Acetate supplementation has been shown to increase milk fat yield in diets with low risk of biohydrogenation-induced milk fat depression. The interaction of acetate supplementation with specific dietary factors that modify rumen fermentation and short-chain fatty acid (FA) synthesis has not been investigated. The objective of this experiment was to determine the effect of acetate supplemented as sodium acetate at 2 dietary fiber levels. Our hypothesis was that acetate would increase milk fat production more in animals fed the low-fiber diet. Twelve lactating multiparous Holstein cows were arranged in a 4 × 4 Latin square design balanced for carryover effects with a 2 × 2 factorial arrangement of dietary fiber level and acetate supplementation with 21-d experimental periods. The high-fiber diet had 32% neutral detergent fiber and 21.8% starch, and the low-fiber diet had 29.5% neutral detergent fiber and 28.7% starch created by substitution of forages predominantly for ground corn grain. Acetate was supplemented in the diet at an average 2.8% of dry matter (DM) to provide approximately 10 mol/d of acetate as anhydrous sodium acetate. Acetate supplementation increased DM intake by 6%, with no effect on meal frequency or size. Furthermore, acetate supplementation slightly increased total-tract apparent DM digestibility and tended to increase organic matter digestibility. Acetate supplementation increased milk fat concentration and yield by 8.6 and 10.5%, respectively, but there was no interaction with dietary fiber. The increase in milk fat synthesis was associated with 46 and 85 g/d increases in the yield of de novo (<16C) and mixed source (16C) FA, respectively, with no changes in yield of preformed FA (>16C). There was a 9% increase in the concentration of milk mixed-source FA and a 7% decrease in milk preformed FA with acetate supplementation, regardless of dietary fiber level. Acetate supplementation also increased the concentrations of plasma acetate and β-hydroxybutyrate, major metabolic substrates for mammary lipogenesis. Overall, acetate supplementation increased milk fat yield regardless of dietary fiber level through an increase mostly caused by an increase in longer-chain de novo FA, suggesting stimulation of mammary lipogenesis. The heightened mammary de novo lipogenesis was supported by an increase in the concentration of metabolic substrates in plasma.

Key words: lipogenesis, milk fat, de novo
Additionally, a positive linear relationship has been reported between milk fat content and dietary physically effective NDF (Zebeli et al., 2008). The interaction between dietary factors and acetate supplementation in the absence of biohydrogenation-induced milk fat depression (BH-MFD) is relatively unknown, and this information would be helpful in managing for optimal milk fat yield.

Measuring ruminal short-chain FA production in vivo is expensive and experimentally challenging, and it has only been reported a few times (summarized by Sutton et al., 2003). Acetate production has been shown to be unresponsive to dietary fiber level in cows (Davis, 1967; Bauman et al., 1971; Sutton et al., 2003), but those experiments were done with diets unlike modern diets and with cows that had very low DMI and milk yield. The technical challenges of observing acetate production make it difficult to directly identify dietary scenarios in which acetate supply might limit milk fat synthesis in the mammary gland. However, acetate can be easily supplemented in diets and provides an opportunity to test the response to increasing acetate supply.

The objective of this study was to test the interaction of acetate and dietary fiber level on milk fat synthesis. Our hypothesis was that acetate supplementation would increase milk fat yield more in cows fed a low-fiber diet, because this diet may result in lower rumen acetate synthesis. Additionally, recent experiments feeding NaAcet have used feeding periods of 14 d or less (Urrutia et al., 2019; Matamoros et al., 2021). Treatment periods in the current experiment were 21 d in order to assess the longer-term effects of supplementation on feeding behavior, apparent total-tract (TT) digestibility, milk synthesis, and plasma metabolites.

MATERIALS AND METHODS

Experimental Design and Animals

All experimental procedures were approved by The Pennsylvania State University Institutional Animal Care and Use Committee (PROTO201900784). Twelve lactating multiparous Holstein cows (134 ± 12 DIM and milk yield of 45.3 ± 7.6 kg/d at the start of the first experimental period; mean ± SD) were arranged in a 4 × 4 Latin square design balanced for carryover effects with a 2 × 2 factorial design of treatments. Experimental periods were 21 d, with an initial pretrial period of 14 d, in which cows were fed the low-fiber diet (detailed below). Cows were housed in a tiestall barn at the Pennsylvania State University Dairy Production Research and Teaching Center, and the experiment was conducted from January to May 2020. Ten of the stalls were equipped with a feed intake monitoring system consisting of a feed tub hanging from a load monitor as described by Rottman et al. (2015). Eating behavior was monitored during the last 7 d of each experimental period, and the number and characteristics of meals and the rate of feed intake across the day were analyzed as described by Niu et al. (2014).

Treatments and Experimental Diets

Treatment main effects were dietary fiber level and acetate supplementation. The high-fiber (HF) diet was formulated to 32% NDF, and the low-fiber (LF) diet was formulated to 28% NDF. Briefly, NDF was decreased by substitution of corn silage and alfalfa haylage with ground corn and a small amount of canola meal to maintain diet CP. Small differences in other dietary ingredients were mostly due to differences in forage DM used in ration balancing and that observed during the feeding period. Both diets were formulated to meet or exceed nutrient requirements in NDS Professional 3.9.8.01 (RUM&N Sas; https://www.rumen.it/en) based on CNCPS 6.55 (Cornell University; https://blogs.cornell.edu/cncps/). Acetate was supplemented in the diet as a percent of DM (2.92 and 2.67% for HF and LF, respectively; Table 1), targeting 10 mol/d of acetate as anhydrous NaAcet (Niacet Corp.). Acetate inclusion for LF and HF was adjusted based on the previous day’s average DMI and mixed for a minimum of 5 min before feeding with an apron chain mixer (I. H. Rissler 1050 Mobile TMR Mixer). Cows were fed once a day at 0600 h at 110% of expected intake, and orts were measured daily before feeding. Orts were subsampled during the last 3 d of each period (1/8 of total orts) and composited within cow by period with the coning and quartering method. Composited ort samples were analyzed for DM (AOAC International, 2000; method 930.15), NDF (Van Soest et al., 1991), ADF (AOAC International, 2000; method 973.18), CP (AOAC International, 2000; method 990.03), starch (Hall et al., 2015), and ash (AOAC International, 2000; method 942.05) by Cumberland Valley Analytical Services Inc. (Waynesboro, PA).

