Postpartum supplementation with fermented ammoniated condensed whey altered nutrient partitioning to support hepatic metabolism

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ABSTRACT

Our previously published paper demonstrated that fermented ammoniated condensed whey (FACW) supplementation improved feed efficiency and metabolic profile in postpartum dairy cows. The objective of this study was to further explore the effects of FACW supplementation on liver triglyceride content, hepatic gene expression and protein abundance, and plasma biomarkers related to liver function, inflammation, and damage. Individually fed multiparous Holstein cows were blocked by calving date and randomly assigned to postpartum (1 to 45 d in milk, DIM) isonitrogenous treatments: control diet ($n = 20$) or diet supplemented with FACW (2.9% dry matter of diet as GlucoBoost; Fermented Nutrition, Luxemburg, WI, replacing soybean meal; $n = 19$). Liver biopsies were performed at 14 and 28 DIM for analysis of mRNA expression, protein abundance, and liver triglyceride content. There was marginal evidence for a reduction in liver triglyceride content at 14 DIM in FACW-supplemented cows compared with the control group. Cows supplemented with FACW had greater mRNA expression of glucose-6-phosphatase at 14 DIM relative to control. Supplementation with FACW increased mRNA expression of pyruvate carboxylase (*PC*), but did not alter cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), resulting in a 2.4-fold greater *PC:PCK1* ratio for FACW-supplemented cows compared with control. There was no evidence for a FACW effect on mRNA expression of propionyl-CoA carboxylase nor on mRNA expression or protein abundance of lactate dehydrogenase A or B. Cows supplemented with FACW had lower plasma urea nitrogen compared with control. Plasma L-lactate was greater for FACW-supplemented cows compared with control at 2 h before feeding time at 21 DIM.

There was no evidence for altered expression of *IL1B* or *IL10*, or blood biomarkers related to liver function and damage. Greater glucose-6-phosphatase and *PC* gene expression, together with greater blood glucose and similar milk lactose output, suggests that FACW increased the supply of glucose precursors, resulting in greater gluconeogenesis between 3 and 14 DIM. Greater hepatic *PC:PCK1* ratio, together with previously reported decreased plasma β -hydroxybutyrate and the marginal evidence for lower liver triglyceride content at 14 DIM, suggests greater hepatic capacity for complete oxidation of fatty acids in FACW-supplemented cows compared with control. Overall, improvements in metabolite profile and feed efficiency observed with postpartum supplementation of FACW may be attributed to increased gluconeogenic and anaplerotic precursors, most likely propionate, due to modulated rumen fermentation.

Key words: transition cow, gluconeogenesis, fatty liver, lactate

INTRODUCTION

The high energy demand for milk production, coupled with low DMI, results in mobilization of body reserves during the postpartum transition period. Excessive mobilization of fatty acids (FA) from adipose tissue can exceed hepatic capacity for complete oxidation of FA. This leads to an excessive production of ketone bodies, resulting in hyperketonemia (HYK), and concomitant fat accumulation in the liver, resulting in fatty liver (Drackley, 1999; Herdt, 2000). Fatty liver and HYK are associated with liver inflammation (Lor et al., 2007; Du et al., 2018) and negative effects on production, fertility, and health in dairy cows (Bobe et al., 2004; Esposito et al., 2014).

Given the negative association with downstream health events and production (Bobe et al., 2004; Ospina et al., 2010; McArt et al., 2012), HYK and fatty liver have a large negative economic effect on dairy farms. Currently, fatty liver can only be diagnosed by liver biopsy, which makes estimation of its associated costs

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difficult (Bobe et al., 2004). However, the estimated total cost of HYK, a metabolic disorder that is closely associated with fatty liver, was reported to be \$289 per case (McArt et al., 2015). Treatment of HYK cows with propylene glycol has shown to decrease time of resolution (McArt et al., 2011; Mann et al., 2017) and risk of displaced abomasum, and to increase milk production relative to those diagnosed with HYK but not treated (McArt et al., 2011). Despite this, early detection and treatment only recovers a fraction of the total cost per case (McArt et al., 2014), highlighting the value of effective management practices and nutritional strategies to prevent metabolic disorders after calving.

Propionate, lactate, glycerol, and gluconeogenic amino acids are the primary hepatic gluconeogenic and anaplerotic precursors in dairy cows (Aschenbach et al., 2010). GlucoBoost (Fermented Nutrition Corporation, Luxemburg, WI) is a fermented ammoniated condensed whey (**FACW**) product rich in ammonium lactate. Previously, diets containing liquid FACW have shown in vitro (Wagner et al., 2018) and in vivo (Caputo Oliveira et al., 2019) to decrease rumen concentration of acetate and increase butyrate and propionate. Postpartum supplementation with FACW has also been shown to increase plasma glucose and insulin, and reduce plasma BHB in dairy cows 3 to 7 d postpartum, with marginal evidence for reduced plasma FA during the same period (Caputo Oliveira et al., 2019). It is possible that FACW supplementation may have aided hepatic metabolism by stimulating insulin release by the pancreas, reducing lipolysis in the adipose tissue, which reduces the supply of FA to the liver, ultimately reducing ketogenesis (Caputo Oliveira et al., 2019) and potentially reducing fat accumulation in the liver. Although not thoroughly understood, it has also been proposed that the etiology of fatty liver and HYK could be related to the diversion of oxaloacetate (**OAA**) from the tricarboxylic acid (**TCA**) cycle, reducing the capacity for complete oxidation of FA in the hepatocytes (Baird et al., 1968; Aschenbach et al., 2010; White, 2015). Postpartum supplementation of FACW may increase the complete hepatic oxidation of FA by directly providing substrates and upregulating gene expression of enzymes related to oxidation of FA. Our hypothesis was that the provision of ammonium lactate through FACW supplementation would alter regulation of gluconeogenesis and complete oxidation of FA, resulting in decreased fat accumulation in the liver and explaining the decreased ketogenesis reported previously. Our objective was to further explore the effects of FACW supplementation on liver triglyceride content (**lvTG**), hepatic gene expression and protein abundance, and plasma biomarkers related to liver function, inflammation, and damage.

