Identification of high and low branched-chain fatty acid producing phenotypes in Holstein cows following high forage and low forage diets in a cross-over designed trial\textsuperscript{1,2,3}

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John W. Finley is an Editor on Current Developments in Nutrition and played no role in the Journal’s evaluation of the manuscript.

\textsuperscript{1}Funding: This work was funded by USDA-ARS Projects 3062-53000-001-00D (MJP), 5090-31000-025-00D (KK), 80-8040-05-01-0000-0000 (JH, NKF) and supported by the USDA-ARS Grand Challenge Synergy project “Dairy Agriculture for People and Planet”.

\textsuperscript{2}Conflicts of interest: Matthew J. Picklo, Kenneth F. Kalscheur, Andrew Magnuson, Michael R. Bukowski, James Harnly, Naomi K. Fukagawa, and John W. Finley have no conflicts of interest.

Running Head: BCFA content in milk

Data share statement: The fatty acid data described in the manuscript will be made publicly and freely available without restriction as a supplemental data file with this manuscript.
List of Abbreviations: BCFA, branched-chain fatty acids; DIM, days in milk; HF:C, high forage and low concentrate; (LF:C), low forage and high concentrate; LCSFA, long chain saturated fatty acid; OCFA, odd-chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acid; sPLSDA, sparse partial least squares discriminant analysis; SFA, saturated fatty acid; 13-Me14:0, 13-methyltetradecanoic acid (13-Me14:0; iso 15:0), 12-Me13:0, 12-methyltridecanoic acid (iso 14:0); 13-Me15:0, 13-methylpentadecanoic acid (anteiso 16:0); 14-Me15:0 14-methylpentadecanoic acid (iso 16:0).

Abstract (296 words)

Background: Branched chain fatty acids (BCFA) are rumen-derived fatty acids comprising about 2% of bovine milk fatty acids. BCFA possess anti-inflammatory properties and enriching the BCFA content of bovine milk may provide human health benefits.

Objective: We determined whether forage content impacts the BCFA content of milk from Holstein cows and identified fatty acid phenotypes in high vs low BCFA-containing milks.

Methods: Holstein cows (n = 62), fed for 67 days in a cross-over design, consumed a diet with high forage and low concentrate (HF:C) and a diet with low forage and high concentrate (LF:C). Milk samples were collected at the end of each treatment period and fatty acid content determined. Paired t-tests, one-way ANOVA, sparse partial least squares discriminant analysis (sPLSDA), and Pearson’s correlation analysis were used to analyze the data.

Results: The total milk fatty acid concentration for cows fed the HF:C diet was greater than that of cows fed the LF:C diet (4.2 ± 0.7 g/100 mL vs 3.9 ± 0.9 g/100 mL). sPLSDA demonstrated separation of the dietary treatments, with BCFA and odd-chain fatty acids as primary determinants. Total BCFA content in milk was elevated by HF:C intake vs LF:C intake (1.80...
Quintile separation of high vs low BCFA milks resulted in 4 groups, HF:C /low BCFA, HF:C /high BCFA; LF:C /low BCFA, and LF:C /high BCFA. Milks from the high BCFA quintiles had lower palmitic acid content (29.6% vs 34.4%) but higher oleic acid content than milks from the low BCFA quintiles (19.7% vs 17.0%). Some cows were identified as high BCFA producers or low BCFA producers regardless of diet.

Conclusions: BCFA content of milk is diet-sensitive but variation in responses exists. The potential to produce milk with high BCFA content and lower SFA content needs further study.

**Summary:** Branched chain fatty acid (BCFA) content of milk from Holstein cows is diet-sensitive but modified by other factors. BCFA content is negatively associated with palmitic acid content in milk fat.

**Keywords:** Branched chain fatty acids, saturated fatty acids, forage, dairy
Introduction

Bovine milk fat is a complex mixture of mammalian and microbial rumen-derived fatty acids, incorporated mainly into triacylglycerols, comprising > 95% of total milk fat, and phospholipids (1). Of the microbial-derived lipids in bovine milk, branched-chain fatty acids (BCFA) have been studied extensively because of their ruminal genesis, providing insight into metabolic dynamics of the rumen, its microbial populations, and host interactions (2-6). BCFA constitute about 2% of total bovine milk fatty acids but are observed at different concentrations in the milk from other ruminal species (7, 8).

BCFA are comprised of a series of fatty acids in which a methyl group is present at either the second to last carbon of the fatty acid chain, an iso BCFA, or the third to last carbon of the fatty acid chain, an anteiso BCFA [for reviews see (5, 9, 10)]. These fatty acids are derived from the amino acids leucine and valine (for iso BCFA) and isoleucine (for anteiso BCFA) that are metabolized to their respective CoA derivatives and serve as the starting point for fatty acids elongation. Labeling studies indicate that this synthetic process occurs in the rumen versus the mammary gland of the cow (5). A number of rumen bacteria including Ruminococcus flavefaciens, R. albus, Butyrivibrio fibrisolvens, and Prevotella contain substantial quantities (>50% of total fatty acids) of BCFA (5, 9). The ruminal content of these bacteria are influenced by the number of days in milk (DIM) and the amount of forage in the diet (11, 12).