Feed and Fecal Sampling

Feed samples were taken weekly for DM determination (48 h at 55°C) for dietary adjustments. Samples were also taken on the last 3 d of each period, composited within period, and analyzed for DM, CP, starch, NDF, 240-h undigestible NDF, ADF, and ash as described above. The FA content of feeds was determined as described by Rico et al. (2014a). Ingredient
composition, corrected for the daily mixing report for each experimental diet, and final nutrient composition are reported in Table 1.

Fecal samples were collected 8 times over the last 3 d of each experimental period to represent every 3 h of a 24-h period (0200, 0500, 0800, 1100, 1400, 1700, 2000, and 2300 h) and stored at −20°C before being composited by cow within period. Composited samples were dried for 72 h in a forced-air oven at 55°C and analyzed for DM, NDF, 240-h undigestible NDF, and ash as described above. Undigestible NDF was used as an internal marker to calculate apparent TT digestibility for DM, NDF, and OM, as described by Huhtanen et al. (1994).

### Table 1. Ingredients and nutrient composition of experimental diets

| Variable                     | HF   | LF   | HF+NaAcet | LF+NaAcet |
|------------------------------|------|------|-----------|-----------|
| Ingredient                   |      |      |           |           |
| Corn silage                  | 39.3 | 28.2 | 38.2      | 27.2      |
| Alfalfa haylage              | 27.8 | 22.1 | 27.0      | 21.6      |
| Ground corn                  | 7.1  | 21.3 | 6.9       | 20.8      |
| Canola meal                  | 10.9 | 12.0 | 10.6      | 11.7      |
| Roasted soybeans             | 2.7  | 3.0  | 2.6       | 2.9       |
| Molasses                     | 2.2  | 2.0  | 2.2       | 2.0       |
| Grass hay                    | 4.2  | 4.9  | 4.0       | 4.8       |
| Mineral-vitamin mix          | 2.0  | 2.2  | 2.0       | 2.2       |
| Corn gluten meal             | 1.7  | 1.9  | 1.7       | 1.9       |
| Expeller SBM                 | 2.0  | 2.1  | 1.9       | 2.1       |
| NPN                          | 0.09 | 0.09 | 0.09      | 0.09      |
| Sodium acetate               | —    | —    | 2.92      | 2.67      |
|Nutrient composition          |      |      |           |           |
| NDF                          | 33.5 | 29.5 | 32.5      | 28.6      |
| uNDF                         | 11.1 | 9.2  | 10.8      | 8.6       |
| ADF                          | 23.3 | 20.5 | 22.6      | 19.3      |
| CP                           | 16.6 | 16.8 | 16.2      | 16.4      |
| Fatty acids                  | 2.5  | 3.3  | 2.4       | 3.2       |
| Starch                       | 21.8 | 28.7 | 21.1      | 27.9      |
| Ash                          | 7.57 | 7.10 | 9.22      | 8.63      |

1The high-fiber (HF) treatment was formulated to have 32% NDF, and the low-fiber (LF) treatment was formulated to have 28% NDF. Sodium acetate (NaAcet) supplementation was designed to provide 10 mol/d of acetate as NaAcet. Both diets were formulated to meet or exceed nutrient requirements in NDS Professional 3.9.8.01 (RUM&N Sas; https://www.rumen.it/en) based on CNCPS 6.55 (Cornell University; https://blogs.cornell.edu/cncps/).

2Corn silage contained, on average, 37.8% NDF, 11.2% undigestible NDF (uNDF), 23% ADF, 6.6% CP, and 2.2% fatty acids on a DM basis.

3Alfalfa haylage contained, on average, 38.1% NDF, 16.5% uNDF, 31% ADF, 18.1% CP, and 1.8% fatty acids on a DM basis.

4Grass hay contained, on average, 69.6% NDF, 25.0% uNDF, and 45.2% ADF on a DM basis.

5Contained (% as-fed basis): 45.8 dried corn distillers grains with solubles; 35.8 limestone (38% Ca); 8.3 magnesium oxide (54% Mg); 6.4 salt; 1.73 vitamin ADE premix; 1.09 selenium premix (0.06% selenium); and 0.88 trace mineral mix. Composition (DM basis): 11% CP; 40% NDF; 6.9% ADF; 14.9% Ca; 0.35% P; 4.58% Mg; 0.41% K; 0.31% S; 357 mg/kg of Cu; 1,085 mg/kg of Zn; 181 mg/kg of Fe; 6.67 mg/kg of Se; 125,875 IU/kg of vitamin A (retinyl acetate); 31,418 IU/kg of vitamin D (activated 7-dehydrocholesterol); and 946 IU/kg of vitamin E (d-l α-tocopheryl acetate).

6Expeller soybean meal (SoyPlus, Landus Cooperative)

7NPN was fed as a slow-release urea (Optigen II, Alltech Inc.; 259% CP on a DM basis).

