Effects of dietary rumen-protected choline supplementation to periparturient dairy cattle on inflammation and metabolism in mammary and liver tissue during an intramammary lipopolysaccharide challenge

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ABSTRACT

The objective of this experiment was to examine the effects of supplementation and dose of rumen-protected choline (RPC) on markers of inflammation and metabolism in liver and mammary tissue during an intramammary lipopolysaccharide (LPS) challenge. Parous Holstein cows were blocked by calving month and randomly assigned within block to receive 45 g/d of RPC (20.4 g/d of choline ions; CHOL45), 30 g/d of RPC (13.6 g/d of choline ions; CHOL30), or no RPC (CON) as a top-dress starting 24 d before expected calving until 21 d postpartum. Cows were alternately assigned within treatment group to either receive an intramammary LPS challenge (200 μg in each rear quarter; Escherichia coli O111:B4) or not at 17 DIM (CHOL45, n = 9; CHOL30, n = 11; CHOL30-LPS, n = 10; CON, n = 10; CON-LPS, n = 9). Hepatic and mammary tissues were collected from all cows on d 17 postpartum. Hepatic and mammary tissues were collected at ~7.5 and 8 h, respectively, after the LPS challenge. An additional mammary biopsy was conducted on LPS-challenged cows (CHOL45-LPS, CHOL30-LPS, and CON-LPS) at 48 h postchallenge. Hepatic and mammary RNA copy numbers were quantified for genes involved in apoptosis, methylation, inflammation, oxidative stress, and mitochondrial function using NanoString technology. Targeted metabolomics was conducted only on mammary tissue samples (both 8 and 48 h biopsies) to quantify 143 metabolites including choline metabolites, amino acids, biogenic amines and derivatives, organic acids, carnitines, and glucose. Hepatic \( \text{IFNG} \) was greater in CHOL45 as compared with CON in unchallenged cows, suggesting an improvement in type 1 immune responses. Hepatic \( \text{CASP3} \) was greater in CHOL45-LPS as compared with CON-LPS, suggesting greater apoptosis. Mammary \( \text{IL6} \) was reduced in CHOL30-LPS cows as compared with CHOL45-LPS and CON-LPS (8 and 48 h). Mammary \( \text{GPX4} \) and \( \text{COX5A} \) were reduced in CHOL30-LPS as compared with CON-LPS (8 h), and \( \text{SDHA} \) was reduced in CHOL30-LPS as compared with CON-LPS (8 and 48 h). Both CHOL30-LPS and CHOL45-LPS cows had lesser mammary \( \text{ATP5J} \) than CON-LPS, suggesting that dietary RPC supplementation altered mitochondrial function following LPS challenge. Treatment did not affect mammary concentrations of any metabolite in unchallenged cows, and only 4 metabolites were affected by dietary RPC supplementation in LPS-challenged cows. Mammary concentrations of isobutyric acid and 2 acyl-carnitines (C4:1 and C10:2) were reduced in CHOL45-LPS as compared with CHOL30-LPS and CON-LPS. Taken together, reductions in medium- and short-chain carnitines along with an increase in long-chain carnitines in mammary tissue from CHOL45-LPS cows suggests less fatty acid entry into the \( \beta \) oxidation pathway. Although the intramammary LPS challenge profoundly affected markers for inflammation and metabolism in liver and mammary tissue, dietary RPC supplementation had minimal effects on inflammatory markers and the mammary metabolome.

Key words: mastitis, metabolomics, methyl donor, nutritional immunology

INTRODUCTION

During the peripartum period, dairy cattle are more susceptible to numerous diseases including mastitis. Indeed, the proportion of clinical mastitis is greatest during early lactation (Olde Riekerink et al., 2008). Immune dysfunction and chronic inflammatory responses may play key roles in the enhanced susceptibility to infectious diseases following calving (Bradford and Swartz, 2020). As such, additional investigations into strategies to improve immunological resistance to mastitis pathogens are needed in dairy cattle.
Acute coliform mastitis, such as cases caused by *Escherichia coli*, are known to cause a rapid onset of clinical symptoms, impair milk production, and reduce milk quality (Burvenich et al., 2003). Lipopolysaccharide is a component of the outer membrane of gram-negative pathogens and is commonly used to induce inflammation. In dairy cattle, an intramammary LPS challenge induced both local and systemic inflammatory responses (Hoeben et al., 2000; Minuti et al., 2015; Humer et al., 2018; Shangraw et al., 2020). Moreover, intramammary LPS challenges altered systemic metabolic functions (Minuti et al., 2015; Humer et al., 2018) including altered liver function, specifically impaired fatty acid metabolism, which consequently increased lipid accumulation in the liver (Minuti et al., 2015). Because of this, developing nutritional strategies that can simultaneously reduce inflammatory responses to LPS and mitigate the negative systemic effects of inflammation on liver function and metabolism may be an effective solution to this complex problem.

Choline is a trimethylated molecule (trimethyl-β-hydroxyethylammonium) that plays a vital role in numerous biological functions including cell membrane integrity, methyl group metabolism, cell signaling pathways, lipid transport, and synthesis of phospholipids, very low-density lipoproteins, and the neurotransmitter acetylcholine (Zeisel and Da Costa, 2009). Dietary rumen-protected choline (RPC) supplementation to healthy periparturient dairy cattle reduced transcript abundance of pro-inflammatory cytokines (TNFα and IL1B) from peripheral blood leukocytes when whole blood was challenged with LPS (Zenobi et al., 2020). Using in vitro methods, increasing dose of choline linearly decreased TNFα secretion from bovine monocytes challenged with LPS (Garcia et al., 2018). Moreover, dietary RPC supplementation is also known to enhance antioxidant defenses in periparturient dairy cows (Pinotti et al., 2003; Sun et al., 2016a), which may reduce tissue damage associated with immune responses. Choline also helps regulate lipid metabolism and hepatic export of very low-density lipoproteins. In some studies, dietary RPC supplementation reduced fat accumulation in the liver in feed-restricted dry cows (Cooke et al., 2007; Zenobi et al., 2018), although these effects were not consistent across different lactational stages (Bollatti et al., 2020). Finally, numerous studies have found that dietary RPC supplementation enhanced milk yield during the postpartum period (meta-analysis, Arshad et al., 2020).

Although some of the dietary RPC supplementation effects on periparturient dairy cattle are well-defined, the effects of dietary RPC supplementation during an intramammary LPS challenge had yet to be examined (Swartz et al., 2023). Therefore, the objective of this experiment was to elucidate the effects of dietary RPC supplementation and dose to peripartum dairy cattle on markers of inflammation, oxidative stress, and metabolism in liver and mammary tissue during an intramammary LPS challenge. A secondary objective was to further describe the effects of an intramammary LPS challenge on inflammation and metabolism in liver and mammary tissue. We hypothesized that dietary RPC supplementation would increase choline metabolite concentrations in mammary tissue, attenuate inflammation, reduce oxidative stress, and mitigate the effects of an intramammary LPS challenge on metabolic functions occurring in both mammary and liver tissue in periparturient dairy cattle.

**MATERIALS AND METHODS**

Experimental procedures were conducted from January through August 2021 at the Michigan State University Dairy Cattle Teaching and Research Center in East Lansing, Michigan, in accordance with the protocol (PROT202000184) approved by the Michigan State University Institutional Animal Care and Use Committee.