MATERIALS AND METHODS

Animal Use, Treatments, and Handling

All animal use and handling protocols were approved by the University of Wisconsin–Madison College of Agricultural and Life Sciences Animal Care and Use Committee. Multiparous Holstein cows ($n = 40$) were housed in a tie-stall facility and fed individually from -28 d relative to the expected calving date to 45 DIM at the University of Wisconsin–Madison Dairy Cattle Instruction and Research Center. Before -28 d relative to the expected calving date, cows were blocked by expected calving date and were randomly assigned to postpartum treatments, defined as either control ($n = 20$) or FACW ($n = 20$). One cow assigned to the FACW treatment had uterine torsion at calving and was removed from the study. After calving, cows were offered ad libitum access to isonitrogenous lactating cow rations containing 2.9% DM of diet as liquid FACW (GlucoBoost; 72.7% lactate, 55.5% CP, pH = 6.5; replacing the equivalent DM percent of soybean meal) or control, once daily at 0800 h until 45 DIM. Further descriptions of animal use, diets, dietary ingredient sample collections and analysis, the production response, rumen fermentation profile, and metabolic profiles of cows have been detailed previously (Caputo Oliveira et al., 2019).

Blood Sample Collection and Metabolite Analysis

Blood samples from the coccygeal vessels were obtained from all cows at 3, 7, 14, and 28 DIM within 1 h before feeding time for BUN, albumin, aspartate aminotransferase (**AST**), and alanine aminotransferase (**ALT**) quantification. Blood samples from the coccygeal vessels were also obtained from a subset of rumen fistulated cows ($n = 3$ per treatment) at 2 h before, and at 1.5, 9, and 18 h after feeding time on 7 and 21 DIM for plasma L- and D-lactate quantification, which aligns with timing of rumen fluid sample collection for pH and VFA quantification reported previously (Caputo Oliveira et al., 2019). Blood samples were collected in tubes containing potassium oxalate and 4% sodium fluoride (BD Vacutainer, Franklin Lakes, NJ) and kept on ice in a cooler until centrifugation. Plasma was separated from blood after centrifuging at $2,000 \times g$ at 4°C for 15 min. Plasma samples were aliquoted into tubes and stored at -20°C . Quantification of BUN (mg/mL), L-lactate (mM), albumin (g/dL), ALT (1 U of ALT = amount of ALT that generates 1.0 μmol of pyruvate per min at 37°C), and AST (1 U of ALT = amount of AST that generates 1.0 μmol of glutamate per min at 37°C) concentrations in plasma samples were based on

previously described chemistries (Talke and Shubert, 1965; Doumas et al., 1971; Bergmeyer et al., 1977) and detected using Catachem VETSPEC reagents on the Catachem Well-T AutoAnalyzer (Catachem, Oxford, CT) as detailed in the Supplemental File S1 (<https://doi.org/10.3168/jds.2019-17790>). Plasma D-lactate (μM) was quantified enzymatically using D-lactate dehydrogenase (D-lactate Colorimetric Assay Kit, MAK058; Sigma Aldrich, St. Louis, MO) and read at 450 nm on a Synergy Hybrid Spectrophotometer (BioTek, Winooski, VT). For all assays, intra-assay CV never exceeded 10%. Additionally, a pooled sample was run with all sample sets and interassay CV were 7.75, 5.26, 5.48, 7.35, 2.40, and 2.34% for BUN, albumin, AST, ALT, L-lactate, and D-lactate, respectively.

Liver Sample Collection

Liver samples (500–1,000 mg) were obtained by blind percutaneous biopsy with local anesthesia (10 mL of 2% lidocaine hydrochloride solution, s.c.), using a 6-mm outside diameter, 4-mm inside diameter trocar biopsy tool at 14 and 28 DIM, as described previously (Greenfield et al., 2000; Lucy et al., 2009). Samples were rinsed with saline and subsampled into empty RNA-free tubes for subsequent lvTG measurement, into RNA-free tubes containing TRIzol (Life Technologies, Carlsbad, CA) for mRNA abundance quantification, and into empty RNA-free tubes for protein abundance analysis. All subsamples were frozen in liquid nitrogen and stored at -80°C until further analysis.

Liver Triglyceride Quantification

For lvTG, samples were thawed on ice and blotted dry, and approximately 100 mg of tissue was homogenized in 3 mL of saline:methanol (1:1). An aliquot was used for total lipid extraction by the method of Folch et al. (1957). Concentration of lvTG was determined using a colorimetric assay (Foster and Dunn, 1973), and a second aliquot of the homogenate was used to measure DNA content of the sample (Labarca and Paigen, 1980).

Gene Expression

Total RNA was extracted from approximately 200 mg of liver stored in TRIzol, using a phenol, chloroform extraction (Chomczynski, 1993) and then purified utilizing the Aurum Total RNA 96 Kit (7326800; Bio-Rad Laboratories, Hercules, CA; Miller et al., 2011) through a modified version of the manufacturer's protocol. Briefly, 350 μL of the aqueous phase after chloroform extraction was mixed with an equal volume of molec-