8Sodium acetate anhydrous (Niacet Corp.)

9Undigestible NDF estimated after an in vitro digestion for 240 h.

### Milk Sampling and Analysis

Cows were milked twice daily at 0600 and 1800 h. Milk yield was determined using an integrated milk meter (AfiMilk; SAE Afikim) and corrected using a stall deviation, as described by Andreen et al. (2020). Milk was sampled at both milkings at d 10 and during the last 3 d of each experimental period. One sample was stored at 4°C with preservative (Bronolab-WII, Advanced Instruments Inc.) until analyzed for fat and protein by Fourier transform infrared spectroscopy (Fossomatic Milko-Scan FT+ and FC; Foss Electric; Dairy One, Ithaca, NY). A second milk sample was composited by cow on d 10 and for the last 3 d for
each period according to milk yield, and the fat cake was extracted by centrifugation at 1,300 × g for 15 min at 4°C and stored at −20°C until further analysis. Milk FA profile was determined after FA were extracted with hexane/isopropanol (Hara and Radin, 1978), transmethylated with sodium methoxide (Chouinard et al., 1999), and measured using a GC equipped with a flame-ionization detector, as described by Urrutia and Harvatine (2017a). A third milk sample was composited according to milk yield for the last 2 milkings of each experimental period into a sterile 50-mL conical tube and centrifuged at 1,300 × g for 15 min at 4°C. The fat cake was carefully removed from the sample, and the skim milk was subsampled into a 15-mL sterile conical tube and stored at −20°C until analysis by nuclear magnetic resonance (NMR)-based metabolomics.

**Milk NMR-Based Metabolomics**

The skim milk sample was thawed at room temperature and then kept on ice. The sample was vortexed for 30 s, and a 600-µL aliquot was transferred to a sterile 15-mL conical tube. Small polar metabolites were extracted from the sample as described by Praticò et al. (2014). Briefly, 3 mL of methanol:chloroform (2:1 vol/vol) was added and vortexed for 30 s. Next, 400 µL of nanopure distilled water and 1 mL of chloroform were added sequentially to the sample, vortexed for 30 s, and kept overnight at 4°C (~16 h). The polar and organic phases were separated by centrifugation at 6,000 × g at 4°C for 20 min. The polar phase was collected in a 2-mL microcentrifuge tube, dried with a vacuum concentrator, and kept at −80°C until NMR analysis. The dried samples were reconstituted by ultrasonication for 10 min in 600 µL of 0.1 M PBS in D2O with 1 mM sodium 3-(trimethylsilyl) propionate-2,3-d4 (TSP) as a chemical shift reference for 1H NMR. The reconstituted sample was centrifuged at 20,000 × g at 4°C for 10 min, and 500 µL was transferred to a 5-mm NMR glass tube for analysis.

All NMR experiments were performed at 298 K on a Bruker Avance NEO 600 MHz spectrometer equipped with a 5-mm TCI (HCN) Z-axis gradient cryoprobe with enhanced sensitivity for 1H and 13C (Bruker BioSpin Corp.). A typical one-dimensional NMR spectrum was acquired for all samples using the NOESY pulse sequence (NOESYGPPPR1D). The 90° pulse length was adjusted to approximately 10 µs for each sample, and 64 transients were collected in 64k data points with a spectral width of 20 ppm. For assignments of metabolites, a series of 2-dimensional NMR experiments were performed for selected milk samples, including 1H–1H total correlation spectroscopy (TOCSY), 1H–13C correlation spectroscopy (COSY), 1H–1H J-resolved spectroscopy (JRES), 1H–13C heteronuclear single quantum correlation (HSQC), and 1H–13C heteronuclear multiple bond correlation spectra (HMBC). Metabolite identification was done with a combination of 1-dimensional (1D) and 2-dimensional NMR experiments, chemical shift, peak multiplicity, J couplings measurements, and online databases (such as HMDB: https://hmdb.ca/). The Chenomx software package (version 8.4; Chenomx Inc.) was used to improve the 1H NMR spectral quality by manual phase correction, baseline correction, and calibration referenced to TSP (0.0) and to quantitate the metabolites. Peak-fitting with reference to the internal TSP signal (0.29 mJ) enabled determination of concentrations for identified metabolites in milk extracts. A total of 15 metabolites (Supplemental Table S1; https://scholarsphere.psu.edu/resources/e1755bb7-e5ce-4d1e-b1ee-33e462b355cd) were identified, and an overrepresentation analysis was performed with the names of all metabolites identified in milk in the Pathway Analysis module of MetaboAnalyst 5.0 (Pang et al., 2021; https://dev metaboanalyst.ca/) with the Bos taurus Kyoto Encyclopedia of Genes and Genomes pathway as a reference (bta map; https://www.genome.jp/kegg/pathway.html). A representative 600 MHz 1D 1H NMR annotated spectrum is provided in Supplemental Figure S1 (https://scholarsphere.psu.edu/resources/e1755bb7-e5ce-4d1e-b1ee-33e462b355cd).

**Plasma Samples and Analysis**

Blood samples were collected from tail vessels using vacuum tubes containing potassium EDTA and sodium heparin (Vacuette Greiner Bio-One North America Inc.); fecal samples were collected at the same time. Blood samples were placed on ice and immediately transported to the laboratory for plasma collection. Plasma was collected after centrifugation for 1,300 × g at 4°C for 15 min. Three aliquots were taken from the potassium EDTA tubes and 2 from the sodium heparin tubes; all were kept at −20°C until further analysis. Plasma glucose, BHB, and nonesterified FA (NEFA) were assayed as described in Matamoros et al. (2021). Plasma acetate was determined by GC-MS after propyl esterification at the Penn State University Metabolomics Core Center, as described in Cai et al. (2017).