**Experimental Design and Treatments**

Experimental procedures related to treatments and study enrollment have been reported elsewhere (Swartz et al., 2022a,b, 2023). Close-up dry Holstein parous cows (i.e., cows that had completed at least one lactation before the study; parity, mean ± SD, 1.9 ± 1.1 at study enrollment; n = 67) were blocked by expected calving month and randomly assigned within block to receive one of 3 treatments. Dietary treatments were top-dressing of 45 g/d of RPC (20.4 g/d of choline ions; CHOL45, n = 23), 30 g/d of RPC (13.6 g/d of choline ions; CHOL30, n = 22), or no RPC (CON, n = 22) starting approximately 24 ± 3 d before expected calving until 21 d postpartum. Mean (±SD) duration of prepartum supplementation was 23 (±4.1), 21 (±5.5), and 21 (±5.5) d for CHOL45, CHOL30, and CON, respectively. The RPC supplement (Balchem Corporation) used in this study is not currently commercially available in the United States. This RPC supplement contained a choline chloride core (60.8% of the supplement) and a lipid coating with a ruminal protection level of 74.9%, determined using a ruminal in situ procedure over a 12-h time frame. Research staff mixed the RPC supplement with ground corn and the supplement was top-dressed for a total weight of 150 g/d (as-is basis). Control cows
received 150 g/d of ground corn (as-is basis). As previously reported (Swartz et al., 2023), sample size estimation was based on the number of animals for determining treatment effects between LPS-challenged cows (PROC POWER, SAS 9.4, SAS Institute Inc.). Using SCS [SCS = log2(SCC/100,000) + 3] as our primary outcome, a power analysis was conducted using $\alpha = 0.05$, $\beta = 0.80$, SD of 1 unit, and a 1- and 1.5-unit difference between CON-LPS and CHOL30-LPS and between CON-LPS and CHOL45-LPS, respectively; based on this analysis, 10 LPS-challenged cows were needed per treatment group.

Cows with nonfunctional mammary quarters were excluded from study enrollment. Moreover, cows with clinical postpartum diseases after enrollment were excluded from the trial due to animal welfare concerns related to compounding disease issues with the LPS challenge (Excluded cows: CHOL45, n = 5; CHOL30, n = 11; CHOL15, n = 3). Within each treatment group, cows were alternately assigned to receive either an intramammary LPS challenge at 17 d postpartum or to be left unchallenged. As such, final sample sizes were as follows: CHOL45, n = 9; CHOL45-LPS, n = 9; CHOL30, n = 11; CHOL30-LPS, n = 10; CON, n = 10; CON-LPS, n = 9. All cows were vaccinated 3 times against E. coli mastitis (Enviracor J-5, Zoetis Animal Health) and were alternately assigned to receive either an intramammary LPS challenge at 17 d postpartum or to be left unchallenged. As such, final sample sizes were as follows: CHOL45, n = 9; CHOL45-LPS, n = 9; CHOL30, n = 11; CHOL30-LPS, n = 10; CON, n = 10; CON-LPS, n = 9. All cows were vaccinated 3 times against E. coli mastitis (Enviracor J-5, Zoetis Animal Health). These vaccinations were administered at dry-off, 5 wk prepartum, and 10 d postpartum.

## Diets, Housing, and Milking

Dietary treatments were provided once daily in the morning (1030 h). Just before the delivery of the TMR, headlocks were set to restrain close-up dry cows such that the feed was being dispensed from the mixer, cows would be caught in the headlocks. Once all cows were restrained, dietary treatments were provided as a topdress and the cows were restrained in the headlocks for approximately 45 min. The lactating TMR was provided once daily in the morning (1000 h) and dietary treatments were top-dressed following feeding. Supplemental rumen-protected methionine (Smartamine M, Adisseo North America) was included in close-up and lactating diets during the transition period to evaluate choline effects in the context of a diet that was enriched with methionine. Cows were milked 3 times daily (0630, 1430, and 2230 h) in a double-7 herringbone parlor.

### Intramammary Challenge

The intramammary challenge was previously described (Swartz et al., 2023). Cows that were assigned to receive an intramammary LPS challenge were infused with 200 μg of LPS (E. coli O111:B4, Sigma) in 10 mL of sterile PBS into each rear quarter (total of 400 μg of LPS per challenged cow). Approximately 1 h following the morning milking at 17 d postpartum (0730 h), teats were sprayed with a 0.4% chlorhexidine aerosol (Fight bac, Deep Valley Farm) and wiped with a paper towel. Teat ends were then scrubbed with cotton balls soaked in 70% ethanol. The challenge dose (10 mL in each quarter) was administered in the rear quarters via a teat cannula (Jorgensen Laboratories Inc.) and a 20-mL syringe. The infusion was dispensed through the teat canal and massaged upward to aid in the dispersion of the LPS into the mammary gland. Afterward, teats were sprayed with a 0.4% chlorhexidine aerosol.

### Liver Biopsies

Liver biopsies were collected on d 17 (1500 h) from both unchallenged and challenged cows. To provide a point of reference relative to the LPS challenge, this biopsy occurred at 7.5 h following the challenge. Two cows (both CHOL45-LPS) were unable to be sampled. Cows were sedated with i.v. administration of xylazine (0.01 mg/kg of BW) into the coccygeal vessel. The biopsy site was located between the 10th and 11th ribs, and 5 cm dorsal to a line between the olecranon and tuber coxae. A scalpel blade (number 10, MedHelp) was used to make a stab incision into the body wall of no more than 5 mm in length. Liver samples were collected using a 12-gauge × 10 cm biopsy needle (MN1210, Becton Dickinson and Company). The biopsy needle was inserted cranioventrally toward the liver and approximately 200 mg of tissue was collected (total of 10 biopsies per cow with approximately 20 mg of liver tissue per biopsy). Approximately half of the sample was placed into a cryovial containing Trizol (Thermo Fisher Scientific) and the other half was placed into an empty cryovial tube. The cryovials were then submerged in liquid N₂. Samples were kept in liquid N₂ until the samples were returned to the laboratory where they were stored at −80°C.

### Mammary Biopsies

Mammary biopsies (approximately 0.75 to 1 g of tissue per biopsy) were taken from both unchallenged

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and challenged cows. A single biopsy from the left rear quarter was conducted on unchallenged cows at 17 d postpartum (1530 h). Cows receiving an intramammary LPS challenge were biopsied twice, once on d 17 (1530 h) and again on d 19 (0730 h) postpartum. To provide a point of reference relative to the LPS challenge, the first biopsy occurred in the left rear quarter at 8 h following the LPS challenge to coincide with the expected peak inflammatory response, whereas the second biopsy occurred in the right rear quarter at 48 h postchallenge when inflammation was expected to be resolving. Biopsy procedures were done similarly to Daley et al. (2018). Cows were sedated with i.v. administration of xylazine (0.01 mg/kg of BW) into the coccygeal vessel (on d 17, when liver biopsies were conducted just before mammary biopsy, xylazine was administered only once just before the liver biopsy). The biopsy site was located at the upper part of the rear udder approximately one-third down from the top of the rear udder. The biopsy site was clipped and cleaned using alternating betadine surgical scrub and 70% ethanol once, followed by subcutaneous administration of a local anesthetic (6 mL of 2% lidocaine) just above the incision site, and then 2 more rounds of alternating surgical scrubs. Using aseptic technique, a 2- to 3-cm vertical incision was made using a number 10 scalpel. The biopsy tool was attached to a cordless drill and inserted approximately 7.5 cm deep into the mammary tissue. A core sample was taken using the drill and tweezers were used to remove the core sample from the biopsy tool. The sample was immediately blotted on a Petri dish to remove blood, cut into smaller pieces, and aliquoted into 2 cryovials (one cryovial containing Trizol and the other cryovial was empty). The cryovials were then submerged into liquid N2. Samples were kept in liquid N2 until the samples were returned to the laboratory where they were stored at −80°C. Immediately after the biopsy procedure, pressure on the incision site was applied for approximately 20 min using a sterile towel with an ice pack to achieve hemostasis. Approximately 5 to 8 stainless steel surgical staples were used to oppose the skin incision, an aluminum bandage was applied, and staples were removed approximately 14 d postbiopsy.