BCFA have unique physicochemical and biological properties. Data indicate that BCFA allow for enhanced membrane fluidity while being resistant to oxidative damage (9). Recent data indicate that BCFA have anti-inflammatory properties in the gut. BCFA reduce necrotizing enterocolitis in when fed to neonatal rats and reduce the expression of IL-8, a pro-inflammatory cytokine, in intestinal epithelial cells in vitro when applied at a 25 µmol/L concentration (13-15). Other data indicate that BCFA may have anti-tumorigenic activity in vitro; however concentrations between
40 µmol/L and 200 µmol/L are needed depending the cell line (16-18). It is estimated that people eating beef and dairy consume approximately 500 mg of BCFA daily, roughly 2 mmol based upon the mass of 16:0 (7). Enriching the BCFA content of bovine milk may provide health benefits for human consumption, akin to enrichment of n-3 polyunsaturated fatty acids (n-3 PUFA) in foods.

The BCFA content in bovine milk, through modifying ruminal metabolism, can be manipulated by feed [see reviews (5, 10)]. Elevated fiber content of the diet yields higher BCFA generation (19). Accordingly, a high forage:concentrate (HF:C) ratio feed will yield an increase in BCFA versus a low F:C (LF:C) ratio feed, and will also influence the production of iso BCFA vs anteiso BCFA (6, 12). Feeding studies also demonstrate that maize silage vs grass silage modifies BCFA content in a BCFA-dependent manner (5, 20). Other data indicate that breed and DIM modify the BCFA content in milk (21).

A potential means of BCFA enrichment is to identify the extent to which cows exhibit high vs low BCFA content in their milk under controlled dietary conditions. In this work using a large experimental cohort of Holstein cows, we examined the extent to which dietary conditions (HF:C vs LF:C diets) impacted the BCFA content of milk in Holstein cows, explored the variability of BCFA concentration, and identified other dairy fatty acids that were associated with BCFA content.
Methods

Experimental Design

This study was conducted at the USDA-ARS U.S. Dairy Forage Research Center Dairy Farm (Prairie du Sac, WI, USA) under protocols approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. The study initially included 76 lactating Holstein cows. Among those 76 cows, 3 cows were removed for health reasons and the data of 11 cows were removed because of technical issues related to accurately estimating their feed intake.

The study was a crossover design starting with a pre-experimental period of 31 days, where all cows were fed a common pre-experimental diet, followed by two experimental periods (HF:C or LF:C treatments) of 56 d each where the first 11 d were used for diet transition; each period was 67 d in total. The common diet fed to all cows during the pre-experimental period was formulated to be half-way between both experimental diets, and included 27.9% corn silage, 29.0% alfalfa silage, 12.2% high moisture corn, 4.1% roasted soybeans, 5.9% beet pulp, 6.1% soybean hulls, 6.1% canola meal, 6.0% corn distiller’s grain with solubles and 2.7% minerals and vitamins mix. After the conclusion of the pre-experimental period, cows were assigned to either: 1) a high-forage, low-concentrate (HF:C) diet, or 2) a low-forage, high-concentrate LF:C diet. The LF:C diet was formulated to have a forage:concentrate ratio of 47:53, a starch concentration of 27.1% (dry matter (DM) basis) and a neutral detergent fiber concentration (NDF) of 29.0% (DM basis), whereas the HF:C diet was formulated to have a forage:concentrate ratio of 66:34, a starch concentration of 12.9% (DM basis) and NDF concentration of 36.9% (DM basis). All diets were similar for crude protein (CP) and were slightly different in metabolizable energy. All diets had the same ingredients but differed by the percentage of ingredients in each diet (Table 1). The fatty acid content of the diets is presented in Supplementary Table S1.
The first experimental period (P1) started on December 11th 2017 and ended on February 4th 2018, whereas the second period (P2) started on February 19th 2018 and ended on April 15th 2018. During the first three days of the transition period, cows were gradually adapted from the previous diet to the new diet with a daily addition of 25% of the next diet and 25% removal of the previous diet. The cows were assigned to 2 cohorts based on parity, dry matter intake, net energy in milk, body weight (BW), body condition score, BW loss, and BW gain estimated over the first 3 weeks of the common diet period. DIM was on average 136 ± 23 d (mean ± standard deviation) for one cohort and 139 ± 29 d for the second cohort at the start of the first experimental period. Starting mean BW was 645 kg and 652 kg for the two cohorts. The current study is based on the data of 62 cows (29 primiparous and 33 multiparous cows) with 14 and 15 primiparous cows distributed into the two cohorts, respectively.

**Sample Collection and Analysis**

Forages were sampled once a day and concentrates were sampled once a week to measure weekly DM. Feed samples used for DM determination were dried 48 h at 55°C, followed by 24 h at 105°C. Feed samples used for chemical analysis were dried 48 h at 55°C and ground through a 1-mm screen (Wiley mill, Arthur H. Thomas Co., Philadelphia, PA). Diets were composited on a DM basis into 4-wk samples for each experimental period. All feed samples were analyzed for nutrient composition by Dairyland Laboratories Inc. (Arcadia, WI). Ash (method 942.05), crude protein (method 990.03), neutral detergent fiber determined gravimetrically using heat-stable α-amylase and sodium sulfite (method 2002.04), acid detergent fiber and lignin (method 973.18), ether extract (method 920.39) and starch concentration (method 996.11) were determined using methods described by AOAC (2005) (22).