ular-grade ethanol and applied to the RNA binding plate. The plate was centrifuged for 2 min at $1,500 \times g$ to remove impurities, and bound-RNA subsequently was washed with a low stringency buffer, and the plate was centrifuged under the same conditions. On-column DNA digestion was performed by incubation with DNase I (7326828, Bio-Rad Laboratories) before subsequent washing with a high and then low stringency buffer with centrifugation steps as described above. RNA was eluted from the RNA binding plate into a PCR clean 96-well plate (1402–9890; USA Scientific). Total RNA was quantified, and quality assured (ratio absorbance of 260/280 nm between 1.9 and 2.1) using the Synergy Hybrid Spectrophotometer (BioTek). Additionally, RNA integrity number (RIN) was assessed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and RIN mean $\pm$ SD was 6.73 ± 0.84 . Purified RNA (1 μg) was reverse transcribed using iScript Reverse Transcription Supermix (1708840; Bio-Rad Laboratories) by incubating RNA, supermix, and nuclease-free water for 5 min at 25°C , 20 min at 46°C , and 1 min at 95°C in a C1000 Touch Thermo Cycler (Bio-Rad Laboratories). Gene expression was determined via real-time qPCR (RT-qPCR) using SsoAdvanced Universal SYBR Green Supermix (1725270, Bio-Rad Laboratories) in a CFX384 Real-Time System (Bio-Rad Laboratories). Primers were either identified in previous publication and verified, or designed to span exon-to-exon junctions using NCBI Primer-BLAST primer designing tool (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17790>). In all cases, a single PCR product was confirmed via melt curve using the following protocol: 1 cycle at 95°C for 30 s; 45 cycles at 95°C and 55°C for 5 and 15 s, respectively; and a melt curve run from 65°C to 95°C by 0.5°C increments for 5 s. Expression of β -actin (*ACTB*), 18S, ubiquitin B (*UBB*), ribosomal protein S9 (*RPS9*), ribosomal protein L32 (*RPL32*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), pyruvate carboxylase (*PC*), glucose-6-phosphatase (*G6PC*), lactate dehydrogenase A (*LDHA*), lactate dehydrogenase B (*LDHB*), cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), mitochondrial phosphoenolpyruvate carboxykinase (*PCK2*), propionyl-CoA carboxylase A (*PCCA*), propionyl-CoA carboxylase B (*PCCB*), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*), *IL10*, *IL1B*, arginase isoform 1 (*ARG1*), argininosuccinate synthase isoform 1 (*ASS1*), ornithine transcarbamoylase (*OTC*), and carbamoyl phosphate synthetase isoform 1 (*CPS1*) was quantified with 2 or 4 μL of cDNA, SsoAdvanced SYBR, respective forward and reverse primers (Supplemental Table S1), and nuclease-free water for a total reaction volume of 10 μL . The analysis of RT-qPCR was performed according to the following protocol: 1 cycle at 95°C for 30 s, and

45 cycles at 95°C and 55°C for 5 and 15 s, respectively. Efficiency of all reactions were between 90 and 110%, based on the 5-point standard curve of the cDNA pool comprised of equal quantities from each sample diluted 1:4. Cycle data were transformed to starting quantity data with Bio-Rad CFX Software (Bio-Rad) based on the standard curve. The best-suited reference genes were chosen based on statistical algorithm geNorm (Vandesompele et al., 2002) and subsequent analysis to ensure no differences across time and between treatments. The mRNA abundance of each target gene was the starting quantity of the target gene expressed as arbitrary units normalized to the geometric mean starting quantity of 2 reference genes (*RPS9* and *GAPDH*) expressed as arbitrary units within each sample.

Protein Isolation and Western Blot Analysis

For the hepatic genes regulated at the protein level, specifically lactate dehydrogenase A (**LDHA**) and B (**LDHB**), semiquantitative Western blotting was used to analyze their protein abundance. Protein was isolated from liver samples of at least 200 mg by homogenization and centrifugation in lysis buffer in the presence of protease inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, Rockford, IL). Equal volumes of isolated protein samples were pooled together to create a protein pool for quality-control standards, referred to herein as “the pool.” Isolated protein samples and pool aliquot concentration was determined by bicinchoninic acid (Pierce Biotechnology Inc., Rockford, IL) assay analysis. A standardized total protein quantity of 25 µg of the pool and all samples was used for analysis under denaturing conditions. Protein samples and a Precision Plus WesternC standard (Bio-Rad Laboratories) were loaded into a 4–20% Criterion TGX Stain-Free Protein Gels (Bio-Rad Laboratories; Elbaggari et al., 2008) and separated by gel electrophoresis. After electrophoresis, gels were activated on a ChemiDoc XRS+ (Bio-Rad Laboratories) for 1 min for downstream total lane protein imaging and for transfer to a polyvinylidene fluoride membrane utilizing the TransBlot Turbo transfer system (Bio-Rad Laboratories; Hammond et al., 2013). Membranes were blocked for 2 h at room temperature before addition of primary antibodies (1:1,000) for either 1 h at room temperature (LDHA; host rabbit, LS-C143436, LSBio) or overnight at 4°C (LDHB; host goat, LS-C305874, LSBio). After primary incubation, blots were washed and secondary antibodies (1:5,000; LDHA, chicken anti-rabbit, ab6829, Abcam, Cambridge, MA; LDHB, donkey anti-goat, ab6885, Abcam) and HRP conjugate for WesternC ladder (161–0381; Bio-Rad Laboratories) were applied for 1 h at room temperature.

Images of blots were captured on the ChemiDoc Imager (Bio-Rad Laboratories) using Image Lab 5.0 software. Total Lane Protein image for downstream normalization was obtained by imaging without substrate using the Stain-Free setting (Bio-Rad Laboratories; Taylor et al., 2013; Taylor and Posch, 2014). To image bands of interest, SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Thermo Scientific, Rockford, IL) was applied to each blot and the ChemiHi sensitivity setting used. After imaging, blots were rinsed, stripped, and re-blocked overnight at 4°C before the next protein quantification. This process was repeated until both proteins of interest were completed on one blot via primary antibody requirements. Previous research in other species has suggested differential roles of dimeric and tetrameric forms of LDHA and LDHB (Yamamoto and Storey, 1988; Urbńska and Orzechowski, 2019) so forms were quantified separately and expressed as total monomeric units for analysis. Monomeric, dimeric, tetrameric forms, and total monomeric units of LDHA and LDHB were quantified from images. Interassay CV of the pool were calculated across blots and were less than 10% for monomeric, dimeric, and tetrameric forms of LDHA and LDHB, respectively. Intra-assay CV of the pool never exceeded 10%. Additional details and example blots are provided in Supplemental File S1 and Figure S1, respectively (<https://doi.org/10.3168/jds.2019-17790>).