**RNA Isolation**

Total RNA was isolated from mammary and liver tissue samples frozen in Trizol. Samples were placed into a mortar that was cooled with liquid N2 and then pulverized using a pestle. Total RNA was isolated using the RNeasy Lipid Tissue kit (Qiagen). RNA purity was assessed (Take3, Agilent Technologies) using the 260/280 ratio, required to be ≥1.8. RNA was quantified using a fluorometer (Qubit 2.0, Life Technologies). RNA integrity was assessed on a random subset of samples (liver, n = 15; mammary, n = 16) using the Agilent Bioanalyzer 2100 (Agilent Technologies). RNA integrity for liver tissue averaged 6.0 ± 0.93 (mean ± SD); RNA integrity for mammary tissue was poor, averaging 2.6 ± 0.81.

**RNA Quantification**

Because mRNA transcript quantification is known to be influenced by RNA integrity when using RT-PCR, we chose to directly quantify RNA copy numbers using a hybridization-based technique that uses target-specific, color-coded probe-pairs (NanoString nCounter, Geiss et al., 2008). NanoString technologies do not require cDNA amplification and therefore can be used to overcome the challenges of working with RNA samples containing substantial degradation (Reis et al., 2011). The methods detailing how NanoString quantifies RNA copy numbers can be found in Geiss et al. (2008), and were conducted by personnel affiliated with the Genomics Core within the Research Technology Support Facility at Michigan State University. Briefly, multiplexed probes were designed with 2 adjacent sequence-specific probes for each RNA target. The capture probe is coupled to biotin; whereas the second probe (the reporter probe) is coupled to a color-coded tag that provides the detection signal. Target and reference RNA are identified by the unique color code generated by the fluorescent tags on the reporter probe, allowing for the quantification of multiple targets in one sample. Using 200 ng of RNA from each sample, RNA copy numbers were measured by counting the number of barcodes for each target using an automated fluorescence microscope. The customized set of probes consisted of 4 reference genes and 30 bovine target genes. Specific information on probe design is provided in Supplemental Table S2 (https://doi.org/10.6084/m9.figshare.24802647.v1; Swartz, 2023). Four reference genes (RPS9, RPS15, UXT, and YWHAZ) were screened and the most stable reference genes (lowest CV) that were also not influenced by treatment or by LPS were selected for normalization. Liver RNA copy numbers were normalized to RPS15 and UXT, whereas mammary RNA copy numbers were normalized to RPS9 and RPS15. The 30 target genes were selected based on their roles in: (1) apoptosis (BAX, BCL2, and CASP3); (2) DNA methylation and transmethylation pathways (DNMT1, DNMT3α, and DNA); (3) inflammation and oxidative stress (CCL5, CXCL8, GPX4, HP, IFNG, IL1B, IL2, IL4, IL6, IL10, IL17A, IL21, IL22, LTF, NOS2, SAA3, TGFβ1, TLR4, and TNF); and (4) mitochondrial function (ATP5J, COX5A, NDUFV3, PINK1, and...
SDHA). Six internal positive controls were included to determine hybridization efficiency and 8 internal negative controls were included to determine nonspecific background. The negative controls were used to correct for background by subtracting the mean + 2 SD of the 8 internal negative controls from the RNA copy numbers. Finally, raw target RNA copy numbers were adjusted for background, normalized to the reference genes, and then transformed \((\log_2)\) using the nSolver 4.0 software (NanoString Technologies).

**DNA Isolation and RT-PCR**

Relative mitochondrial DNA (mtDNA) copy number was quantified by real-time PCR in liver and mammary tissue. Isolation of DNA was performed using the DNeasy Blood and Tissue Kit (Qiagen). Mammary and liver tissues (25 mg) were cut up into small pieces and placed into a 1.5-mL microcentrifuge tube with 180 μL of animal tissue lysis buffer and 20 μL of proteinase K. The mix was incubated at 56°C on a shaker for 2 h. After incubation, DNA was isolated per the manufacturer’s protocol. Isolated DNA was quantified, and purity was assessed using a microvolume plate (Take3, Agilent Technologies) and a plate reader (Synergy HTX, Agilent Technologies). Quantitative real-time PCR was performed (QuantaStudio 7 Flex Real-Time PCR System, Applied Biosystems) in duplicate using 2 μg of DNA with 200 nM gene-specific forward and reverse primers with iTaq Universal SYBR Green Supermix (Bio-Rad Lab. Inc.). Primers known to not amplify RNA transcripts were designed to quantify nuclear DNA (nDNA; cyclophilin A, forward: GGTCTGGGACTTCTGTCAT, reverse: TGGCAGTGCAATGAAAAACTG) and mtDNA (NADH dehydrogenase subunit 6, forward: TCGAAGAAAACCCCACAAAACC, reverse: GGGTGGGACTTTCGAAATGTTAGT). Primer efficiency was calculated using a 5-point curve (nDNA, 88%; mtDNA, 93%). Relative mtDNA abundance was expressed as the ratio between mtDNA/nDNA using the Pfaffl (2001) method.

**Targeted Metabolomics**

Mammary tissue samples were submitted to The Metabolomics Innovation Center (TMIC; University of Alberta). Mass spectrometric analysis was performed on an ABSciex 4000 Qtrap tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies) equipped with an Agilent 1260 series UHPLC system (Agilent Technologies). Data analysis was done using Analyst 1.6.2. Samples were screened for 143 metabolites including choline metabolites (37), amino acids (23), biogenic amines and derivatives (24), organic acids (18), carnitines (40), and glucose using a combination of direct injection mass spectrometry with a reverse-phase LC-MS/MS custom assay (TMIC PRIME Assay). Specific choline metabolites included choline, betaine, trimethylamine N-oxide (TMAO), numerous phosphatidylcholines (PC; 2 acyl-alkyl and 8 diacyl PC), numerous lysophosphatidylcholines (LPC; 14), and numerous sphingomyelins (SM; 5 SM and 5 hydroxysphingomyelins). It should be noted that some PC, LPC, and SM correspond to multiple unique lipid structures.

**Statistical Analyses**

For metabolomics data analyses, if the concentration of a metabolite was below the limit of detection (LOD), an imputed value (LOD divided by 2) was used (Hornung and Reed, 1990). Metabolites with more than 50% missing values were deemed as not detectable and statistical analyses were not conducted. Because of the complexity of the statistical models, metabolites were analyzed individually using the GLIMMIX procedure (SAS 9.4, SAS Inst.). Data were analyzed using 2 statistical models. For the mammary tissue collected at 8 h, we assessed the fixed effects of treatment, LPS (challenged vs. unchallenged), and the 2-way interaction, along with the random effects of block. In our second model, we assessed treatment effects in only LPS-challenged cows, which were biopsied twice (8 and 48 h postchallenge). This model included the fixed effects of treatment, time (repeated measure), and the 2-way interaction, along with the random effects of block and cow. Parity (2 vs. 3+) and BCS recorded just before applying treatment (−24 d) were tested as covariates. To limit type 1 error, the Benjamini-Hochberg procedure was used to determine the false discovery rate (FDR). PROC MULTTEST, SAS 9.4. When treatment FDR ≤0.10, treatment least squares means were separated using the PDIFF statement with a Tukey adjustment. Significance was declared at \(P \leq 0.05\).

For hepatic RNA and mtDNA abundance, linear mixed models were conducted using the GLIMMIX procedure (SAS 9.4). The model included the fixed effects of treatment, LPS (challenged vs. unchallenged), and the 2-way interaction, along with the random effects of block. Parity (2 vs. 3+) and BCS recorded just before applying treatment (−24 d) were tested as covariates.