Cows were housed in individual tie-stalls and were fed *ad libitum* once a day, adjusting offered feed to allow for approximately 5% refusals. Individual intake was determined daily. Cows were
milked three times a day (04:00, 10:30 and 18:00), with milk production being recorded at each milking. Milk samples were collected at the 04:00 milking for 2 consecutive days on day 27 and 28 of each experimental period and saved for later analysis.

**Fatty acid analysis**

*Milk.* Individual milk samples were analyzed for fatty acid content in duplicate using fatty acid methyl ester analysis (FAME). A direct-dilution and transesterification methods based upon that of LePage and Roy was used (23). Nonadecanoic acid (19:0, 150 µg; Nu-Chek Prep. Inc., Elysian, MN) was added as an internal standard to 100 µl of milk in a 13 x 100 mm glass screw top test tube. Transesterification reagent (methanol:acetyl chloride 19:1 v/v, 2mL) was added to the milk samples. Tubes were capped, sealed with Teflon® tape, and placed on a rotator overnight (~16 hours) at 37°C. The reaction was quenched with 0.5 ml of 1.4 M potassium carbonate. Hexane (1 mL) was added, and the samples were vortexed for 30 seconds then centrifuged 2000 x g for 15 minutes at room temperature. The top organic layer (hexane) was removed and the fatty acid content of the organic extract and its resulting fractions were determined using gas chromatography with flame ionization detection (GC-FID). Whole milk powder NIST® SRM® 1549a (Sigma, St. Louis, MO) was processed and measured with each set of samples as an external quality control. Samples were quantified as grams/100 mL of milk and subsequently processed as the percent of the total fatty acid concentration. Nonadecanoic acid, methyl nonadecanoate, fatty acid standard mixtures GLC 462 and GLC 569 were purchased from Nuchek Prep. (Elysian, MN, USA) and used for determination of retention times and response factors for quantitation. BCFA (14-methylhexadecanoic acid (14-Me16:0), 12-methyltridecanoic acid (12-Me13:0), 16-methylheptadecanoic acid (16-Me17:0), 18-methyleneicosanoic acid (18-Me20:0), 14-methylpentadecanoic acid (14-Me15:0), 12-methyltetradecanoic acid (12-Me14:0), 15-methylhexadecanoic acid (15-Me16:0), 13-methyltetradecanoic acid (13-Me14:0) were ordered from Matreya LLC, (State College, PA.)
and Larodan AB, (Solna, Sweden) In cases where a purified standard was not available, carbon number and desaturation level were confirmed by GCMS and double bond position was inferred by retention time based upon literature precedent (24-26).

In order to identify species that were not covered in the standard set, fatty acid methyl esters from a random subset of samples were also analyzed by GMCS. Samples were analyzed on a Shimadzu 8040TQ GCMS (Shimadzu Scientific Instruments, Columbia, MD, USA) with a 75 m x 0.18 mm x 0.14 µm SP-2560 capillary column (Sigma Aldrich, St. Louis, MO, USA) using helium carrier gas with a flow rate of 1.71 mL/min on-column (linear velocity = 30 cm/sec). Samples were injected via autosampler (1 µL) into an injector operating at 250 °C with a 10:1 split. The temperature profile was: 50 °C, hold 1 minute; ramp to 160 °C at 6 °C/min, hold at for 6 min; ramp to 240 °C at 5 °C/min, hold 4 minutes. The ion source temperature and interface temperature were 270 °C and 250 °C, respectively, and the source was operated in electron impact mode (70 eV). Scans from m/z = 45 to 500 were collected. Unknown species were identified by spectral similarity search using the NIST 11 Version 2 Mass Spectral Library (NIST, Gaithersburg, MD, USA.)

Diet. The fatty acid composition of the diets were determined using a modification of a previously used method (27). Approximately 1 g of diet, in duplicate, was extracted with hexane :isopropanol (10 ml, 3:2, v/v) (HIP) containing 50 µM butylated hydroxytoluene on a rotator overnight at room temperature. Samples were centrifuged (2000 x g, 10 m, 23°C) and the supernatant was placed in a new glass test tube. Additional HIP (10 mL) was added to the pellet which was then resuspended by vortexing and centrifuged. The supernatant was removed and combined with the original supernatant. Combined supernatants were dried under argon at which point 2 ml HIP was added for storage at -20°C. Samples were removed from freezer,
warmed to room temperature, centrifuged and the supernatant was transferred to a new tared glass test tube. Remaining pellet (chlorophyll) was rinsed with 2 ml HIP, centrifuged, and the supernatants were combined. Combined supernatants were dried under argon and remaining lipid was weighed. Lipid was then diluted with 5 ml hexane.

Diet fatty acid composition was determined by transesterification of extracted lipids in acidified methanol (27). FAME derivatization was performed on 50 µl of diluted lipid sample by the addition of FAME reagent (2 mL, anhydrous methanol: acetyl chloride, 19:1, v/v) and 50 µL of 3 mM nonadecanoic acid in anhydrous methanol as an internal standard. Samples were incubated overnight at 35°C. The reaction was quenched with 500 µl of 1.4 M potassium carbonate solution. Fatty acid methyl esters were extracted in 2 mL hexane. Resulting FAME were quantified by GC-FID.