**Statistical Analysis**

Milk production data, milk FA, and plasma metabolites were analyzed in PROC MIXED of SAS 9.4 (SAS Institute Inc.) with repeated measures, using a model that included the fixed effect of fiber, acetate, day, and their 2- and 3-way interactions and the random effect of cow and period. Subject was defined as cow by period,
denominator degrees of freedom were adjusted using the Kenward-Roger method, and the heterogeneous autoregressive structure was used. Data without repeated measures were analyzed using a reduced version of the model above that did not include the effect of day or time. Differences for main effects were declared at $P \leq 0.05$ and tendencies acknowledged at $0.05 < P \leq 0.10$. Differences for interactions were declared at $P \leq 0.10$ and tendencies acknowledged at $0.10 < P \leq 0.15$.

Linear regressions were done in the Fit Linear module of JMP Pro 15.0.0 (SAS Institute Inc.). The best fitting model was determined by the lowest value of the corrected Akaike and Bayesian information criteria and the adjusted coefficient of determination (Schwarz, 1978; Sugiura, 1978). High-resolution figures were created using Daniel’s XL Toolbox (Kraus, 2014).

## RESULTS AND DISCUSSION

### Intake, Feeding Behavior, and Apparent TT Digestibility

Acetate was supplemented in its neutralized form as a sodium salt because acetic acid has been reported to decrease DMI (Gualdrón-Duarte and Allen, 2018). Acetate supplementation was designed to provide 10 mol/d of acetate as NaAcet, and it delivered 11.6 and 11.9 mol/d for the HF and LF treatments, respectively, based on actual average DMI.

Table 2. The effect of feeding a low-fiber (LF) or high-fiber (HF) diet with or without sodium acetate (NaAcet) supplementation on DMI, feeding behavior, and apparent digestibility of select nutrients

| Variable                  | Treatment1 | SEM | $P$-value2 |
|---------------------------|------------|-----|------------|
|                           | HF         | LF  | HF+NaAcet | LF+NaAcet |
| Eating behavior           |            |     |            |            |
| DMI, kg/d                 | 30.9       | 34.5| 32.7       | 36.5       | 1.71       | <0.001 | 0.02 | 0.89 |
| Meals, bouts/d            | 9.2        | 9.6 | 9.4        | 10.3       | 0.37       | 0.02   | 0.13 | 0.39 |
| Meal length, min/meal     | 37.0       | 34.5| 37.3       | 32.0       | 1.67       | 0.02   | 0.35 | 0.23 |
| Meal size, kg/meal        | 3.29       | 3.48| 3.33       | 3.46       | 0.24       | 0.13   | 0.93 | 0.77 |
| Apparent digestibility, % |            |     |            |            |
| DM                        | 67.0       | 69.6| 67.7       | 71.1       | 0.55       | <0.001 | 0.01 | 0.39 |
| OM                        | 67.8       | 70.5| 68.2       | 71.5       | 0.54       | <0.001 | 0.09 | 0.39 |
| NDF                       | 46.9       | 46.3| 46.7       | 47.7       | 1.96       | 0.77   | 0.38 | 0.24 |

1The high-fiber (HF) treatment was formulated to have 32% NDF, and the low-fiber (LF) treatment was formulated to have 28% NDF. Sodium acetate (NaAcet) supplementation was designed to provide 10 mol/d of acetate as NaAcet, and it delivered 11.6 and 11.9 mol/d for the HF and LF treatments, respectively, based on actual average DMI.

2$P$-value for the fixed effect of fiber, acetate, and their two-way interaction in a mixed model that also includes the random effect of period and cow, n = 12 cows per treatment.

Overall daily DMI is a function of hunger and satiety signals that dictate meal frequency and size (Allen, 2014), and feeding behavior provides insight into the physiological regulation driving DMI. The mechanism by which acetate increased DMI is not clear because we observed no effect of acetate on meal frequency, length, or size ($P = 0.13, 0.35, \text{ and } 0.93$, respectively; Table 2). There was a main effect of fiber level on meal frequency and length ($P = 0.02$ for both) and there was no interaction with acetate ($P = 0.39$ and 0.23, respectively; Table 2). Meal frequency was increased $\sim 7\%$ and meal length decreased $\sim 12\%$ in LF diets ($P = 0.02$). Oba and Allen (2003) similarly reported an increase in meal frequency and a decrease in meal length when fiber level was decreased. Because meal size was not changed, it appears that LF diets increased DMI through increased meal frequency. There was no effect of fiber, acetate, interaction between fiber and acetate, or a 3-way interaction with time of day on the rate of intake expressed as kilograms per hour or percent...
of intake per hour (Figure 1). Salfer et al. (2018) also reported no effect of dietary NDF or starch concentration on the daily pattern of feed intake.

Apparent TT DM digestibility was increased 1.6% by acetate regardless of dietary fiber level \((P < 0.001; \text{ Table 2})\). There was also a main effect of fiber \((P = 0.01)\) with LF increasing DM digestibility 4.5% compared with HF. There was an effect of fiber and a tendency for an effect of acetate on apparent TT OM digestibility \((P < 0.001 \text{ and } 0.09, \text{ respectively; Table 2})\). Low-fiber diets had 4.4% higher OM digestibility compared with HF diets. Apparent TT NDF digestibility was not changed by fiber, acetate, or their interaction \((P = 0.77, 0.38, \text{ and } 0.24, \text{ respectively; Table 2})\). Supplementation of NaAcet increases DCAD, and the meta-analysis by Iwaniuk and Erdman (2015) reported a positive linear relationship between DCAD and DM digestibility. Furthermore, Sheperd and Combs (1998) reported increases in DM, OM, and NDF apparent TT digestibility of a greater magnitude than the current results, but their acetate dose was 3 times higher (36 mol/d). The increase in DM digestibility associated with the decrease in NDF:starch ratio is similar to that in previous reports (Beckman and Weiss, 2005).