For mammary RNA copy numbers and mtDNA abundance, data were analyzed using 2 statistical approaches. For the mammary tissue collected at 8 h, we assessed the fixed effects of treatment, LPS (challenged vs. unchallenged), and the 2-way interaction, along with the random effects of block. In our second approach, we assessed treatment effects in only LPS-challenged cows,
which were biopsied twice (8 and 48 h postchallenge). This model included the fixed effects of treatment, time (repeated measure), and the 2-way interaction, along with the random effects of block and cow. Parity (2 vs. 3+) and BCS recorded just before applying treatment (−24 d) were tested as covariates.

For all analyses, backward elimination was used to remove nonsignificant terms until all variables in the model had a $P \leq 0.05$ or were part of a significant interaction term except for treatment, LPS (when applicable), and time (when applicable), which were forced into the model. For repeated measures analyses, the spatial power error structure was used. Treatment least squares means were separated using the SLICE-DIFF or PDIFF statement with a Tukey adjustment. Outliers were removed if the absolute value of the studentized residual was greater than 4. If an outcome variable was non-normally distributed, a logarithmic transformation was used for relative mtDNA abundance and for certain metabolites. Significance was declared at $P \leq 0.05$.

RESULTS

**Hepatic RNA Copy Numbers and mtDNA Abundance**

Treatment LSM, SEM, and probability levels are provided in Table 1 for hepatic RNA copy numbers. There was a significant treatment by LPS interaction for hepatic $CASP3$ ($P = 0.02$). The CHOL45-LPS cows had greater hepatic $CASP3$ than CHOL30-LPS ($P < 0.01$) and CON-LPS ($P = 0.01$) cows. Unchallenged cows had greater hepatic $CASP3$ than LPS-challenged cows within every treatment group ($P \leq 0.01$).

Although we observed a significant treatment by LPS interaction for hepatic $AHCY$ ($P = 0.04$), no treatment effects were identified ($P \geq 0.11$). Unchallenged cows had greater hepatic $AHCY$ than challenged cows within every treatment group ($P < 0.001$).

We observed a significant treatment by LPS interaction for hepatic $IFNG$ ($P = 0.03$). Unchallenged CHOL45 cows had greater hepatic $IFNG$ than CON ($P < 0.01$); no difference was found between CHOL45 and CHOL30 ($P = 0.21$), or between any of the treatment groups within LPS-challenged groups ($P \geq 0.39$). Unchallenged CHOL45 cows also had greater hepatic $IFNG$ than CHOL45-LPS cows ($P < 0.01$). No LPS effects were found for hepatic $IFNG$ within the other treatment groups (CHOL30-LPS vs. CHOL30, $P = 0.09$; CON-LPS vs. CON, $P = 0.68$).

We observed a significant treatment by LPS interaction for hepatic $TLR4$ ($P = 0.03$). Unchallenged CHOL30 cows had greater hepatic $TLR4$ than CON ($P < 0.01$), but no difference was found between CHOL45 and CHOL30 ($P = 0.23$) or between CHOL45 and CON ($P = 0.36$). For LPS-challenged cows, hepatic $TLR4$ did not differ between treatment groups ($P \geq 0.52$). Challenged cows had greater hepatic $TLR4$ than unchallenged cows within every treatment group ($P < 0.001$).

We observed a significant treatment by LPS interaction for hepatic $SDHA$ ($P = 0.02$). Cows in the CHOL45-LPS group had greater hepatic $SDHA$ than CHOL30-LPS ($P = 0.02$), but no difference was found between CHOL45-LPS and CON-LPS ($P = 0.07$) or between CHOL30-LPS and CON-LPS ($P = 0.89$). Furthermore, no treatment effects were found within the unchallenged groups ($P \geq 0.12$). Unchallenged cows had greater hepatic $SDHA$ than challenged cows within every treatment group ($P < 0.01$).

The LPS challenge also increased hepatic $DNMT3A$ ($P < 0.001$), $CCL5$ ($P = 0.03$), $CXCL8$ ($P < 0.001$), $GPX4$ ($P < 0.001$), $ATP5J$ ($P = 0.001$), $NDUFB5$ ($P < 0.001$), and $COX5A$ ($P < 0.01$) as compared with unchallenged cows. In contrast, the LPS challenge reduced hepatic $DNMT1$ ($P < 0.001$), $NOS2$ ($P < 0.001$), $ATP5J$ ($P < 0.001$), $NDUFB5$ ($P < 0.001$), and $PINK1$ ($P < 0.001$). Finally, neither treatment ($P = 0.06$, Figure 1A) nor LPS challenge ($P = 0.78$) affected hepatic mtDNA abundance relative to nuclear DNA.

**Mammary RNA Copy Numbers and mtDNA Abundance at 8 h**

Treatment LSM, SEM, and probability levels are provided in Table 2 for mammary RNA copy numbers at 8 h. We observed a significant treatment by LPS interaction for mammary $GPX4$ at 8 h ($P = 0.05$). Cows in the CHOL30-LPS group had lesser mammary $GPX4$ than CON-LPS ($P = 0.02$); no difference was found between CHOL45-LPS and CHOL30-LPS ($P = 0.39$) or between CHOL45-LPS and CON-LPS ($P = 0.38$). No treatment effects were found within the unchallenged groups ($P \geq 0.61$). Both CHOL45-LPS and CON-LPS cows had greater mammary $GPX4$ than their unchallenged counterparts (CHOL45-LPS vs. CHOL45, $P = 0.04$; CON-LPS vs. CON, $P < 0.01$); however, LPS effects were not found between CHOL30-LPS and CHOL30 cows ($P = 0.92$).

We observed a significant treatment by LPS interaction for mammary $COX5A$ at 8 h ($P < 0.01$). Cows in the CHOL30-LPS group had lesser mammary $COX5A$
Table 1. Effects of peripartum dietary supplementation of rumen-protected choline at 45 g/d (CHOL45, n = 9; CHOL45-LPS, n = 7), 30 g/d (CHOL30, n = 11; CHOL30-LPS, n = 10), or no supplementation (CON, n = 10; CON-LPS, n = 9) on liver RNA copy numbers (log2 [counts])

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2,3Within a row, means with different lowercase superscripts denote significant differences (P ≤ 0.05) within LPS-challenged treatment groups. If there was a significant interaction between treatment and LPS, the interaction term was sliced by LPS to assess treatment effects within challenge groups.

4Within a row, means with different uppercase superscripts denote significant differences (P ≤ 0.05) within unchallenged treatment groups.

Cows were either challenged with 200 μg of LPS in each rear quarter (400 μg of LPS in total per cow) at 17 d postpartum or were left unchallenged. Liver biopsies were conducted at 7.5 h after challenge.

BCL2, IL2, IL4, IL6, IL17A, and IL21 were poorly expressed in all groups (data not shown).

AHCY = adenosylhomocysteinase; ATP5J = ATP synthase peripheral stalk subunit F6; BAX = BCL2 associated X, apoptosis regulator; BCL2 = B-cell lymphoma 2; CASP3 = caspase 3; CCL5 = C-C motif chemokine ligand 5; COX5A = cytochrome C oxidase subunit 5A; CXCL8 = C-X-C chemokine ligand 8; DNMT1 = DNA methyltransferase 1; DNMT3A = DNA methyltransferase 3 α; GPX4 = glutathione peroxidase 4; HP = haptoglobin; IFNG = interferon gamma; IL = interleukin; LTF = lactoferrin; NDUFB5 = NADH:ubiquinone oxidoreductase subunit B5; NOS2 = nitric oxide synthase 2; PINK1 = PTEN induced kinase 1; SAA3 = serum amyloid A 3; SDHA = succinate dehydrogenase complex flavoprotein subunit A; TLR4 = toll-like receptor 4; TGBF1 = transforming growth factor β 1; TNF = tumor necrosis factor.