**Data analysis.**

Fatty acid composition (expressed as percent of total fatty acid concentration) from the two sequential sampling days per treatment period were averaged. Resulting data for each treatment period were analyzed with MetaboAnalyst software, using a paired t-test with a false discovery rate (FDR) applied (28, 29). Data were normalized using Pareto scaling. In order to further refine our understanding of this variability, values for total BCFA content within each treatment were sorted into quintiles from highest to lowest concentration of BCFA. The milk samples with the highest and lowest 20% BCFA values \((n = 12)\) from each of the HF:C and the LF:C treatments were identified. Following this segregation of cows into high- vs low-BCFA producers for each treatment, data were compared using a one-way ANOVA with Tukey’s posttest with an FDR applied using the MetaboAnalyst software. \(P < 0.05\) was taken as statistical significance. Relationships and differences between treatments were explored using
Pearson’s correlation analysis and sparse partial least squares discriminant analysis (sPLSDA), respectively.

**Results**

FAME analysis quantified 74 fatty acids ranging from butyric acid (4:0) to docosahexaenoic acid (22:6n-3). The total fatty acid concentration of the HF:C treatment was greater than that of the LF:C treatment (4.2 ± 0.7 g/100 mL vs 3.9 ± 0.9 g/100 mL). Values for all milk fatty acids are provided in *Supplementary Data File S1*. Subsequent analyses of fatty acid differences were based upon concentration values.

Sparse partial least squares discriminant analysis (sPLSDA) was used to assess differences in fatty acid composition between the two treatment groups (Figure 1). sPLSDA demonstrated clear separation of the dietary treatments (Figure 1A). Component 1-based separation was mainly due to elevations in cis-vaccenic acid (18:1n-7,2), the odd chain fatty acids (OCFA) 13:0, 11:0 and 9:0, and CLA derivatives by the LF:C treatment and increases in 16:1n-2 and the BCFAs 13-methyltetradecanoic acid (13-Me14:0; iso 15:0), 12-methyltridecanoic acid (12-Me13:0, iso 14:0) and 13-methylpentadecanoic acid (13-Me15:0; anteiso 16:0) by the HF:C diet (Figure 1B). Component 2-based separations were due mainly to elevations in multiple OCFA by the LF:C treatment (Figure 1C).

Given the identification of BCFAs and OCFA as determinants of treatment separation, the values for individual and total BCFAs and OCFA are presented in Table 2. Total BCFA content of milk was elevated by HF:C diet (1.80 ± 0.13 %) vs LF:C diet (1.68 ± 0.14%); whereas total OCFA content was elevated by the LF:C diet (2.58 ± 0.52 %) vs HF:C diet (2.26 ± 0.29 %). The most abundant BCFA in both treatments were 14-Me16:0 and 15-Me16:0, comprising approximately 24% of total BCFA, respectively, but these BCFAs did not change by treatment. On the other
hand, the less abundant BCFA were elevated by the HF:C treatment over the LF:C treatment. The OCFA 15:0 comprised approximately 53% of the total OCFA for both treatments and was decreased by the HF:C treatment compared to the LF:C treatment. The smaller, minor abundance OCFA 7:0, 9:0, 11:0, and 13:0 were decreased by HF:C intake. 23:0 was elevated by HF:C diet compared to LF:C diet.

Plotting the total BCFA content in the resulting milk demonstrated overlap between treatments. In the HF:C treatment, BCFA content ranged from 1.53% to 2.34% (mean of 1.80%) of total fatty acids and 1.39% to 2.14% (mean of 1.68%) for the LF:C treatment (Figure 2A). In order to further refine our understanding of this variability, values for total BCFA content within each treatment were sorted into quintiles. The resulting samples (n = 12) from the top and bottom quintiles for each treatment yielded four groups, HF:C diet/low BCFA, HF:C diet/high BCFA; LF:C diet/low BCFA, and LF:C diet/high BCFA. The total BCFA content of the low BCFA milk was elevated by the H:FS diet vs the LF:C diet, 1.63 ± 0.05% vs 1.50 ± 0.04%, respectively (Figure 2B). Similarly, the total BCFA content of the high BCFA milk was elevated by the H:FC diet vs the LF:C diet, 1.89 ± 0.10% vs 1.99 ± 0.11%, respectively. Elevated content was observed for 15-Me16:0, 14-Me16:0, and 12-Me14:0. 12-Me13:0, 13-Me14:0, and 14-Me15:0 were elevated in the HF:C/high BCFA samples vs the other groups. 16-Me18:0 and 15-Me18:0, were not different between quintiles (Table 3).