**Milk Yield and Milk Components**

The LF diets increased milk yield by 11.6% compared with HF diets \((P < 0.001 \text{ for the effect of fiber})\), but there was no effect of acetate or interaction between fiber and acetate or interaction with time on treatment \((P = 0.36, 0.96, \text{ and } 0.32, \text{ respectively; Figure 2A})\). Other recent experiments have not observed an effect of dietary supplementation of acetate on milk yield, even when acetate increased DMI (Urrutia et al., 2019; Matamoros et al., 2021).

Acetate supplementation increased milk fat concentration 8.6% and milk fat yield 10.5% \((P < 0.001 \text{ for both; Figure 2C})\), with no interaction with dietary fiber level or day on treatment. Interestingly, milk fat yield was not different between LF and HF \((P = 0.44)\), but HF increased milk fat concentration 9.7% \((P < 0.001)\), likely due to dilution of milk fat in the greater milk yield in LF. We did not directly measure changes in ruminal short-chain FA production in the current experiment, but the lack of difference in milk fat yield and the increased milk yield in LF compared with HF may indicate that the diets did not result in an acetate deficiency. Instead, LF diets may have affected propionate production to a greater extent, consistent with the limited data available on ruminal short-chain FA yield (summarized by Sutton et al., 2003). This indicates that acetate supplementation increases milk fat production to a similar extent in diets that differ in fiber level and result in an increase in milk yield. Furthermore, the increase in milk fat synthesis was consistent over the 21-d period, indicating a robust mechanism without feedback inhibition. Previous experiments investigating ruminally infused NaAcet and dietary supplementation of NaAcet have reported rapid responses occurring on the first day of treatment and persisting for up to 14 d at a similar dose (Urrutia and Harvatine, 2017b; Urrutia et al., 2019; Matamoros et al., 2021) and 21 d at a dose >3-fold higher than used the current experiment (Sheperd and Combs, 1998).

The response in milk fat yield relative to supplemented acetate can be compared through calculation of the apparent mass transfer, defined as the observed
increase in milk fat yield divided by acetate supplied. The apparent mass transfer of acetate to milk fat was 21% and 28% for HF and LF diets, respectively. Previous studies have reported transfers ranging from ~20 to 42%, and mass transfer appears to be related to dose and delivery method (Urrutia and Harvatine, 2017a,b).
Urrutia et al., 2019; Matamoros et al., 2021). Milk fat yield is quadratically increased with acetate supplementation, with a maximal response observed at 10 mol/d (Urrutia and Harvatine, 2017b). The greater amount of acetate supplemented in this experiment (11.6 and 11.9 mol/d for HF and LF, respectively) might explain why transfer rates in the current experiment were lower than in other studies.

There was a 3-way interaction between fiber, acetate, and day on treatment for milk protein concentration ($P < 0.05$; Figure 2D). Milk protein concentration was decreased 3.2% by NaAcet in LF but not in HF on d 10 [least significant difference (LSD) $P = 0.01$ and 0.12, respectively], whereas, on d 21, milk protein concentration decreased 3.2% by NaAcet in HF but not in LF (LSD $P = 0.03$ and 0.30, respectively). Overall milk protein yield was 11% higher in LF diets compared with HF diets ($P < 0.001$; Figure 2E) and was not modified by acetate supplementation. The effect of acetate supplementation on milk protein is inconsistent in reports in the literature. Urrutia and Harvatine (2017a) found no effect on milk protein synthesis when ruminally infusing 7 mol/d of acetate as NaAcet. Urrutia and Harvatine (2017b) observed a quadratic effect of acetate infusion on milk protein yield, with a maximal response at 10 mol/d of acetate. Urrutia et al. (2019) observed an increase in milk protein yield from feeding 10 mol/d of acetate as NaAcet on the third day of supplementation, but not on the seventh day of supplementation. Matamoros et al. (2021), Danes et al. (2020), and Sheperd and Combs (1998) found no effect on milk protein synthesis when feeding 8.4 mol/d, abomasally infusing 15.5 mol/d, or ruminally infusing 36 mol/d of acetate, respectively. Acetate has been shown to stimulate milk protein synthesis in primary bovine mammary epithelial cells through the mammalian target of rapamycin and Janus kinase 2/signal transducer and activator of transcription 5 signaling pathways (Verbeke et al., 1997; Zhao et al., 2019). Despite this, the variability and small magnitude of the protein response to acetate supplementation in vivo limits the potential for acetate regulation of milk protein synthesis.

**Milk FA**

The increase in milk fat from acetate supplementation is mostly associated with an increase in the yield of mixed source FA (16C) and, to a lesser extent, an increase in de novo FA (<16C), but an increase in preformed FA (>16C) has also been reported (Urrutia and Harvatine, 2017a,b; Urrutia et al., 2019; Matamoros et al., 2021). The de novo and mixed-source FA increase more than preformed FA with acetate supplementation, resulting in an increase in the concentrations of <16 and 16C FA. In the current experiment, acetate increased the yield of de novo FA by 46 g/d ($P < 0.001$; Figure 3A). There was no effect of fiber, interaction between acetate and fiber, or a 3-way interaction with the effect of day on the yield of de novo FA ($P = 0.42$, 0.28, and 0.92, respectively). Despite the increase in yield of de novo FA with acetate supplementation, there was no effect of acetate on the concentration of de novo FA ($P = 0.22$; Supplemental Figure S2A; https://scholarsphere.psu.edu/resources/e1755bb7-e5ce-4d1eb1ee-33e462b355cd). There was an effect of fiber on the concentration of milk FA <16 C: LF diets had 2.8% higher de novo FA concentration than HF diets ($P = 0.02$). When risk of BH-MFD is low, increasing dietary starch concentration has been shown to increase the concentration of milk de novo FA (Alzahal et al., 2009), consistent with our results.