Statistical Analysis

Continuous response variables were analyzed with general linear mixed models using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute, Cary, NC). For L- and D-lactate, the linear predictor included the fixed effects of treatment, sampling day, sampling time relative to feeding, and all interactions, as well as the random effects of block and cow nested within treatment. Repeated measures of cow within treatment over time were accounted for. In each case, based on Bayesian information criteria and Akaike's information criteria, the model with spatial power covariance structure was the best-fit and was used for further inference. For all other response variables, the linear predictor included the fixed effects of treatment, sampling day, and all interactions, as well as the random effects of block and cow nested within treatment. Repeated measures of cow within treatment over time were accounted for. In each case, the model with the best-fitting covariance structures was selected based on Bayesian information criteria and Akaike's information criteria and used for further inference. Contrasts for 3 to 14 DIM and 17 to 28 DIM were analyzed for previously reported metabolites of BHB, FA, and glucose. Analyses of studentized

Table 1. Estimated mean (95% CI) plasma metabolites in dairy cows treated with control diet or supplemented with fermented ammoniated condensed whey (FACW)

Metabolite ¹	Treatment ²		<i>P</i> -value
	Control	FACW	
Glucose			
3–14 DIM	49.37 (46.78, 51.96)	52.11 (49.69, 54.52)	0.02
17–28 DIM	52.04 (49.84, 54.25)	52.61 (50.55, 54.65)	0.70
Fatty acids			
3–14 DIM	0.80 (0.76, 0.83)	0.76 (0.73, 0.80)	0.11
17–28 DIM	0.69 (0.65, 0.72)	0.70 (0.67, 0.74)	0.40
BHB			
3–14 DIM	0.76 (0.68, 0.83)	0.74 (0.67, 0.81)	0.45
17–28 DIM	0.71 (0.64, 0.77)	0.74 (0.68, 0.80)	0.34

¹3–14 DIM = analysis of blood samples collected on 3, 5, 7, 9, 11, and 14 DIM; 17–28 DIM = analysis of blood samples collected on 17, 21, and 28 DIM.

²Treatments: FACW-supplemented diet (n = 19) containing 2.9% DM of diet in a liquid FACW (Glucoboost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet (n = 20) containing the equivalent percentage as soybean meal, from calving to 45 DIM.

residuals were performed to check that model assumptions were reasonably met. The following data transformations were used to meet residual assumptions: the variables *lvTG*, plasma L-lactate, the gene expression (normalized to geometric mean of reference genes) of *G6PC*, *LDHA*, *PC*, *PCK1*, *IL10*, *IL1B*, *PC:PCK1* ratio, *PC:PCK2* ratio, *PCCA*, *PCCB*, *HMGCS2*, *ARG1*, *ASS1*, *OTC*, and *CPS1* were natural log-transformed, and gene expression of *LDHB* and *PCK2* normalized to reference genes was square root-transformed.

When treatment by time interaction effects $P \leq 0.10$, treatment differences were evaluated within each time point (i.e., simple-effect comparisons). All post hoc simple-effect comparisons were Bonferroni adjusted to prevent inflation of Type I error due to multiplicity (Bello and Renter, 2018). Means were considered significantly different when $P \leq 0.05$ and marginal evidence for difference when $0.05 < P \leq 0.10$. Estimated treatment means are presented as least square estimates and 95% confidence intervals (95% CI) in the observed data scale, following back-transformation when appropriate. Supplemental Table S2 and Figures S2, S3, and S4 (<https://doi.org/10.3168/jds.2019-17790>) present data in the transformed scale.

RESULTS

The effect of FACW supplementation on milk yield, DMI, and blood and rumen metabolites from this study can be found in Caputo Oliveira et al. (2019). Additionally, supplementation of FACW resulted in improved feed and N efficiency, as assessed by measures of milk output relative to feed intake (Caputo Oliveira et al., 2019). Briefly, cows supplemented with FACW had greater plasma glucose and insulin, and lower plasma BHB and FA concentrations from 3 to 7 DIM (Caputo

Oliveira et al., 2019). For reference to liver biopsy time points in the current manuscript, contrasts for glucose, FA, and BHB from 3 to 14, and 17 to 28 DIM are provided (Table 1). Glucose concentration was greater ($P = 0.02$) from 3 to 14 DIM, but not 17 to 28 DIM, in FACW-treated cows compared with control. No differences ($P \geq 0.11$) were detected for FA or BHB for these contrast periods.

For plasma L-lactate, a 3-way interaction between treatment, DIM and sampling time relative to feeding ($P = 0.01$) was explained by a 2.9-fold greater ($P < 0.01$) plasma L-lactate concentration for FACW compared with control at 2 h before feeding time at 21 DIM (Figure 1). There was no evidence for any treatment or treatment by time interaction ($P \geq 0.16$) on plasma D-lactate [$13.8 \mu\text{M}$ (95% CI: 7.24, 20.51) vs. $13.4 \mu\text{M}$ (95% CI: 6.83, 19.9) for control and FACW, respectively]. Cows supplemented with FACW had lower ($P = 0.05$) BUN compared with control (Figure 2). There was no evidence ($P \geq 0.22$) for any FACW-driven effects on plasma AST, ALT, or albumin (Table 2).

For hepatic *G6PC* mRNA expression, the treatment by time interaction effect ($P < 0.01$) was explained by a greater ($P < 0.001$) mRNA expression at 14 DIM for FACW-supplemented cows compared with control (Figure 3). Cows supplemented with FACW had greater ($P = 0.01$) mRNA expression of *PC* (Table 3). There was no evidence for any treatment or treatment by time interaction effect ($P \geq 0.16$) on mRNA expression of *PCK1* and *PCK2* (Table 3). Together, the greater mRNA expression of *PC*, with no detected FACW-driven changes in *PCK*, resulted in a greater ($P \leq 0.02$) *PC:PCK1* and *PC:PCK2* in cows supplemented with FACW compared with control (Table 3). There was no evidence ($P \geq 0.16$) for any treatment effects or treatment by time interaction effects on mRNA ex-

Figure 1. Estimated mean $\pm$ 95% CI plasma L-lactate in dairy cows treated with control (open bar) or fermented ammoniated condensed whey (FACW; closed bar) 2 h before and 1.5, 9, and 18 h after feeding time on 7 and 21 DIM. Treatments: FACW-supplemented diet ($n = 19$) containing 2.9% DM of diet in a liquid FACW (Glucoboost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet ($n = 20$) containing the equivalent DM percentage as soybean meal from calving to 45 DIM. Plasma L-lactate concentration was natural log-transformed. Estimated treatment means and 95% CI were back-transformed to be presented in the observed data scale. Treatment $P = 0.15$ and 3-way interaction between treatment, DIM, and sampling time relative to feeding $P = 0.01$. Brackets represent Bonferroni-adjusted simple-effect comparisons between treatment means within sampling time relative to feeding.