In order to evaluate whether other fatty acids segregated within these quintile responses, sPLSDA was performed. Four distinct groups were apparent following sPLSDA (Figure 2C). Along component 1 of separation, total BCFA content and a number of BCFA including total BCFA were identified as main determinants of separation as expected (Loadings 1, Figure 2D). 18:0, a long chain saturated fatty acid (LCSFA), was determined as a component 1 separation factor via its elevation in the high BCFA containing milk. Component 2 yielded separation based upon the HF:C and LF:C diet treatments, not BCFA content. The separation along component 2
was due to differences in the content of a number of fatty acids including MUFA and PUFA (Loadings 2, Figure 2E). Albeit smaller in impact, lower concentrations of total LCSFA, defined as the sum of 14:0, 16:0, 18:0 20:0, 22:0, and 24:0, were identified as lower in the high BCFA-containing vs low BCFA-containing milk.

Because of the potential relationships of total LCSFA and 18:0 with total BCFA, a Pearson’s correlation analysis was performed. Table 4 highlights the associations determined between LCSFA, and major monounsaturated fatty acids (MUFA), total BCFA. These LCSFA and MUFA comprise >70% of the total fatty acid content of the milk. The mean total LCSFA concentration was lower in the high BCFA milk than the low BCFA milk. This decrease was driven by the decrement in 16:0 in the high BCFA milk (30.2 ± 4.0% and 28.9 ± 2.3% for LF:C and HF:C, respectively) than the low BCFA milk (33.9 ± 4.0% and 35.0 ± 1.8% for HF:C and LF:C, respectively). On the other hand, 18:0 (stearic acid) was elevated by intake of the HF:C diet as well as by high BCFA content.

Conversely, oleic acid content was elevated with mean value of 19.7% in the high BCFA milk versus 17.0% in the low BCFA milk. Concentrations of palmitoleic acid 16:1n-7 were not different between BCFA quintiles; however, the concentrations of cis-vaccenic acid (18:1n-7Z) were elevated by the LF:C intake. Complete fatty acid concentrations of the highest and lowest BCFA quintiles for HF:C and LF:C treatments and the Pearson’s correlation values are provided in the Supplementary Data File S1.
Discussion

Recently, BCFA have gained additional attention as potential bioactive fatty acids present in dairy products that may provide additional nutritional benefit to dairy intake [see current review (10)]. The objective of this study was to define the impact of HF:C (low starch) vs LF:C (high starch) diets fed to Holstein cows upon the BCFA content of resulting milk and then determine the fatty acid differences between high and low BCFA-containing milk. Our data demonstrate that cows fed the HF:C diet had a higher mean BCFA content in the resulting milk overall, but that a large amount of overlap remained between dietary treatments. Following quintile distribution based on the BCFA content of HF:C and LF:C milk, similar amounts and distributions of BCFA were found in the highest BCFA-containing milk fat regardless of forage content. It also was observed that the milk with a higher BCFA composition possessed a higher amount of oleic acid and a lower amount of palmitic acid than low BCFA-containing milk.

_In vitro_ studies demonstrate an anti-inflammatory effect of multiple BCFA in enterocyte models at concentrations of 25 µmol/L, and these data indicate BCFA may have differential effects upon blocking IL-8 expression vs NF-κB expression (13). Our data show that the content of several BCFA having _in vitro_ efficacy can be elevated in milk by feeding a high-forage diet or by selection of high-BCFA producing cows. Of these, 12-Me14:0, 14-Me16:0, and 15-Me16:0 have the highest abundance and comprise 1.3% of the total fatty acids in milk in the high-BCFA containing milk from cows fed the high-forage diet. Administration of BCFA in vivo to rat pups at a concentration of 20% w/w of an artificial rat formula reduced the severity of necrotizing enterocolitis in a rodent model of necrotizing enterocolitis (15). We note however that subsequent studies are needed to determine the necessary dietary intake of BCFA for in vivo efficacy in other inflammatory states such as obesity.
Individual and total content of BCFA of the milk are comparable with that described in previous studies (3, 7, 21). The elevation of BCFA content in milk resulting from cows fed the HF:C diet agrees with previous studies (6, 12). Given the larger dataset (n = 62) with which to work, we were able to examine the extent to which differences in fatty acid concentrations occurred between milk that contained the highest vs lowest quintile of BCFA concentrations. The BCFA of 16 carbons and less, 12-Me13:0, 12-Me14:0, 13-Me14:0, 14-Me15:0, 13-Me15:0, and were elevated by intake of the HF:C diet. As expected, this diet-dependence remained following quintile separation. On the other hand, 14-Me16:0 and 15-Me16:0 (the most abundant BCFA) were not different between diet treatments when comparing the entire cohort (Table 2) but were greater in the high BCFA quintiles vs the low BCFA quintiles independent of diet (Table 3). The elevations in these BCFA may be result of additional synthesis of these BCFA in the bovine mammary gland or enhanced elongation of ruminal BCFA by the cow given that BCFA are substrates for mammalian fatty acid elongases (2, 30).