*Trt = treatment.

*Within a row, means with an asterisk denote significant LPS effects (P ≤ 0.05) within a treatment group (i.e., the comparisons are CHOL45 vs. CHOL45-LPS, CHOL30 vs. CHOL30-LPS, and CON vs. CON-LPS). If there was a significant interaction between treatment and LPS, the interaction term was sliced by LPS to assess LPS effects within treatment groups.

than CON-LPS (P = 0.05); no difference was found between CHOL45-LPS and CHOL30-LPS (P = 0.76) or between CHOL45-LPS and CON-LPS (P = 0.22). No treatment effects were found within the unchallenged groups (P ≥ 0.06). Both CHOL45-LPS and CON-LPS cows had greater mammary COX5A than their unchallenged counterparts (P < 0.01); however, LPS effects were not found between CHOL30-LPS and CHOL30 cows (P = 0.91).

We observed a significant treatment by LPS interaction for mammary SDHA at 8 h (P = 0.02). Cows in the CHOL30-LPS group had lesser mammary SDHA than CON-LPS (P < 0.01); no difference was found between CHOL45-LPS and CHOL30-LPS (P = 0.41) or between CHOL45-LPS and CON-LPS (P = 0.09). No treatment effects were found within the unchallenged groups (P ≥ 0.27). The LPS challenge decreased mammary SDHA within the CHOL30 treatment group (CHOL30-LPS vs. CHOL30, P < 0.001); however, LPS effects were not found between CHOL45-LPS and CHOL45 cows (P = 0.06) or between CON-LPS and CON cows (P = 0.42).
The LPS challenge also increased mammary BAX (P < 0.001), CASP3 (P < 0.001), CCL5 (P = 0.01), CXCL8 (P < 0.001), HP (P < 0.001), IFNG (P < 0.01), IL1B (P < 0.001), IL6 (P < 0.001), IL10 (P < 0.001), LTF (P < 0.01), NOS2 (P < 0.001), SAA3 (P < 0.001), TLR4 (P < 0.001), TNF (P < 0.001), AHCY (P < 0.001), and ATP5J (P = 0.02) as compared with unchallenged cows at 8 h. The LPS challenge reduced mammary DNMT1 (P < 0.001) and DNMT3A (P < 0.001). Neither treatment (P = 0.33, Figure 1B) nor LPS (P = 0.86) affected mammary relative mitochondrial abundance at 8 h.

Mammary RNA Copy Numbers and mtDNA Abundance for LPS-Challenged Cows at 8 and 48 h

Treatment LSM, SEM, and probability levels are provided in Table 3 for mammary RNA copy numbers for LPS-challenged cows biopsied at 8 and 48 h following the LPS challenge. Treatment affected mammary IL6 (P < 0.01), ATP5J (P = 0.02), and SDHA (P < 0.001) in LPS-challenged cows. Cows in the CHOL30-LPS group had lesser IL6 than CHOL45-LPS (P = 0.01) and CON-LPS (P = 0.03). Both CHOL30-LPS and CHOL45-LPS cows had lesser ATP5J than CON-LPS (both P = 0.04). Also, CHOL30-LPS had lesser SDHA than CON-LPS (P < 0.001), although no difference was found between CHOL45-LPS and CON-LPS (P = 0.06). Finally, treatment did not affect mammary relative mtDNA abundance (P = 0.07, Figure 1C).

Mammary Metabolites at 8 h

Of the 143 metabolites measured, 117 were detectable in mammary tissue. Of the 117 detectable metabolites, 26 metabolites had samples that were below the LOD, and these values were imputed (n = number of samples below LOD out of 86 total samples; PC aa C38:0, n = 13; PC aa C40:6, n = 3; PC aa C40:2, n = 3; LPC a C14:0, n = 11; LPC a C18:0, n = 13; LPC a C26:0, n
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<sup>a</sup>Within a row, means with different lowercase superscripts denote significant differences (P ≤ 0.05) within LPS-challenged treatment groups. If there was a significant interaction between treatment and LPS, the interaction term was sliced by LPS to assess treatment effects within challenge groups.

<sup>b</sup>Within a row, means with different uppercase superscripts denote significant differences (P ≤ 0.05) within unchallenged treatment groups.

<sup>c</sup>Cows were either challenged with 200 μg of LPS in each rear quarter (400 μg of LPS in total per cow) at 17 d postpartum or were left unchallenged. Mammary biopsies were conducted at 8 h after challenge.

<sup>d</sup>BCL2, IL2, IL4, IL17A, IL23 and IL32 were poorly expressed in all groups (data not shown).

<sup>e</sup>AHCY = adenosylhomocysteinase; ATP5J = ATP synthase peripheral stalk subunit F6; BAX = BCL2 associated X, apoptosis regulator; BCL2 = B-cell lymphoma 2; CASP3 = caspase 3; CCL5 = C-C motif chemokine ligand 5; COX5A = cytochrome C oxidase subunit 5A; CCL8 = C-C motif chemokine ligand 8; DNMT1 = DNA methyltransferase 1; DNMT3A = DNA methyltransferase 3 α; GPNJ2 = glutathione peroxidase 4; HP = haptoglobin; IFNG = interferon γ; IL1B = interleukin 1 β; IL6 = interleukin 6; IL10 = interleukin 10; LTF = lactoferrin; NOS2 = nitric oxide synthase 2; PINK1 = PTEN induced kinase 1; SAA3 = serum amyloid A 3; SDHA = succinate dehydrogenase complex flavoprotein subunit A; TLR4 = toll-like receptor 4; TGFB1 = transforming growth factor β 1; TNF = tumor necrosis factor.

<sup>f</sup>Trt = treatment.

<sup>*</sup>Within a row, means with an asterisk denote significant LPS effects (P ≤ 0.05) within a treatment group (i.e., the comparisons are CHOL45 vs. CHOL45-LPS, CHOL30 vs. CHOL30-LPS, and CON vs. CON-LPS). If there was a significant interaction between treatment and LPS, the interaction term was sliced by treatment to assess LPS effects within treatment groups.

= 33; LPC a C28:0, n = 6; SM C20:2, n = 6; SM(OH) C22:2, n = 36; SM(OH) C22:1, n = 2; homocysteine, n = 22; lysine, n = 2; ornithine, n = 7; asymmetric dimethylarginine, n = 4; DOPA, n = 1; kynurenine, n = 1; sarcosine, n = 28; serotonin, n = 1; C3:1, n = 1; C6, n = 23; C5:1DC, n = 11; C12:1, n = 9; C14, n = 1; C16:2, n = 35, C16:1OH, n = 39; C18:1, n = 1). Treatment LSM, SEM, and probability levels (false discovery rate; FDR) are provided in Supplemental Table S3 (https://doi.org/10.6084/m9.figshare.24802647.v1; Swartz, 2023) for mammary metabolite concentrations at 8 h.

**Choline Metabolites.** The predominant choline metabolite in mammary tissue was choline, followed by betaine, total LPC, total SM, and total PC. All choline metabolite concentrations were unaffected by treatment (FDR >0.10). Mammary TMAO was undetectable in 59% of samples collected at 8 h (61% CHOL45, 38% CHOL30, and 79% CON samples had undetectable levels of TMAO).
The LPS challenge had no effect on the concentrations of water-soluble choline metabolites in mammary tissue (choline, $P = 0.18$; betaine, $P = 0.13$), but it reduced concentrations of fat-soluble choline metabolites (total LPC, $P < 0.0001$; total SM, $P = 0.01$; total PC, $P < 0.01$). These LPS effects were somewhat dependent on the fatty acyl group for PC and SM; however, all detectable LPC fatty acyl isomers were affected by the LPS challenge (see Supplemental Table S3).