Figure 2. Estimated mean $\pm$ 95% CI; BUN concentration in dairy cows treated with control (open triangles) or fermented ammoniated condensed whey (FACW; closed circles) on 3, 7, 14, and 28 DIM. Treatments: FACW-supplemented diet ($n = 19$) containing 2.9% DM of diet in a liquid FACW (Glucoboost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet ($n = 20$) containing the equivalent DM percentage as soybean meal from calving to 45 DIM. Treatment $P = 0.05$ and treatment $\times$ time interaction $P = 0.45$. Overall treatment LSM and 95% CI: 15.01 μ M (95% CI: 13.77, 16.26) versus 13.59 μ M (95% CI: 12.45, 14.74) for control and FACW, respectively.

pression of *PCCA*, *PCCB*, *LDHA*, *LDHB*, *HMGCS2*, *IL1B*, and *IL10* (Table 3). There was no evidence ($P \geq 0.34$) for any treatment effects or treatment by time interaction effects on mRNA expression of the target genes *ARG1*, *ASS1*, *OTC*, and *CPS1* involved in the urea cycle (Table 3).

For lvTG content, the treatment by time interaction effect ($P < 0.01$) was explained by marginal evidence for lower ($P = 0.09$) lvTG content at 14 DIM for FACW-fed cows compared with control (Figure 4). There was no evidence ($P \geq 0.25$) for any treatment effects or treatment by time interaction effects on liver LDHA or LDHB protein abundance (Table 4).

DISCUSSION

Postpartum supplementation with FACW in dairy cows resulted in greater blood insulin and glucose, and reduced plasma FA and BHB, indicating improved plasma metabolite profile especially in the first week postpartum (Caputo Oliveira et al., 2019). These findings are suggestive that supplementation of FACW modulated rumen fermentation to generate gluconeogenic and anaplerotic precursors available for absorption and subsequent liver uptake. Availability of substrates could alter regulation of gluconeogenesis and complete oxidation of FA, resulting in decreased fat accumulation in the liver and partially explaining

Table 2. Estimated mean (95% CI) plasma metabolites and enzyme activity related to liver function and damage in dairy cows treated with control diet or diet supplemented with fermented ammoniated condensed whey (FACW)

Item ¹	Treatment ²		P-value ³		
	Control	FACW	Trt	DIM	Trt $\times$ DIM
Albumin, g/dL	3.75 (3.61, 3.89)	3.83 (3.68, 3.97)	0.22	<0.01	0.78
ALT, U/L	12.09 (10.53, 13.87)	11.93 (10.36, 13.74)	0.84	0.02	0.80
AST, U/L	78.75 (67.23, 92.24)	83.70 (71.18, 98.44)	0.48	<0.01	0.31

¹ALT = plasma alanine aminotransferase; 1 U ALT = amount of ALT that generates 1.0 μ mol of pyruvate per min at 37°C; AST = plasma aspartate aminotransferase; 1 U AST = amount of AST that generates 1.0 μ mol of glutamate per min at 37°C.

²Treatments: FACW-supplemented diet ($n = 19$) containing 2.9% DM of diet in a liquid FACW (Glucoboost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet ($n = 20$) containing the equivalent percentage as soybean meal, from calving to 45 DIM.

³Trt = treatment; blood samples collected on 3, 7, 14, and 28 DIM.

the decreased ketogenesis (White, 2015) reported previously (Caputo Oliveira et al., 2019). Therefore, the objective of this study was to further explore the effects of FACW supplementation on IvTG concentration, hepatic gene expression and protein abundances, and plasma biomarkers related to liver function, inflammation, and damage.

The ammonium lactate product contains 72.7% of a racemic mix composed of equal proportions of D- and L-isomers of lactate (Wagner et al., 2018). Once in the rumen, lactate can be fermented to propionate and other VFA by lactate-utilizing microbes (Baldwin et al., 1962; Brossard et al., 2004; Nagaraja and Titgemeyer, 2007) or absorbed directly as lactate (Schwaiger et al., 2013; Qumar et al., 2016). During the first weeks postpartum, increased hepatic capacity for utilization of lactate may be of great value to dairy cows because propionate, although still the main gluconeogenic precursor, is limited during this phase (Reynolds et al., 2003; Aschenbach et al., 2010). Greater hepatic lactate

uptake and its increased use for gluconeogenesis around calving has been well documented (Reynolds et al., 2003; Aschenbach et al., 2010; Larsen and Kristensen, 2013) with increased hepatic utilization of lactate peaking around the first 2 wk of lactation (Reynolds et al., 2003; Larsen and Kristensen, 2009). Increased rumen absorption of lactate can only definitively be quantified in portal blood or by measuring net hepatic uptake directly; however, increased hepatic uptake of lactate may be reflected in the hepatic proteins involved in lactate metabolism. Transcriptional regulation of the *LDH* gene has been found to be regulated by lactate as well as by many other factors including estrogen and a variety of protein factors reviewed by Valvona et al. (2016). In the current study, there was no apparent differences between treatments, nor across time, on *LDHA* or *LDHB* mRNA expression, the enzyme that catalyzes the inter-conversion of pyruvate and lactate, nor the abundance of the resulting LDHA and LDHB proteins.

Supplementation with FACW in the current study only minimally affected plasma lactate, as observed with only a treatment by time interaction of L-lactate to be increased 2 h before feeding at 21 DIM. It is possible that fermentation of lactate to propionate and other VFA in the rumen may have limited lactate absorption and thus reduced the FACW supplementation response on increasing plasma D- and L-lactate, supported by the shifts in the molar proportions of VFA previously reported (Caputo Oliveira et al., 2019). The relative contribution of lactate to hepatic metabolism may also be confounded by glucose and propionate availability, given that glucose and propionate infusion has been demonstrated to decrease net hepatic uptake of lactate (Baird et al., 1968). This should be considered here because there was marginal evidence for an increase in molar proportion of propionate in rumen fluid at 7 DIM and blood glucose was increased in the first week postpartum with FACW supplementation (Caputo Oliveira et al., 2019). Taken together, it appears that the responses to FACW supplementation may be primarily in response to modulation of rumen fermentation to produce other gluconeogenic precursors such as propionate.