Our data indicate that milk with a higher nutritional quality may be selected on the basis of BCFA content and lower SFA content. The high BCFA-containing milk had approximately 10% lower concentrations of long chain SFA, primarily 16:0 palmitic acid, versus the low BCFA milk regardless of diet treatment. The Dietary Guidelines for Americans recommend lowering intakes of SFA (31). We have previously demonstrated that dairy is a significant contributor to SFA intake (32). The main SFA, 16:0, is viewed as contributing to CVD risk via elevation of LDL-cholesterol and mechanistic data indicate that 16:0 is a pro-inflammatory fatty acid through specific interaction to the Toll-like receptor 4 (TLR4) and TLR2 (33). milk. It is clear however, that the impact of SFA upon disease risks is dependent on the type and amount of amount of SFA eaten, the overall diet context, and, likely, genetic disposition (34). For example, increasing evidence demonstrates that LCSFA types have differing physiologic effects.
18:0 is a LCSFA, clinical studies have demonstrated a neutral effect of 18:0 upon LDL-cholesterol concentrations [(35, 36) and references therein].

That the reduction in SFA was also observed with increases in oleic acid may have functional consequences for human intake. A recent study by Vasilopoulou and colleagues demonstrated that intake of dairy products with a higher oleic acid content reduces indices associated with elevated CVD risk vs dairy products with higher SFA content (37). The elevation of oleic acid in the high BCFA containing milk suggest an increase in stearoyl CoA desaturase (SCD) activity either in the liver or mammary glands. SCD activity is contributor to the production of oleic acid milk. Variation and heritable elevations in SCD activity are documented in Holstein-Friesian, Jersey, Piedmontese and Valdostana dairy breeds, and also in Wagyu and Hanwoo beef breeds (38-42). The discrepancy between differences in oleic acid but not palmitoleic acid (16:1n-7) agree with those reported by Garnsworthy and colleagues (42). This difference may be the influence of SCD activity in the mammary gland vs the liver (43). Heritable changes in milk lipid composition is also documented with variations in the bovine SREBF1 gene whose protein is a transcription factor regulating the expression of lipid synthesis genes (44). The mechanisms linking elevated oleic acid to BCFA content are not clear, but our data lead us to speculate the existence of microbial influences upon bovine fatty acid metabolism as demonstrated in mice or that elevations in host oleic acid production elevate ruminal production of BCFA (45).

Our analyses demonstrated that a small subset of the cows in the study were inherently either high BCFA or low BCFA producers. Following quintile separation, 4 cows were identified as low BCFA producers and 5 cows as high BCFA producers regardless of the HF:C, LF:C, or the pre-experimental lead-in diets (Supplementary Data File S1). These data suggest the potential for selecting cows as high BCFA producers. To our knowledge, this has not been observed or explored. The underpinnings of this observation, for example differences in ruminal microbial
populations, remain to be determined. Our current study is limited in that examination of the ruminal microbiome was not performed.

Recent studies have characterized breed-type dependency and DIM-dependency for BCFA production in Holstein, Jersey and first generation Holstein x Jersey crossbreeds cows fed an HF:C diet (11, 21). Jersey cows produce milk with a higher content of BCFA than Holstein cows or crossbreeds and increases in BCFA occur with days in milk for Jersey but not Holstein cows (21). The extent to which different breeds may have cows that produce milk high BCFA production and lower SFA regardless of forage concentration or type needs more study. Our findings support the importance of performing cross-over design trials with larger cohorts of animals in order to identify and provide selection for phenotypic traits related to the nutritional quality of milk.

Acknowledgments

USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The authors thank Joseph Idso, Laura Idso, and Benjamin Hanson for their technical assistance. M.J.P. and K.F.K. designed research; M.J.P. and K.F.K. conducted research; M.J.P., M.R.B., and A.D.M. analyzed data; and M.J.P, K.F.K, A.D.M., M.R.B, J.H., N.K.F, and J.W.F wrote the paper. M.J.P. had primary responsibility for final content. All authors read and approved the final manuscript.
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Table 1. Ingredient and chemical composition of experimental diets.

| Ingredients (% of diet DM) | High F:C¹ | Low F:C |
|----------------------------|------------|---------|
| Alfalfa silage             | 33.9       | 24.2    |
| Corn silage                | 32.4       | 23.2    |
| High moisture corn         | 0          | 24.5    |
| Beet pulp pelleted         | 8.9        | 2.8     |
| Canola meal                | 2.7        | 9.7     |
| Corn distillers grain      | 9.2        | 2.7     |
| Roasted soybean            | 4.1        | 4.1     |
| Soybean hulls              | 6.1        | 6.1     |
| Mineral and vitamin mix²   | 2.7        | 2.7     |

| Chemical composition (% of diet DM) | High F:C¹ | Low F:C |
|-------------------------------------|------------|---------|
| DM, % of diet                       | 45.9       | 50.1    |
| CP                                  | 16.5       | 16.2    |
| NDF                                 | 36.9       | 29.0    |
| Forage NDF                          | 24.9       | 17.7    |
| ADF                                 | 27.4       | 21.1    |
| Lignin                              | 3.9        | 3.0     |
| Ether extract                       | 5.1        | 4.9     |
| Ash                                 | 7.4        | 5.7     |
| Starch                              | 13.0       | 26.7    |