### AA and Biogenic Amines

No effect of treatment was found on mammary amino acids and biogenic amines (FDR >0.10). The LPS challenge reduced mammary concentrations of aspartate, citrulline, glutamic acid, ornithine, serine, threonine, and spermine ($P \leq 0.05$). In contrast, the LPS challenge increased concentrations of kynurenine and methylhistidine (both $P \leq 0.01$).

### Organic Acids and Glucose

No effect of treatment was found on mammary organic acids or glucose concentrations (all FDR >0.10). The LPS challenge reduced mammary concentrations of α-aminoadipic, β-hydroxybutyric, hippuric, methylmalonic, and propionic acids ($P \leq 0.05$). In contrast, the LPS challenge increased concentrations of isobutyric, α-ketoglutaric, lactic, and pyruvic acids ($P < 0.01$).

### Carnitines

Treatment did not affect mammary carnitine concentrations (FDR >0.10). The LPS challenge reduced mammary concentrations of hydroxy-
propionylcarnitine (C3OH), valeryl carnitine (C5), malonyl carnitine (C4OH), glutaryl carnitine (C5DC), dodecanoyl carnitine (C12), and octadecanoyl carnitine (C18; \( P \leq 0.05 \)). Mammary concentrations of octadecenoyl carnitine (C18:1) were increased by the LPS challenge (\( P = 0.04 \)).

**Mammary Metabolites, LPS-Challenged Cows at 8 and 48 h**

**Choline Metabolites.** Treatment LSM, SEM, and probability levels (FDR) are provided in Supplemental Table S4 ([https://doi.org/10.6084/m9.figshare.24802647.v1](https://doi.org/10.6084/m9.figshare.24802647.v1); Swartz, 2023) for mammary metabolite concentrations collected from LPS-challenged cows (8 and 48 h). All choline metabolite concentrations were unaffected by treatment (all FDR >0.10). Mammary TMAO was undetectable in 7% of samples collected from LPS-challenged cows (67% CHOL45, 65% CHOL30, and 89% CON samples were undetectable).

**Organic Acids and Glucose.** Isobutyric acid concentrations were affected by treatment (FDR <0.01), which was reduced in mammary tissue from CHOL45-LPS cows as compared with CHOL30-LPS (\( P < 0.001 \)) and CON-LPS (\( P < 0.0001 \)). Treatment LSM for isobutyric acid were \(-1.87, -1.46, \) and \(-1.37 \)(natural log, nmol/g of tissue; SE = 0.11) for CHOL45-LPS, CHOL30-LPS, and CON-LPS, respectively. All other organic acids as well as glucose concentrations in mammary tissue were not affected by treatment in LPS-challenged cows (FDR >0.10).

**Carnitines.** Butenylcarnitine (C4:1; FDR <0.01), decadienoylcarnitine (C10:2; FDR <0.01), and octadecadienyl carnitine (C18:2; FDR = 0.09) were affected by treatment. Mammary tissue concentrations of C4:1 were reduced in CHOL45-LPS cows as compared with CHOL30-LPS (\( P < 0.001 \)) and CON-LPS (\( P < 0.001 \)). Treatment LSM for C4:1 were 0.16, 0.21, and 0.22 nmol/g of tissue (SE = 0.0083) for CHOL45-LPS, CHOL30-LPS, and CON-LPS, respectively.

Mammary tissue concentrations of C10:2 were reduced in CHOL45-LPS cows as compared with CHOL30-LPS (\( P < 0.01 \)) and CON-LPS (\( P < 0.001 \)). Treatment LSM for C10:2 were 0.041, 0.42, and 0.48 (natural log, nmol/g of tissue; SE = 0.071) for CHOL45-LPS, CHOL30-LPS, and CON-LPS, respectively.

Finally, mammary tissue concentrations of C18:1 were increased in CHOL45-LPS and CHOL30-LPS cows as compared with CON-LPS (both \( P \leq 0.01 \)). Treatment LSM for C18:1 were 0.62, 0.62, and 0.42 nmol/g of tissue (SE = 0.088) for CHOL45-LPS, CHOL30-LPS, and CON-LPS, respectively.

**DISCUSSION**

The objective of our experiment was to elucidate the effects of dietary RPC supplementation and dose to periparturient dairy cattle on inflammation and metabolism in liver and mammary tissue during an intramammary LPS challenge. Because past studies found an anti-inflammatory effect of choline using either in vitro (Garcia et al., 2018) or ex vivo (Zenobi et al., 2020) methods, we hypothesized that dietary RPC supplementation would attenuate inflammation during an intramammary LPS challenge. Although the intramammary LPS challenge induced inflammation in both liver and mammary tissue, dietary RPC supplementation did not have a broad effect on inflammatory responses in either tissue. Dietary RPC supplementation also did not enhance concentrations of choline metabolites in mammary tissue. As such, we speculate that the lack of treatment effects on choline metabolite concentrations in mammary tissue may be the reason why dietary RPC supplementation did not attenuate inflammation during an intramammary LPS challenge.

**Liver Responses: Effects of Dietary RPC Supplementation in Unchallenged Cows**

Hepatic TLR4 was greater in unchallenged CHOL30 cows as compared with CON, whereas no differences were found between CHOL45 and CON. In agreement with our results, in vitro studies using bovine neutrophils found that choline dose had a quadratic relationship with TLR4 (Garcia et al., 2018; Lopreiato et al., 2019). Transcripts of TLR4 encode the LPS pattern recognition receptor and when TLR4 is activated, transcription of pro-inflammatory cytokines is upregulated. Although CHOL30 cows had greater hepatic TLR4, pro-inflammatory cytokines, such as IL1B and TNF, were not affected by treatment and therefore the implications of this may be limited.

Interferon-γ is a critical cytokine for innate and adaptive immunity. It is produced primarily by T-helper 1 cells (Th1), cytotoxic T cells, and natural killer cells to activate innate immune cells such as macrophages to provide protection against invading pathogens. Hepatic IFNG was greater in unchallenged CHOL45 cows as compared with CON, suggesting greater Th1 responses in the liver of unchallenged cows. In support of this, past studies have found that dietary RPC supplementation to periparturient dairy cattle enhanced plasma
Liver Responses: Effects of Dietary RPC Supplementation in LPS-Challenged Cows

Hepatic CASP3 and SDHA were the only 2 transcript abundances affected by treatment in LPS-challenged cows. The treatment effect was inconsistent for SDHA, as differences were only found between CHOL45-LPS and CHOL30-LPS, and no differences were found between CHOL45-LPS and CHOL30-LPS versus CON-LPS. As such, the implications of this are limited. Caspase 3 is an enzyme that regulates cellular death. Hepatic CASP3 was greater in CHOL45-LPS as compared with CON-LPS, suggesting greater hepatic apoptosis in CHOL45-LPS cows. In support of these results, our companion paper also suggested that CHOL45-LPS had greater systemic inflammatory responses as vaginal temperatures were greater during the LPS challenge as compared with CON-LPS cows (Swartz et al., 2023). A possible mechanism behind this could be related to TMAO. Indeed, we found that colostrum from CHOL30 and CHOL45 cows had greater TMAO concentrations than CON, and plasma TMAO concentrations were greater in CHOL45-LPS as compared with CON-LPS during the challenge (Swartz et al., 2022b, 2023). Trimethylamine N-oxide is derived from microbial metabolism of choline to trimethylamine in the gut, which is then oxidized by flavin-containing monoxygenases in the liver (Wang et al., 2011). Elevated concentrations of TMAO have been associated with numerous human diseases (Ufnal et al., 2015) and greater mortality rates with nonalcoholic fatty liver disease (Flores-Guerrero et al., 2021), although the direct effects remain unclear in cattle (Myers et al., 2021). Using either in vitro methods or mouse models, TMAO has been found to exert a pro-inflammatory effect (Sun et al., 2016b; Boini et al., 2017; Zhou et al., 2022). Trimethylamine N-oxide may induce pyroptosis (Zhou et al., 2022), an inflammatory lytic programmed cellular death, by activating inflammasomes (Sun et al., 2016b; Boini et al., 2017; Zhou et al., 2022). Activation of inflammasomes results in the upregulation of caspases, including CASP3, to induce cell lysis (Bertheloot et al., 2021).