In the current study, supplementation with FACW increased hepatic gene expression of *PC* compared with control throughout the entire period analyzed, and it has been demonstrated that gene expression of *PC* has a strong correlation with its enzyme activity (Greenfield et al., 2000). In the liver of ruminant animals, *PC* serves as an anaplerotic enzyme that catalyzes the ATP-dependent carboxylation of pyruvate, primarily from lactate and amino acids, to OAA in the mitochondria, replenishing the pool of OAA in the TCA cycle (White, 2015). Previously, *PC* mRNA expression

Figure 3. Estimated mean $\pm$ 95% CI mRNA expression of glucose-6-phosphatase (*G6PC*) normalized to the geometric mean starting quantity of 2 reference genes within each sample (ribosomal protein S9 and glyceraldehyde-3-phosphate dehydrogenase) in dairy cows treated with control (open bars) or fermented ammoniated condensed whey (FACW; closed bars) on 14 and 28 DIM. Treatments: FACW-supplemented diet ($n = 19$) containing 2.9% DM of diet in a liquid FACW (Glucoboost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet ($n = 20$) containing the equivalent DM percentage as soybean meal from calving to 45 DIM. The gene expression of *G6PC* normalized to reference genes was natural log-transformed. Estimated means and 95% CI were back-transformed to be presented in the observed data scale. Treatment $P < 0.001$ and treatment $\times$ time interaction $P < 0.01$. Brackets represent Bonferroni-adjusted simple-effect comparisons between treatment means within DIM.

has been shown to be increased with reduced insulin: glucagon ratio (She et al., 1999; Hammon et al., 2009) and during feed restriction in dry (Lor et al., 2006) and mid-lactation dairy cows (Velez and Donkin, 2005). In bovine, activity of *PC* is regulated at the transcriptional level and further augmented through direct allosteric activation by acetyl-CoA (Greenfield et al., 2000; Mayes et al., 2003). Profile and increased concentration of blood FA seemed to explain most of the effect of feed restriction and negative energy balance on upregulating *PC* promoter 1 in dairy cows (White et al., 2011); however, propionate (Xu and Wang, 2006; Zhang et al., 2016) and pyruvate (Xu and Wang, 2006) also induce mRNA expression of *PC* in bovine calf hepatocytes. In the current study, the postpartum supplementation with FACW resulted in marginal evidence for a higher molar ratio of rumen propionate on d 7 postpartum and marginal evidence for a reduction in plasma FA concentration from 3 to 7 DIM, and greater plasma insulin throughout the supplementation period (Caputo Oliveira et al., 2019). Considered together, it is possible that the greater *PC* mRNA expression in FACW-fed cows was stimulated by a potential increased supply of propionate through FACW supplementation.

Cows supplemented with FACW had 2.1-fold greater gene expression of *G6PC* at 14 DIM compared with control cows. The hepatic *G6PC* gene encodes the enzyme glucose 6-phosphatase, which is responsible for the last step of gluconeogenesis, the release of glucose from the hepatocytes (Tanaka et al., 2006). In nonruminants, the expression of *G6PC* is known to be upregulated at the transcriptional level by multiple hormones and metabolites such as glucose (Argaud et al., 1997; Massillon et al., 1998), propionate and butyrate (Massillon et al., 2003), and glucagon, glucocorticoids, and FA (Pedersen et al., 2007; Hutton and O'Brien, 2009). The mechanism in which intracellular glucose and its metabolites upregulates *G6PC* gene expression seems to be by a transcriptional activation of the *G6PC* gene promoter, and a decrease in the degradation of *G6PC* mRNA (Massillon, 2001). Conversely, in ruminants, propionate did not induce *G6PC* gene expression in bovine calf hepatocytes (Zhang et al., 2016); however, its upregulation seems to be part of the adaptation of increased hepatic gluconeogenesis after calving in dairy cows (Cedeño et al., 2008). Supplementation with FACW increased molar ratio of propionate and plasma glucose from 3 to 7 DIM (Caputo Oliveira et al., 2019).

Table 3. Estimated mean (95% CI) hepatic gene expression relative to the geometric mean of reference genes¹ in dairy cows treated with control diet or diet supplemented with fermented ammoniated condensed whey (FACW)

Gene ²	Treatment ³		P-value ⁴		
	Control	FACW	Trt	DIM	Trt × DIM
<i>G6PC</i>	1.40 (1.15, 1.70)	2.21 (1.76, 2.78)	<0.01	0.09	<0.01
<i>PC</i>	0.85 (0.64, 1.12)	1.20 (0.90, 1.60)	0.01	0.49	0.77
<i>PCK1</i>	1.20 (0.73, 1.97)	0.82 (0.48, 1.41)	0.14	0.81	0.95
<i>PCK2</i>	1.07 (0.87, 1.44)	1.09 (0.89, 1.52)	0.77	0.06	0.16
<i>PC:PCK1</i>	0.65 (0.35, 1.23)	1.56 (0.78, 3.14)	0.01	0.58	0.86
<i>PC:PCK2</i>	0.66 (0.45, 0.96)	1.06 (0.70, 1.61)	0.02	0.55	0.78
<i>PCCA</i>	3.75 (2.91, 4.50)	4.19 (3.24, 4.99)	0.32	<0.01	0.78
<i>PCCB</i>	1.96 (1.47, 2.39)	2.02 (1.54, 2.49)	0.72	<0.01	0.68
<i>LDHA</i>	11.43 (8.06, 16.13)	11.10 (7.52, 15.79)	0.79	0.44	0.16
<i>LDHB</i>	9.91 (5.07, 16.16)	10.20 (4.51, 18.02)	0.91	0.28	0.94
<i>HMGCS2</i>	0.86 (0.65, 1.07)	0.92 (0.69, 1.15)	0.59	0.24	0.94
<i>IL10</i>	2.26 (0.98, 5.35)	4.04 (1.55, 10.72)	0.21	0.92	0.59
<i>IL1B</i>	0.68 (0.47, 0.97)	0.63 (0.42, 0.94)	0.72	0.65	0.87
<i>ARG1</i>	1.25 (0.98, 1.60)	1.11 (0.88, 1.41)	0.50	0.55	0.70
<i>ASS1</i>	2.45 (2.00, 3.02)	2.79 (2.30, 3.39)	0.36	<0.01	0.34
<i>OTC</i>	1.81 (1.35, 2.41)	1.86 (1.41, 2.46)	0.88	0.12	0.87
<i>CPS1</i>	1.57 (1.26, 1.95)	1.73 (1.41, 2.13)	0.51	<0.01	0.73

¹Ribosomal protein S9 and glyceraldehyde-3-phosphate dehydrogenase.

