted that included all available treatment means in the literature corrected to account for the loss of short-chain FA associated with acid methylation procedures. The fixed effect of method

and the interaction of method and the predictor was included in the model. There was a significant interaction ($P < 0.05$) between the individual corrected FA and summation of corrected de novo FA and method for all traits except *cis*-9 C14:1 (*cis*-9 C14:1 $\times$ method; P -value = 0.22) suggesting heterogeneous slopes for each one of the models (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-18129>). However, the magnitude of the differences in the slopes was very small and of little practical importance within relevant methods in the literature (derivatization based on methyl ester formation). Parameter estimates for the NaM method were similar to the above when modeled alone and were selected for plotting to allow comparison between analyses as it is expected to be the method of choice for commercial laboratories (Figure 4). The individual in-

Figure 3. Relationship between individual de novo fatty acids (FA) and milk fat percentage in the sodium methoxide treatment means-only data set ($n = 492$ from 94 experiments). The fitted model for each individual de novo FA is represented as a red line in its respective panel. Milk fat percentage has been adjusted for the random effect of study. RMSE = root mean square error.

Figure 4. Relationship between de novo fatty acids (FA) and milk fat percentage in the data set including all methylation methods (180 experiments, 844 means). Milk FA were corrected for the effect of methylation method and heterogeneous slopes were detected for each derivatization method, although the difference was of small magnitude within methylation-based protocols. The model relationship for sodium methoxide methylation is shown as a red line within each panel. Milk fat percentage is plotted adjusted for the random effect of study. RMSE = root mean square error.

tercepts and slopes for each method are reported in the supplement material (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-18129>).

Gas chromatography allows quantification of individual de novo FA in milk with the caveat of a more involved sample preparation procedure and longer measuring time, thus a higher cost per sample compared with Fourier-transform infrared spectroscopy. Relationships between individual short- and medium-chain FA and milk fat percentage were explored in a similar fashion as above. The similar results were obtained when modeling only NaM methylated data and the complete

data set. Each one of the saturated de novo synthesized FA were linearly related to milk fat percentage and many had stronger relationships than total de novo FA. For example, C6:0 and C8:0 had the strongest relationship with milk fat percentage with an R^2 of approximately 0.70, indicating that both models accounted for ~70% of total variation, in NaM data (R^2 of 0.66 with all treatment means; panel A and B in Figure 3 and panel A in Figure 4). The exact mechanism explaining these stronger relationships is hard to determine as modification of the factors affecting elongation of FA during de novo lipogenesis in the mammary gland

by FASN are unclear (Palmquist, 2009). Future work may focus on exploration of the relationship between dietary factors and production of specific de novo FA. For example, Glasser et al. (2008) reported a decrease in short- and medium-chain FA in response to increasing supplementation of oil seeds rich in C18 FA.

CONCLUSIONS

The relationship between *trans*-10 C18:1 and milk fat percentage was described by a 2-component model that considers the responsive and nonresponsive portions of milk fat production to BH-MFD. The fraction of milk fat responsive to BH-MFD described by this model is lower than what has been reported with infusion of purified CLA isomers. The fraction of milk fat responsive to BH-MFD is estimated to be 34%, less than the ~50% maximal reduction reported with *trans*-10, *cis*-12 CLA infusions. Fatty acids <16 C were also linearly related to milk fat percent, but the relationship was weaker than previously reported. This is presumably because de novo FA can also be affected by numerous factors other than BH-MFD. Furthermore, some individual de novo fatty acids have stronger relationships with milk fat percentage than the summation of de novo FA. As milk fatty acid profile becomes a more available tool, the relationship between *trans*-10 C18:1 and milk fat described can be used to differentiate BH-MFD from other causes of low milk fat production in dairy cows and predict milk fat percentage from milk FA profile. For example, the expected milk fat concentration can be predicted from *trans*-10 C18:1 and compared with actual milk fat concentration to determine what portion of a milk fat decrease is due to alternate biohydrogenation. The reported relationships between FA <16 C and milk fat concentration support a relationship, but highlight that multiple factors contribute to the concentration of de novo FA.

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