¹ ADF, Acid detergent fiber; CP, Crude protein, DM, Dry matter; F:C, Forage:concentrate ratio; NDF, Neutral detergent fiber
²The mineral and vitamin mix contained (on a DM basis): 16.0% Ca, 5.85% Mg, 0.54% K, 14.8% Na, 6.67% Cl, 0.73% S, 42.5 mg of Co/kg, 519 mg of Cu/kg, 60.2 mg of I/kg, 778 mg of Fe/kg, 2.601 mg of Mn/kg, 14.6 mg of Se/kg, 2.808 mg of Zn/kg, 292 kIU of vitamin A/kg, 58.5 kIU of vitamin D/kg, 1.36 kIU of vitamin E/kg, and 0.494 g of monensin/kg (Vita Plus Corporation, Madison, WI).
Table 2. Impact of low forage and high forage diets on BCFA and OCFA content in milk

| Fatty acid                                      | Low F:C (n = 62) | High F:C (n = 62) |
|------------------------------------------------|------------------|-------------------|
| **BCFA**                                        |                  |                   |
| 12-Methyltridecanoic acid (iso 14:0)            | 0.079 ± 0.026\(^1\),* | 0.107 ± 0.023     |
| 13-Methyltetradecanoic acid (iso 15:0)          | 0.17 ± 0.02*     | 0.21 ± 0.02       |
| 12-Methyltetradecanoic acid (anteiso 15:0)      | 0.34 ± 0.04*     | 0.36 ± 0.04       |
| 13-Methylpentadecanoic acid (anteiso 16:0)      | 0.040 ± 0.007*   | 0.048 ± 0.008     |
| 14-Methylpentadecanoic acid (iso 16:0)          | 0.19 ± 0.05*     | 0.23 ± 0.04       |
| 14-Methylhexadecanoic acid (anteiso 17:0)       | 0.42 ± 0.05      | 0.41 ± 0.03       |
| 15-Methylhexadecanoic acid (iso 17:0)           | 0.42 ± 0.04      | 0.41 ± 0.03       |
| 16-Methylheptadecanoic acid (iso 18:0)          | 0.017 ± 0.016    | 0.023 ± 0.020     |
| Total BCFA                                       | 1.68 ± 0.14*     | 1.80 ± 0.13       |
| **OCFA**                                        |                  |                   |
| 7:0                                             | 0.040 ± 0.016*   | 0.027 ± 0.012     |
| 9:0                                             | 0.069 ± 0.022*   | 0.045 ± 0.013     |
| 11:0                                            | 0.011 ± 0.05*    | 0.07 ± 0.03       |
| 13:0                                            | 0.17 ± 0.05*     | 0.12 ± 0.03       |
| 15:0                                            | 1.37 ± 0.31*     | 1.18 ± 0.19       |
| 17:0                                            | 0.59 ± 0.07      | 0.60 ± 0.04       |
| 17:1 isomer                                     | 0.18 ± 0.04      | 0.17 ± 0.03       |
| 21:0                                            | 0.024 ± 0.008    | 0.028 ± 0.009     |
| 23:0                                            | 0.018 ± 0.008*   | 0.024 ± 0.009     |
| Total OCFA                                      | 2.58 ± 0.52*     | 2.26 ± 0.29       |

\(^1\)Data are the mean ± SD and are the percent of total fatty acid concentration.

* significant difference (p < 0.05) from values in the same row using a paired t-test with a false discovery rate applied.

BCFA, Branched-chain fatty acids; F:C, Forage:concentrate ratio; OCFA, Odd-chain fatty acids
Table 3. Comparison of BCFA content of milk from the lowest and highest BCFA quintiles for each treatment.

| Fatty acid | Low BCFA Low F:C (n = 12) | Low BCFA High F:C (n = 12) | High BCFA Low F:C (n = 12) | High BCFA High F:C (n = 12) |
|------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 12-Methyltridecanoic acid (*iso* 14:0) | 0.066 ± 0.018a<sup>1,2</sup> | 0.089 ± 0.022b | 0.103 ± 0.034b | 0.131 ± 0.020c |
| 13-Methyltetradecanoic acid (*iso* 15:0) | 0.16 ± 0.01a | 0.19 ± 0.01b | 0.19 ± 0.02b | 0.22 ± 0.02c |
| 12-Methyltetradecanoic acid (*anteiso* 15:0) | 0.31 ± 0.03a | 0.33 ± 0.03ab | 0.39 ± 0.04c | 0.40 ± 0.04c |
| 13-Methylpentadecanoic acid (*anteiso* 16:0) | 0.037 ± 0.007a | 0.042 ± 0.006a | 0.043 ± 0.006ab | 0.054 ± 0.009c |
| 14-Methylpentadecanoic acid (*iso* 16:0) | 0.16 ± 0.04a | 0.20 ± 0.03b | 0.24 ± 0.05c | 0.27 ± 0.03c |
| 14-Methylhexadecanoic acid (*anteiso* 17:0) | 0.38 ± 0.03a | 0.38 ± 0.03a | 0.46 ± 0.06b | 0.47 ± 0.06b |
| 15-Methylhexadecanoic acid (*iso* 17:0) | 0.39 ± 0.04a | 0.39 ± 0.04a | 0.45 ± 0.05b | 0.44 ± 0.03b |
| 16-Methylheptadecanoic acid (*iso* 18:0) | 0.015 ± 0.015 | 0.009 ± 0.014 | 0.022 ± 0.020 | 0.030 ± 0.023 |
| Total BCFA | 1.50 ± 0.04a | 1.63 ± 0.05b | 1.89 ± 0.10c | 1.99 ± 0.11d |

<sup>1</sup> Data are the mean ± SD and are the percent of total fatty acid concentration.