²*G6PC* = glucose-6-phosphatase; *PC* = pyruvate carboxylase; *PCK1* = cytosolic phosphoenolpyruvate carboxykinase; *PCK2* = mitochondrial phosphoenolpyruvate carboxykinase; *PCCA* = propionyl-CoA carboxylase A; *PCCB* = propionyl-CoA carboxylase B; *LDHA* = lactate dehydrogenase A; *LDHB* = lactate dehydrogenase B; *HMGCS2* = 3-hydroxy-3-methylglutaryl-CoA synthase 2; *ARG1* = arginase isoform 1; *ASS1* = argininosuccinate synthase isoform 1; *OTC* = ornithine transcarbamoylase; and *CPS1* = carbamoyl phosphate synthetase isoform 1. The gene expression (normalized to reference genes) of *G6PC*, *LDHA*, *PC*, *PCK1*, *IL10*, *IL1B*, *PC:PCK1* ratio, *PC:PCK2* ratio, *PCCA*, *PCCB*, *HMGCS2*, *ARG1*, *ASS1*, *OTC*, and *CPS1* was natural log-transformed. The gene expression of *LDHB* and *PCK2* normalized to reference genes was square root-transformed. Estimated treatment means and 95% CI from transformed data were back-transformed to be presented in the observed data scale.

³Treatments: FACW-supplemented diet (n = 19) = 2.9% DM of diet in a liquid FACW (GlucoBoost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet (n = 20) = the equivalent percentage as soybean meal, from calving to 45 DIM.

⁴Trt = treatment; liver samples collected on 14 and 28 DIM.

Table 4. Estimated mean (95% CI) hepatic protein abundance of lactate dehydrogenase A (LDHA) and B (LDHB) in dairy cows treated with control diet or diet supplemented with fermented ammoniated condensed whey (FACW)

Protein ¹	Treatment ²		P-value ³		
	Control	FACW	Trt	DIM	Trt × DIM
LDHA, ×10 ⁷					
Tetramer	1.82 (1.47, 2.16)	1.54 (1.15, 1.93)	0.30	<0.01	0.61
Dimer	1.57 (1.29, 1.86)	1.51 (1.19, 1.82)	0.75	0.17	0.40
Monomer	0.35 (0.25, 0.49)	0.33 (0.23, 0.48)	0.82	0.03	0.36
Total	10.85 (8.91, 12.79)	9.16 (6.99, 11.33)	0.25	0.07	0.87
LDHB, ×10 ⁷					
Tetramer	1.64 (1.39, 1.88)	1.49 (1.22, 1.77)	0.44	0.09	0.55
Dimer	1.58 (1.33, 1.84)	1.61 (1.33, 1.89)	0.90	<0.01	0.97
Monomer	0.07 (0.05, 0.11)	0.06 (0.04, 0.09)	0.52	0.64	0.91
Total	9.81 (8.45, 11.16)	9.28 (7.76, 10.80)	0.60	0.96	0.67

¹Protein abundance was normalized to total lane protein and natural log-transformed. Estimated treatment means and 95% CI from transformed data were back-transformed to be presented in the observed data scale. Total monomeric units calculated as total = monomeric + (dimeric × 2) + (tetrameric × 4).

²Treatments: FACW-supplemented diet (n = 19) containing 2.9% DM of diet in a liquid fermented ammoniated condensed whey (Glucoboost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet (n = 20) containing the equivalent percentage as soybean meal, from calving to 45 DIM.

³Trt = treatment; liver samples collected on 14 and 28 DIM.

Figure 4. Estimated mean ± 95% CI; hepatic triglyceride (TG) concentration in dairy cows treated with control (open bar) or fermented ammoniated condensed whey (FACW; closed bar) on 14 and 28 DIM. Treatments: FACW-supplemented diet (n = 19) containing 2.9% DM of diet in a liquid FACW (Glucoboost, Fermented Nutrition Corporation, Luxemburg, WI; 72.7% lactate, 55.5% CP); control diet (n = 20) containing the equivalent DM percentage as soybean meal from calving to 45 DIM. Hepatic triglyceride concentration was natural log-transformed. Estimated treatment means and 95% CI were back-transformed to be presented in the observed data scale. Treatment $P = 0.81$ and treatment by time interaction $P < 0.001$. Brackets represent Bonferroni-adjusted simple-effect comparisons between treatment means within DIM.

Additionally, post hoc analysis of metabolites during the 3 to 14 DIM that would proceed the respective liver biopsies supports increased plasma glucose concentrations between calving and the 14 DIM liver sample (plasma glucose contrast from 3–14 DIM, $P = 0.03$, Table 1), and aligns with the increased *G6PC* mRNA expression. Therefore, greater *G6PC* gene expression, together with previously reported greater blood glucose and similar milk lactose output (Caputo Oliveira et al., 2019), suggests that FACW provided greater gluconeogenic precursors, resulting in greater gluconeogenesis compared with control during this period. This premise is yet to be tested experimentally with direct measure of endogenous glucose production and should be further examined.