Mammary Responses: Effects of Dietary RPC Supplementation in Unchallenged Cows

Despite the fact that cows supplemented with dietary RPC produced more milk (Swartz et al., 2023), mammary RNA copy numbers from unchallenged cows for genes involved with apoptosis, methylation, inflammation, oxidative stress, and mitochondrial function were not affected by treatment. Similarly, mammary mtDNA abundance and metabolite concentrations were also not affected by treatment in unchallenged cows. It is important to note that the metabolomics data described herein is quantifying concentrations of metabolites in mammary tissue and is not measuring flux of metabolites across the mammary tissue into milk. Because cows supplemented with dietary RPC supplementation produced more milk, greater fluxes of metabolites through the mammary epithelial cells would be required to support this additional milk synthesis. Nevertheless, our study failed to elucidate an effect of dietary RPC supplementation on the mammary gland that could explain the mechanism behind enhanced milk yield responses, which are often found (Arshad et al., 2020).
tive kinase 1, is encoded by \textit{PINK1}. This kinase is used to identify and target damaged mitochondria for elimination through a process called mitophagy (\textit{PINK1}/Parkin pathway).

Abundance of \textit{GPX4} was reduced in mammary tissue of CHOL30-LPS cows as compared with CON-LPS at 8 h. No difference was found between CHOL30-LPS and CHOL30 cows; however, upregulation of \textit{GPX4} was evident in LPS-challenged cows in both CHOL45-LPS and CON-LPS groups as compared with their unchallenged counterparts, respectively. The reduction in \textit{GPX4} in CHOL30-LPS as compared with CON-LPS was contrary to our hypothesis, particularly as past studies have found improvements in antioxidant defenses in plasma from dietary RPC supplemented cows (Pinotti et al., 2003; Sun et al., 2016a; Swartz et al., 2023). Nevertheless, a lack of a response to the LPS challenge on \textit{GPX4} abundance in mammary tissue of CHOL30 cows could indicate that antioxidant defenses were already sufficient in CHOL30 cows before the LPS challenge, and therefore upregulation was not needed during the challenge. Conversely, this could also indicate the opposite, where CHOL30-LPS cows experienced more oxidative stress due to inadequate upregulation of \textit{GPX4} during the challenge; however, inflammatory markers were not increased in CHOL30-LPS cows as compared with either CON-LPS or CHOL45-LPS, suggesting that this scenario is unlikely. In fact, CHOL30-LPS cows had less mammary \textit{IL6} than CON-LPS and CHOL45-LPS when assessing treatment effects over both time points.

Copy numbers of \textit{COX5A} were reduced in the mammary tissue of CHOL30-LPS as compared with CON-LPS at 8 h. This transcript encodes a subunit of cytochrome C oxidase, which is the terminal enzyme of the ETC. Cytochrome C oxidase couples the transfer of electrons from cytochrome C to molecular oxygen and contributes to a proton electrochemical gradient across the inner mitochondrial membrane. Similarly, \textit{SDHA} was reduced in CHOL30-LPS as compared with CON-LPS over both time points. This gene encodes a subunit of the succinate dehydrogenase enzyme involved in electron transport in the ETC and oxidizing succinate to fumarate in the TCA cycle; plasma fumarate concentrations were increased in CHOL45-LPS and CHOL30-LPS compared with CON-LPS, as reported in our companion paper (Swartz et al., 2023). Finally, \textit{ATP5J}, which encodes a subunit of ATP synthase, was reduced in mammary tissue from both CHOL45-LPS and CHOL30-LPS as compared with CON-LPS over both biopsy time points. Similar to the results for \textit{GPX4}, the results for \textit{COX5A}, \textit{SDHA}, and \textit{ATP5J} were contrary to our hypotheses. We hypothesized that dietary RPC supplementation would enhance mitochondrial activity and therefore dietary RPC supplementation would increase RNA abundance of those genes in mammary tissue, regardless of the LPS challenge. We speculated this primarily because cows supplemented with dietary RPC produce more milk (Arshad et al., 2020), as also found in the present study (Swartz et al., 2023). Nevertheless, the implications of these findings are unknown and require additional investigation.

Concentrations of isobutyric acid and a few carnitines were affected by treatment in LPS-challenged cows. Typically, branched-chain volatile fatty acids, such as isobutyric acid, are commonly investigated in ruminant nutrition because they are degradation products from branched-chain amino acids by microbial fermentation and can be used to enhance bacterial growth in the rumen (Andries et al., 1987). However, the mammary epithelium can also deaminate valine (a branched-chain AA) to synthesize isobutyric acid (Wohlt et al., 1977). In LPS-challenged cows, isobutyric acid concentrations were reduced in mammary tissue from CHOL45-LPS cows as compared with CON-LPS and CHOL30-LPS. In our companion paper (Swartz et al., 2023), dietary RPC supplementation did not affect plasma concentrations of isobutyric acid. As such, the reduction in isobutyric acid concentrations in mammary tissue from CHOL45-LPS cows is likely due to altered mammary epithelium function rather than reduced supply of isobutyric acid from the gut. Recently, an in vitro study found that valine is used by the goat mammary epithelium to synthesize antimicrobial products, such as lactoferrin among others (Tsugami et al., 2023). Possibly, a reduction in isobutyric acid in mammary tissue of CHOL45-LPS cows could mean less deamination of valine in the mammary gland, and therefore reduced production of antimicrobial products during an intra-mammary challenge. Nevertheless, the implications of this require further investigation.

Aside from isobutyric acid, concentrations of a few carnitines in mammary tissue were affected by treatment. Carnitines are quaternary ammonium compounds that support energy metabolism by transporting long-chain fatty acids into the mitochondria for oxidation. Acyl-carnitines are carnitines with an acyl-ester (short-chain, C2-C5; medium-chain, C6–14; long-chain, C16–20) that are produced when carnitine binds to acyl groups produced during \(\beta\) oxidation of fatty acids in the mitochondria (Dambrova et al., 2022). Plasma concentrations of long-chain acyl-carnitines during the peripartum period have been positively associated with body condition score (Rico et al., 2018). Similarly, long-chain acyl-carnitines were positively associated with plasma nonesterified fatty acid (NEFA) concentrations in postpartum dairy cattle (Humer et al., 2016). Mammary tissue concentrations of octadecadienylcarnitine (C18:1) were increased in CHOL45-LPS and CHOL30-
LPS cows as compared with CON-LPS. In our companion paper (Swartz et al., 2023), plasma NEFA concentrations were greater during the LPS challenge in CHOL45-LPS and CHOL30-LPS cows as compared with CON-LPS. Indeed, serum C18:1 concentrations are positively correlated with serum NEFA concentrations (Yang et al., 2019). Taken together, these data suggest greater supply of fatty acids reaching the mammary gland in cows supplemented with dietary RPC resulting in greater mammary C18:1 concentrations.