<sup>2</sup> Values with different letters are significantly different (*p* < 0.05) from values in the same row by one way ANOVA with a false discovery rate applied.

BCFA, Branched-chain fatty acids, F:C, Forage:concentrate ratio
Table 4. Comparison of long chain saturated fatty acids (LCSFA) and monounsaturated fatty acids of milk from the lowest and highest branched chain fatty acid (BCFA) quintiles for each dietary treatment.

| Fatty acid | Low BCFA Low F:C (n=12) | Low BCFA High F:C (n=12) | High BCFA Low F:C (n=12) | High BCFA High F:C (n=12) | Relation 1 to total BCFA |
|------------|--------------------------|---------------------------|--------------------------|--------------------------|--------------------------|
|            |                          |                           |                          |                          | r | P           |
| 14:0       | 12.1 ± 0.82              | 11.8 ± 0.8                | 12.3 ± 1.2               | 11.7 ± 1.2               | -0.06 | 0.68 |
| 16:0       | 33.9 ± 4.0a              | 35.0 ± 1.8a               | 30.2 ± 4.0b              | 28.9 ± 2.3b              | -0.64 | <0.001 |
| 18:0       | 8.2 ± 1.0a               | 9.5 ± 0.7b                | 9.6 ± 1.3b               | 11.5 ± 2.0c              | 0.61  | <0.001 |
| 20:0       | 0.11 ± 0.02a             | 0.12 ± 0.01a              | 0.16 ± 0.09a             | 0.23 ± 0.11b             | 0.50  | <0.001 |
| 22:0       | 0.06 ± 0.01a             | 0.07 ± 0.01b              | 0.07 ± 0.01b             | 0.08 ± 0.01ab            | 0.56  | <0.001 |
| 24:0       | 0.04 ± 0.01a             | 0.04 ± 0.01a              | 0.04 ± 0.01a             | 0.05 ± 0.01b             | 0.41  | 0.003 |
| Total LCSFA | 54.4 ± 3.0a              | 56.5 ± 1.7b               | 52.3 ± 2.7c              | 52.4 ± 1.5c              | -0.52 | <0.001 |
| 18:1n-9 Z  | 17.2 ± 1.6a              | 16.8 ± 1.2b               | 19.6 ± 2.5c              | 19.8 ± 1.5c              | 0.62  | <0.001 |
| 16:1n-7 Z  | 1.52 ± 0.28              | 1.35 ± 0.22               | 1.26 ± 0.39              | 1.19 ± 0.24              | -0.36 | 0.01  |
| 18:1n-7 Z  | 1.01 ± 0.09a             | 0.74 ± 0.12b              | 1.07 ± 0.16a             | 0.83 ± 0.10b             | 0.05  | 0.72  |

1 Pearson’s correlation coefficients were derived for fatty acids compared to the concentration of total BCFA.

2 Data are the mean ± SD and are the percent of total fatty acid concentration.

3 Values with different letters are significantly different (p < 0.05) from values in the same row by one-way ANOVA with a false discovery rate applied.

BCFA, Branched-chain fatty acids; F:C, Forage:concentrate ratio; LCSFA, Long chain saturated fatty acids
Figure 1. Discriminant analysis of milk fatty acid composition from Holstein cows fed high forage:starch ratio (HF:C) and low forage:starch (LF:C) ratio feeds. (A) Separation of milk fatty acid composition from individual cows following sparse partial least squares discriminant analysis (sPLSDA). Each colored circle represents the milk from an individual cow (n=62 for each treatment). (B) Fatty acids underlying separation along Component 1 of the sPLSDA plot.
(C) Fatty acids underlying separation along Component 2 of the sPLSDA plot. The scale bar provides relative differences between treatments. OCFA, odd-chain fatty acids. 13-Me14:0, 13-methyltetradecanoic acid; 12-Me13:0, 12-methyltridecanoic acid; 13-Me15:0, 13-methylpentadecanoic acid; 14-Me15:0, 14-methylpentadecanoic acid.
Figure 2. Distribution of branched-chain fatty acids (BCFA) and discriminant analysis fatty acid composition of high-BCFA containing and low-BCFA containing milk from Holstein cows fed high forage:starch (HF:C) and low forage: starch (LF:C) rations. (A) Box plot (mean ± SD; n =
62) of total BCFA content of milk from HF:C and LF:C treatment. (B) Separation of milk fatty acid composition from cows high BCFA and low BCFA milk from HF:C and LF:C diets following sparse partial least squares discriminant analysis (sPLSDA). For each condition (n = 12), each colored circle represents the milk from an individual cow. (C) Fatty acids underlying separation along Component 1 of the sPLSDA plot. (D) Fatty acids underlying separation along Component 2 of the sPLSDA plot. The scale bar provides relative differences between treatments. LCSFA, long chain saturated fatty acid; 13-Me14:0, 13-methyltetradecanoic acid; 12-Me13:0, 12-methyltridecanoic acid; 13-Me15:0, 13-methylpentadecanoic acid; 14-Me15Me15:0, 14-methylpentadecanoic acid.