In bovine, the gene expression of *PCK1* and *PCK2* are upregulated in the presence of propionate (Zhang et al., 2016). Rumen propionate production increased in vitro in diets containing FACW (Wagner et al., 2018) and rumen molar proportion of propionate was greater in vivo in dairy cows supplemented with FACW on d 7, but it was not different on d 21 postpartum (Caputo Oliveira et al., 2019). There is evidence that the regulation of *PCCA* mRNA abundance is coordinated with *PCK1* gene expression (Aschenbach et al., 2010). The physiological importance of the upregulation of mRNA expression of *G6PC* and *PC*, but not *PCK* and *PCC*, with FACW supplementation is not understood; however, the higher plasma insulin observed FACW-supplemented cows compared with control (Caputo Oliveira et al., 2019) may have mitigated the potential stimulation of *PCK* mRNA expression by propionate (She et al., 1999; Zhang et al., 2016). Plasma glucose

concentration remained greater at 14 DIM, as noted above, for cows fed with FACW compared with control, as was *PC* and *G6PC* mRNA expression; therefore, it is possible that gene transcription of *PCK* and *PCC* may not have been the rate-limiting steps for gluconeogenesis at that time.

In opposition to *PC*, the cataplerotic enzyme encoded by *PCK1* and *PCK2* drains OAA from the TCA cycle for gluconeogenesis by metabolizing OAA to phosphoenolpyruvate (Aschenbach et al., 2010). In this study, the greater gene expression of *PC*, with no detected FACW-driven changes in *PCK1*, resulted in a 2.4-fold greater *PC:PCK1* in FACW-supplemented cows compared with control. A diversion of OAA from the TCA cycle likely contributes with the etiology of fatty liver and HYK (Baird et al., 1968; Aschenbach et al., 2010; White, 2015); thus, because mRNA abundance of *PC* and *PCK1* are strongly correlated with enzyme activities (Greenfield et al., 2000; Agca et al., 2002; Al-Trad et al., 2010), a greater mRNA abundance of *PC:PCK1* ratio has been suggested to lead to greater hepatic capacity for complete oxidation of FA (White, 2015). Together with the observed marginal evidence for a reduced plasma FA in FACW-supplemented cows from 3 to 7 DIM (Caputo Oliveira et al., 2019), the greater *PC:PCK1* ratio in FACW-supplemented cows may explain in part the lower plasma BHB from 3 to 7 DIM (Caputo Oliveira et al., 2019) and the marginal evidence for lower lvTG content at 14 DIM observed in FACW-supplemented cows compared with control. Interestingly, the proposed mechanism that suggests greater hepatic capacity for complete oxidation of FA in FACW-supplemented cows due to higher *PC:PCK1* ratio is consistent with the marginal evidence for a reduced feed intake observed in cows fed with FACW compared with control after wk 2 postpartum (Caputo Oliveira et al., 2019). Together with the potentially greater supply of anaplerotic substrates into the liver of FACW-fed cows, greater potential for ATP production from increased capacity for complete oxidation of FA in the hepatocytes of FACW-fed cows compared with control may have generated a feedback stimulus via hepatic vagal afferents to the brain, which could stimulate satiety, according to the hepatic oxidation theory (Allen et al., 2009).

Poor adaptation to lactation during the transition period in dairy cows leads to significant increases in plasma FA and liver lipid accumulation, resulting in increased hepatic oxidative stress, which is associated with hepatocellular inflammation and damage (Lor et al., 2007; Du et al., 2018). Given this potential issue, and the improvement in the metabolite profile observed in FACW-supplemented cows (Caputo Oliveira et al., 2019) together with a decreased lvTG accumulation, it

stands to reason that liver inflammation status and cellular damage could have also been affected by FACW supplementation. To examine this, we quantified presence of hepatic enzymes in the blood, specifically AST and ALT, indicating hepatocellular damage (i.e., lysis and necrosis). Moreover, we also assessed indicators of hepatic inflammatory response and resolution through the hepatic gene expression of *IL1B* and *IL10*, respectively; previous studies have shown that mRNA levels correlate closely with the active protein levels for those cytokines (Csak et al., 2011; Ouyang and O'Garra, 2019). Although not a comprehensive investigation of hepatic inflammation, we found no evidence for any treatment effect on the hepatic gene expression or blood enzymes associated with liver inflammation and damage in the markers investigated within.

Cows supplemented with FACW had lower BUN compared with control throughout the entire period analyzed. The hepatic synthesis of urea in dairy cows is derived mainly from rumen ammonia-N and the catabolism of amino acids (Lapierre and Lobley, 2001) which accounted for about 70 and 30% of the urea synthesized in lactating dairy cows, respectively, in a previous study (Reynolds and Huntington, 1988). Supplementation with FACW resulted in marginal evidence for increased rumen fluid ammonia-N concentration in dairy cows (Caputo Oliveira et al., 2019) and increased concentration in vitro (Wagner et al., 2018) when compared with isonitrogenous diets containing soybean meal. Decreased ureagenesis from catabolism of amino acids has been demonstrated with infusions of insulin (Jefferson et al., 1968; Mackle et al., 1999), and plasma insulin was greater in FACW-supplemented cows (Caputo Oliveira et al., 2019). Given the lower BUN and the marginal evidence for greater efficiency of nitrogen utilization in FACW-supplemented cows compared with control (Caputo Oliveira et al., 2019), we suspected that less amino acids may have been catabolized in the liver of cows supplemented with FACW compared with control; however, unaltered expression of genes related to urea cycle enzymes does not support this hypothesis. Further research is needed to understand the mechanism by which FACW potentially influenced nitrogen metabolism.

CONCLUSIONS

Overall, apparent improvements in metabolite profile and feed efficiency observed with postpartum FACW supplementation may be attributed to increased gluconeogenic and anaplerotic precursors, most likely propionate, through modulated rumen fermentation. Greater gene expression of *G6PC* and *PC*, together with greater blood glucose and similar milk lactose output, suggests

that FACW provided greater supply of gluconeogenic precursors to dairy cows, resulting in greater gluconeogenesis compared with control. Greater hepatic *PC* to *PCK1* ratio observed with postpartum FACW supplementation suggests that anaplerotic precursors may have also supported greater hepatic capacity for complete oxidation of FA, which could partially explain the lower lvTG content at 14 DIM and the previously reported lower ketogenesis in the first week postpartum for FACW-supplemented cows compared with control.

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