Acetate supplementation increased the yield of milk mixed-source FA by 85 g/d, regardless of fiber level ($P < 0.001$; Figure 3B). There was no effect of fiber, interaction between acetate and fiber, or a 3-way interaction with day on treatment for yield of mixed FA ($P = 0.19$, 0.98, and 0.88, respectively). At the FA profile level, acetate supplementation increased the concentration of milk mixed-source FA by 9%, regardless of fiber level ($P < 0.001$; Supplemental Figure S2B). There was a tendency for an effect of fiber and interaction between fiber and acetate ($P = 0.08$ and 0.13, respectively). Dietary acetate supplementation has been shown to increase the yield and concentration of longer-chain de novo FA (e.g., >12C; Urrutia et al., 2019; Matamoros et al., 2021). Authors have suggested that the additional acetate provided through acetate supplementation drives mammary de novo lipogenesis toward longer-chain FA, resulting in increased production of 16C FA. Regulation of FA elongation in bovine mammary de novo lipogenesis is poorly understood (Palmquist and Harvatine, 2020), but it appears that substrate availability plays a role in allowing the mammary gland to produce longer-chain de novo synthesized FA.

There was no effect of acetate, fiber, acetate by fiber interaction, or a 3-way interaction with the effect of day on the yield of preformed FA ($P = 0.66$, 0.30, 0.35, and 0.25, respectively; Figure 3C). There was an effect of acetate on the concentration of milk preformed FA ($P < 0.001$), but no effect of fiber, acetate by fiber interaction, or 3-way interaction with time ($P = 0.25$, 0.75, and 0.32, respectively; Supplemental Figure S2C). Diets without acetate supplementation had 7% higher preformed FA concentration in milk compared with diets supplemented with acetate. Considering that there was an increase in the yield and concentrations of FA that at least partially originate from de novo lipogenesis, it is not surprising that acetate supplementation
Figure 3. Time course of the effect of feeding a low-fiber (LF) or high-fiber (HF) diet with or without sodium acetate (NaAcet) supplementation on milk fatty acid (FA) yield: (A) de novo FA, (B) mixed source FA, (C) preformed FA, (D), odd and branched-chain FA (OBCFA), and (E) concentration of milk trans-10 C18:1. Sodium acetate supplementation delivered 11.6 and 11.9 mol/d for the HF and LF treatments, respectively, based on actual average DMI. The effect of fiber (F), acetate (A), their 2-way interaction (F×A), and the 3-way interaction with the effect of day (F×A×Day) is shown on each panel. Data are presented as LSM with SEM bars.
decreased the concentration of milk preformed FA. It is widely recognized that dietary FA supplementation can decrease mammary de novo lipogenesis (Glasser et al., 2008). An exact mechanism of how increased uptake of preformed FA by the mammary gland may inhibit de novo lipogenesis is unknown, but it has been proposed that allosteric regulation of acetyl-CoA carboxylase and FA synthase, or competitiveness between diet-derived FA and FA synthesized within the mammary gland for specific sn positions during triglyceride synthesis, may play a role (Rico et al., 2014b). The opposite appears to not be true according to our results and the previous literature, because stimulating mammary de novo lipogenesis with acetate may decrease the concentration of preformed FA but ultimately does not decrease their yield (Urrutia and Harvatine, 2017a,b; Matamoros et al., 2021).

Acetate supplementation tended to decrease the yield of odd and branched-chain FA (OBCFA; \( P = 0.06; \) Figure 3D). There was an effect of fiber on the yield of OBCFA (\( P = 0.03 \)), such that LF diets increased the yield of OBCFA by 3.9 g/d compared with HF diets. There was an interaction between the effect of fiber and acetate on the concentration of OBCFA (\( P = 0.03; \) Supplemental Figure S2D), but no 3-way interaction with time on treatment (\( P = 0.79 \)). Acetate decreased the concentration of milk OBCFA in LF and HF diets, but milk OBCFA concentration was higher in LF without acetate supplementation, resulting in a larger proportional decrease. A relationship between milk OBCFA concentration and rumen function has been proposed (Vlaeminck et al., 2006), but milk OBCFA also result from mammary de novo lipogenesis starting with odd and branched-chain substrate (Vlaeminck et al., 2015; Palmquist and Harvatine, 2020). The effect of dietary fiber level on the concentration of individual milk OBCFA differs, but high-fiber diets generally have lower milk OBCFA than lower-fiber diets (Vlaeminck et al., 2006; Alzahal et al., 2009). Acetate supplementation provides more even and straight carbon substrate for mammary and ruminal de novo lipogenesis and likely competes with odd and branched-chain carbon substrates in de novo synthesis both in the rumen and mammary gland.