During β oxidation, long-chain fatty acids are progressively broken down into smaller chain fatty acids. Although acyl chains are bound to coenzyme A during β oxidation, acyl-CoA moieties are in equilibrium with the corresponding acyl-carnitines (Bremer, 1983), resulting in medium- and short-chain acyl-carnitines. Surprisingly, mammary tissue concentrations of decadienoylcarnitine (C10:2) and butenylcarnitine (C4:1) were reduced in CHOL45-LPS cows as compared with CHOL30-LPS and CON-LPS. Collectively, an increase in long-chain acyl-carnitines (C18:1) along with a decrease in medium-chain (C10:2) and short-chain (C4:1) carnitines in mammary tissue from CHOL45-LPS cows suggests less entry of fatty acids into the β oxidation pathway. Interestingly, the reduction in medium- and small-chain acyl-carnitines was not found in CHOL30-LPS as compared with CON-LPS, despite that circulating NEFA concentrations and mammary C18:1 were also increased in CHOL30-LPS as compared with CON-LPS (i.e., similar plasma NEFA and mammary C18:1 results for the comparisons of CHOL30-LPS vs. CON-LPS as CHOL45-LPS vs. CON-LPS). As such, these data suggest that the CHOL30 dose may not affect β oxidation; however, larger doses (CHOL45) may reduce mammary β oxidation. Nevertheless, the implications of altering carnitine concentrations in mammary tissue are unknown. Indeed, the mammary gland would likely limit β oxidation to promote milk fat synthesis (Dimenna and Emery, 1980).

**Noteworthy Intramammary LPS Effects**

As expected, the intramammary LPS challenge induced hepatic and mammary inflammation as evidenced by increased RNA copy numbers of numerous pro-inflammatory cytokines. In addition to that, RNA copy numbers for numerous genes involved in mitochondrial function were affected, suggesting altered metabolic processes in both liver and mammary tissues; this was particularly evident in the liver tissue in agreement with a previous study (Minuti et al., 2015). Transcript abundance for genes related to DNA methylation were altered by the LPS challenge in both mammary and liver tissue, likely for remodeling of DNA methylation patterns to alter gene transcription in response to the LPS challenge (Chen et al., 2019).

Intriguingly, we observed a diverging response to the intramammary LPS challenge found for genes related to apoptosis and inflammation (CASP3 and NOS2) between liver and mammary tissues. Specifically, CASP3 and NOS2 were reduced in the liver, but increased in mammary tissue for LPS-challenged cows. We also found diverging responses to the intramammary LPS challenge for genes related to methylation including DNMT3A (increased in liver but reduced in mammary) and AHCY (increased in mammary but reduced in liver), as well as ATP synthesis (ATP5J, increased in mammary but reduced in liver) during the LPS challenge. Although not entirely clear, we suspect that these diverging responses could be related to shifts in cell types, specifically a reduction of immune cell populations in the liver tissue due to greater recruitment of immune cells into the mammary tissue during an intramammary challenge; however, future studies are needed to clarify this.

For mammary choline metabolites, the LPS challenge reduced concentrations of total PC, total LPC, and total SM. In agreement, Humer et al. (2018) found that plasma LPC concentrations declined 24 h following an intramammary LPS challenge in Simmental cattle. The reason why these choline metabolites are reduced is unknown; however, LPC are known to regulate inflammation and immune function through cell signaling (Grzelczyk and Gendaszewska-Darmach, 2013; Law et al., 2019).

The LPS challenge also reduced mammary tissue concentrations of 6 amino acids (Asp, Cit, Glu, Orn, Ser, and Thr), similar to our plasma data (Swartz et al., 2023) as well as results found in plasma from lambs (Hoskin et al., 2016), steers (Waggoner et al., 2009), and Simmental cattle (Humer et al., 2018) challenged with LPS. Potentially, a reduction in uptake of amino acids into the mammary gland was occurring due to reduction in milk protein synthesis or possibly due to a reduction in circulating AA concentrations. Concentrations of methylhistidine, a marker for muscle catabolism (Plaizier et al., 2000), were greater in mammary tissue from LPS-challenged cows, suggesting breakdown of muscle cells in the mammary gland. This finding was the opposite to the results we found in plasma (Swartz et al., 2023), suggesting that an intramammary LPS challenge induces muscle catabolism in the mammary gland, but impairs it in peripheral tissues (likely due to enhanced insulin signaling; Kvidera et al., 2017; Horst et al., 2019). Finally, kynurenine, a tryptophan metabolite, was increased in mammary tissue from LPS-challenged cows, suggesting greater tryptophan catabolism by immune cells to enhance immunological...
tolerance and promote resolution (Moffett and Namboodiri, 2003).

For organic acids, concentrations of α-aminoacidipic acid, a leucine metabolite (Tucker et al., 2017), were reduced in mammary tissue of LPS-challenged cows. Plasma concentrations of leucine and α-aminoacidipic acid were reduced during the LPS challenge in the present study as well (Swartz et al., 2023), likely due to enhanced insulin signaling impairing muscle tissue catabolism and restricting circulating AA supply. Mammary β-hydroxybutyric acid concentrations were also reduced, similar to our plasma β-hydroxybutyric acid and NEFA data reported in our companion paper (Swartz et al., 2023). Again, this is likely due to enhanced insulin signaling (Kvidera et al., 2017; Horst et al., 2019) on adipose tissue reducing plasma NEFA concentrations and subsequently reducing ketogenesis in the liver. It should be noted that the BHB found in mammary tissue would have most likely originated from ketogenesis occurring in the liver and rumen tissue, and not due to synthesis in the mammary gland.

Concentrations of lactic, pyruvic, and α-ketoglutaric acids were increased in mammary tissue of LPS-challenged cows. These results also mirror our findings in plasma (Swartz et al., 2023). Increases in lactic and pyruvic acid indicate an enhancement in glycolytic flux, likely due to immune activation (Ganeshan and Chawla, 2014). Alpha-ketoglutaric acid is a TCA cycle intermediate located between succinyl-coenzyme A and isocitric acid and produced by oxidizing pyruvate originally derived from glucose. It is also produced in anaplerotic reactions by catabolizing glutamate. Moreover, α-ketoglutaric acid is a precursor to amino acids such as glutamate and glutamine (Zdizińska et al., 2017), the former of which was reduced by the LPS challenge.

For volatile fatty acids, mammary concentrations of propionic acid were decreased in LPS-challenged cows, whereas isobutyric acid was increased. These data mirror our findings in plasma where isobutyric acid concentrations increased during the first 8 h following the LPS challenge, whereas plasma propionate and butyrate decreased (Swartz et al., 2023). This may be the result of decreased DMI (Swartz et al., 2023), altered passage rate, differences in microbial fermentation in the rumen, or altered mammary epithelium function. Future studies are needed to provide clarity. Finally, mammary concentrations of methylmalonic acid, a product of propionic acid catabolism, were also similarly reduced as was propionic acid.

The LPS challenge decreased mammary concentrations of 4 short-chain (C3OH, C5, C4OH, C5DC), one medium-chain (C12), and one long-chain acyl-carnitine (C18). Conversely, one long-chain acyl-carnitine (C18:1) was increased by the LPS challenge. Reduced concentrations of medium and short-chain carnitines along with increased concentrations of C18:1 likely reflect reductions in fatty acid oxidation occurring in the mammary tissue. A decrease in C18 may be due to reduction in circulating NEFA concentrations during the LPS challenge. Nevertheless, it is noteworthy that all these carnitines are in relatively small concentrations, and therefore, the implications may be limited.

**CONCLUSIONS**

Although the intramammary LPS challenge clearly induced inflammation and altered metabolic processes, dietary RPC supplementation did not have a clear effect on inflammatory responses in either liver or mammary tissue during an intramammary LPS challenge. Dietary RPC supplementation also did not increase concentrations of choline metabolites in mammary tissue; potentially, this is the reason why dietary RPC supplementation did not attenuate inflammation during the LPS challenge. Furthermore, no differences between treatment groups were found for any outcome in mammary tissue from unchallenged cows, leaving open the mechanistic question of how dietary RPC supplementation affects the mammary gland to enhance milk yield.

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