Milk trans-10 C18:1 was measured as a biomarker of BH-MFD (Matamoros et al., 2020). We detected main effects of fiber and acetate on milk trans-10 C18:1 concentration (\( P < 0.001 \) and \( P = 0.008 \), respectively) and no interaction between fiber and acetate or 3-way interaction with time (\( P = 0.63 \) and 0.60, respectively; Figure 3E). Acetate decreased milk trans-10 C18:1 by 11%, regardless of dietary fiber level or day. Milk trans-10 C18:1 was 17% higher in LF diets than in HF diets. It is important to note that there was no clear evidence of

### Table 3. The effect of feeding a low-fiber (LF) or high-fiber (HF) diet with or without sodium acetate (NaAcet) supplementation on molar concentration of milk polar metabolites

| Metabolite | Treatment | SEM | Fiber | Acetate | F × A |
|------------|-----------|-----|-------|---------|-------|
| Acetate    | HF        | 88  |       | 70      | 77    | 83  | 17  | 0.45 | 0.93 | 0.10 |
|            | LF        | 37  | 31    | 32      | 99a   | 17  |     | 0.009 | 0.34 | 0.01 |
|            | HF+NaAcet | 110 | 58b   | 100b    |       |     |     | 0.007 | 0.80 | 0.27 |
|            | LF+NaAcet | 273 |       | 194     | 223   | 210 | 38  | 0.03  | 0.42 | 0.11 |
| Alanine    | HF        | 110 |       | 92      | 94    | 78  | 20  | 0.24  | 0.30 | 0.94 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |
|            | HF+NaAcet | 11  | 10    | 6       | 8     | 1   | <0.001 | 0.29 | 0.16 |
|            | LF+NaAcet | 188 |       | 142     | 171   | 145 | 26  | 0.05  | 0.72 | 0.57 |
| Choline    | HF        | 44  |       | 30      | 35    | 30  | 6   | 0.006 | 0.17 | 0.17 |
|            | LF        | 57  | 44    | 47      | 37    | 8   |     | 0.02  | 0.09 | 0.64 |
|            | HF+NaAcet | 45  | 27    | 41b     | 48a   | 11  |     | 0.22  | 0.09 | 0.01 |
|            | LF+NaAcet | 42  | 26    | 37      | 30    | 6   |     | 0.01  | 0.95 | 0.32 |
| Citrate    | HF        | 119 |       | 58      | 100   | 99  | 20  | 0.03  | 0.50 | 0.11 |
|            | LF        | 2,118 |      | 3,119   | 2,341 | 3,136 | 481 |     | 0.02  | 0.75 | 0.78 |
| Creatine   | HF        | 110 |       | 92      | 94    | 78  | 20  | 0.24  | 0.30 | 0.94 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |
| Dimethylamine | HF    | 273 |       | 194     | 223   | 210 | 38  | 0.03  | 0.42 | 0.11 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |
| Ethanol    | HF        | 110 |       | 92      | 94    | 78  | 20  | 0.24  | 0.30 | 0.94 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |
| Fumarate   | HF        | 110 |       | 92      | 94    | 78  | 20  | 0.24  | 0.30 | 0.94 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |
| Glutamate  | HF        | 110 |       | 92      | 94    | 78  | 20  | 0.24  | 0.30 | 0.94 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |
| GPC        | HF        | 110 |       | 92      | 94    | 78  | 20  | 0.24  | 0.30 | 0.94 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |
| GPC:PC     | HF        | 110 |       | 92      | 94    | 78  | 20  | 0.24  | 0.30 | 0.94 |
|            | LF        | 73  | 61    | 65      | 70    | 15  |     | 0.60  | 0.99 | 0.25 |

a,bLeast squares means that do not share a superscript differ (\( P \leq 0.05 \)).

The high-fiber (HF) treatment was formulated to have 32% NDF, and the low-fiber (LF) treatment was formulated to have 28% NDF. Sodium acetate (NaAcet) supplementation was designed to provide 10 mol/d of acetate as NaAcet, and it delivered 11.6 and 11.9 mol/d for the HF and LF treatments, respectively, based on actual average DMI.

P-value for the fixed effect of fiber, acetate, and their two-way interaction in a mixed model that also includes the random effect of period and cow (n = 12 cows per treatment).

GPC = glycerophosphocholine, PC = phosphocholine; GPC:PC is their ratio.
BH-MFD, as treatment means ranged from 0.42 to 0.58 \( \text{trans-10 C18:1 g/100 g of milk FA} \). Using the equation reported by Matamoros et al. [2020; milk fat percentage = 2.51 + 1.55 \( e^{-0.503 \times \text{C18:1 trans-10 g/100 g of FA}} \)], the change in milk \( \text{trans-10 C18:1} \) concentration from LF to HF diet and unsupplemented to acetate-supplemented diets would only have accounted for 1.3 and 0.8% changes in milk fat concentration, respectively. This suggests that our observed increase in milk fat production by acetate supplementation is not likely due to a decrease in risk of BH-MFD.

**Milk Polar Metabolites**

A total of 15 polar metabolites were identified in the deproteinized and skimmed aqueous portion of milk (Table 3). Pathway analysis identified 5 metabolic pathways that are overrepresented by the identified metabolites, including those associated with amino acid, glyoxylate and dicarboxylate, pyruvate, and glucose metabolism and the citric acid cycle (Supplemental Table S2; https://scholarsphere.psu.edu/resources/e1755bb7-e5ce-4d1e-b1ee-33e462b355cd). Fiber concentration had the largest effect on milk polar metabolites, as it modified the concentration of 8 of the 15 identified metabolites. Notably, HF diets increased milk citrate 33% compared with LF diets. Milk citrate has been reported to be related to de novo FA synthesis (Garnsworthy et al., 2006). Despite the clear effect of acetate on de novo lipogenesis, there was no effect of acetate on milk citrate concentration (\( P = 0.80 \)). Acetate supplementation only modified milk glycerophosphocholine concentration, where diets supplemented with acetate had an 18.7% lower concentration (\( P = 0.02 \)).

The ratio of glycerophosphocholine and phosphocholine (GPC:PC), 2 intermediary metabolites of phosphatidylcholine metabolism, has been proposed as a biomarker of ketosis in periparturient dairy cows (Klein et al., 2012). Importantly, Klein et al. (2012) noted that cows that had higher milk fat concentration had a lower GPC:PC ratio. In our study, there was an effect of fiber and acetate on GPC:PC (\( P = 0.01 \) and 0.04, respectively; Table 3). Acetate supplementation decreased GPC:PC 33%, and HF diets resulted in a 44% decrease. Similar to Klein et al. (2012), these are also the treatments that had higher milk fat concentration. Furthermore, there was a linear relationship between milk fat concentration and log-transformed GPC:PC [\( P < 0.001 \) and \( R^2 = 0.67 \); milk fat, \% = 4.92–0.44 Ln(GPC:PC); Figure 4A]. There was also a relationship between GPC:PC and milk \( \text{trans-10 C18:1} \) concentration, although it was weaker than expected (\( P = 0.002 \) and \( R^2 = 0.22 \); milk \( \text{trans-10 C18:1, g/100 g of FA} = 0.43–0.005 \) (GPC:PC); Figure 4C) even though \( \text{trans-10 C18:1} \) was more strongly related to milk fat concentration [\( P < 0.001 \) and \( R^2 = 0.53 \); milk fat, \% = 3.14–1.005 Ln(milk \( \text{trans-10 C18:1, g/100 g of FA} \); Figure 4C)]. These relationships suggest that the variation in milk fat concentration explained by GPC:PC is
Plasma Metabolites

Acetate supplementation increased plasma acetate concentration by 29% \((P < 0.001; \text{Figure 5A})\). There was also an effect of fiber on plasma acetate concentration \((P < 0.001)\). Plasma acetate concentration was 15% higher in HF compared with LF \((P < 0.001)\). There was no interaction between acetate and fiber or 3-way interaction with time of day for plasma acetate concentration \((P = 0.34 \text{ and } 0.20, \text{respectively})\). Previous studies show a clear daily pattern in plasma acetate concentration, where the concentration appears to be higher in the portion of the day concomitant with higher rates of intake \((\text{Allen et al., 2005; Matamoros et al., 2021})\). In the current experiment, there was a clear peak in plasma acetate concentration in HF diets supplemented with acetate, consistent with previous reports \((\text{Matamoros et al., 2021})\), but the peak was less prominent in LF diets supplemented with acetate. The factors that regulate plasma acetate concentration are poorly understood. More research is needed to determine factors that regulate ruminal acetate absorption and peripheral acetate utilization.

Plasma BHB concentration is related to ruminal acetate absorption, as there is an interconversion of acetate to butyrate in the rumen, which can then be converted to BHB in the rumen wall \((\text{Sutton et al., 2003})\). There was a 3-way interaction among fiber, acetate, and time of day for plasma BHB concentration \((P = 0.08; \text{Figure 5B})\). Plasma BHB remained high for most of the day, except at 1100 and 1700 h in LF diets supplemented with acetate. Similarly, plasma BHB concentration was higher in the HF diet supplemented with acetate compared with the unsupplemented diet at all time points except 0800 h. Acetate supplementation has consistently increased plasma BHB concentration in recent reports, consistent with the current results \((\text{Urrutia and Harvatine, 2017a,b; Urrutia et al., 2019; Matamoros et al., 2021})\). The daily rhythm of plasma BHB is related to that of plasma acetate \((\text{Matamoros et al., 2022})\) as peak plasma BHB concentration occurs at the same time as peak plasma acetate concentration. Similar to plasma acetate concentration, the amplitude of plasma BHB concentration in HF diets was greater than that of LF diets.

We detected no effect of acetate, interaction between fiber and acetate, or 3-way interaction with the effect of time of day for plasma glucose \((P = 0.30, 0.45, \text{and } 0.72, \text{respectively}; \text{Figure 5C})\) and NEFA concentrations \((P = 0.38, 0.54, \text{and } 0.23, \text{respectively}; \text{Figure 5D})\). There was an effect of fiber on plasma glucose and NEFA concentrations \((P < 0.001 \text{ for both}; \text{Figure 5C, D})\). Plasma glucose was 4% higher in LF diets, regardless of acetate supplementation or time. Plasma NEFA concentration was 14% higher in HF diets, regardless of acetate supplementation. Low-fiber diets have a higher net propionate production in the rumen \((\text{Bauman et al., 1971; Sutton et al., 2003})\). Considering that propionate is a major gluconeogenic precursor in the liver \((\text{Wiltrout and Satter, 1972})\), it is most likely that the increased propionate supply associated with LF diets resulted in an increase in plasma glucose concentration. The decrease in plasma NEFA observed in LF diets is most likely due to a decrease in adipose tissue lipolysis associated with higher insulin concentration in response to higher plasma glucose concentrations \((\text{Oba and Allen, 2003})\).

CONCLUSIONS

Dietary acetate supplementation as NaAcet appeared to increase milk fat production to a similar extent in LF and HF diets. Acetate supplementation increased plasma acetate concentration, increasing the supply of substrates for de novo lipogenesis to the mammary gland. The increase in milk fat synthesis was most likely due to the stimulation of mammary de novo lipogenesis from the additional acetate supply, as it was associated with an increase in yield of de novo and mixed-source milk FA. The small decrease in milk trans-10 C18:1 concentration with acetate supplementation can explain only a small portion of the increase in milk fat yield observed. Sodium acetate, either through an effect of acetate or DCAD, may also affect rumen function based on DM digestibility and milk FA associated with rumen function.

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