ALMA MATER STUDIORUM – UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

XXXV CICLO

Settore Concorsuale di afferenza 07 G1

Settore Scientifico Disciplinare AGR 18

NUTRITIONAL STRATEGIES TO MODULATE METABOLISM AND HEAT STRESS RESILIENCE IN DAIRY CATTLE

Coordinatore

Prof.ssa C. Castagnetti

Relatore

Dott.ssa E. Grilli

Tesi di Dottorato di:

Víctor Sáinz de la Maza Escolà

Correlatore

Chiar.mo Prof. A. Piva

Esame finale anno 2023

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ABSTRACT

Physiological and environmental stressors can disrupt barrier integrity at epithelial interfaces (e.g., uterine, mammary, intestinal, and lung), which are constantly exposed to pathogens that can lead to the activation of the immune system. Unresolved inflammation can result in the emergence of metabolic and infectious diseases. Maintaining cow health and performance during periods of immune activation such as in the peripartum or under heat stress represents a significant obstacle to the dairy industry. Feeding microencapsulated organic acids and pure botanicals (OAPB) has shown to improve intestinal health in monogastric species and prevent systemic inflammation via the gut-liver axis. Feeding unsaturated fatty acids (FA) such as oleic acid (OA) and very-long-chain omega-3 (VLC n-3) FA are of interest in dairy cow nutrition because of their potential to improve health, fertility, and milk production. In the first study, we evaluated the effects of heat stress (HS) conditions and dietary OAPB supplementation on gut permeability and milk production. In parallel with an improved milk performance and N metabolism, cows supplemented with OAPB also had an enhanced hepatic methyl donor status and greater inflammatory and oxidative stress status compared to the HS control group. In a second study, we evaluated the relative bioavailability of VLC n-3 in cows fed a bolus of rumen-protected (RP) fish oil (FO). In a third study, we proved the interaction between RPFO and RP choline to promote the synthesis of phosphatydilcholines. Lipid forms that support hepatic triglyceride export and can prevent steatosis in dairy cows. The last study, demonstrated that algae oil outperforms against a toxin challenge compared to FO and that feeding RPOA modulates energy partitioning relative to n-3 FA-containing oils. Overall, this thesis confirms the need and the effectiveness of different strategies that aimed to improve dairy cows' health and performance under heat stress, inflammation or metabolic disease.

CHAPTER 1. INTRODUCTION

In ruminants, the liver produces around 80% of the glucose via gluconeogenesis (Berman et al., 1974 and Aschenbach et al., 2010). In addition, this organ is crucial for a range of physiological processes, such as lipid and amino acid metabolism, detoxification, and immune defense. The small intestine is responsible for digestion and absorption of nutrients flowing from the pregastric fermentation, other than being an important barrier and immune organ in the body (Van Soest, 1987 and Church, 1993). The enterohepatic circulation connects the liver and the intestines. This connection is bidirectional and allows the intestine to influence the liver and vice versa in a variety of ways. The liver secrets primary bile acids (Bas), immunoglobulin A, and angiogenin into the gastrointestinal tract (GIT). These molecules contribute to preserving gut eubiosis and deterring pathogenic bacterial overgrowth in the intestine (Tripathi et al., 2018). On the other hand, gut microbiota plays a key role in the biotransformation of intestinal primary BAs to secondary BAs and facilitate fat absorption (Holt, 1972). The intestinal barrier is a very important component of this axis as it serves as a physical barrier between the gut and liver. Due to the high metabolic rate and the presence of a large number of bacteria and other microorganisms in the intestine, maintaining gut permeability is vital to the intricate connection between the microbiome, liver function, systemic endotoxemia, and disease development (Turner, 2009). We must acknowledge the potential implications of the gut-liver connection in environmental heat stress. It is well-known that hot exposure causes morphological insults in the intestine that reduce its integrity and contribute to systemic endotoxemia (Collier et al., 1982; Baumgard and Rhoads, 2013). Our group has demonstrated that heat-stressed lactating dairy cows also develop with an increased total-tract gut permeability (Fontoura et al., 2022). This condition leads to leakage of bacteria and their endotoxin (e.g., lipopolysaccharide [LPS]) into the bloodstream, which in turn triggers an immune response. Immune activation, can restrict milk production as glucose is diverted to fight infection (Kvidera et al., 2017).

A critical time in the life cycle of a cow where both liver and intestines play an important role is during the progression from gestation into lactation (i.e., transition period). In early lactation (first 3 to 5 weeks of lactation), nutrient intake often does not meet maintenance and milk production requirements, creating a negative energy balance (NEB; Drackley, 1999). When dairy cows struggle to adjust to the demands of lactation, they may undergo metabolic stress. This stress is characterized by uncontrolled adipose tissue mobilization and a decrease in insulin sensitivity. As a result, lipotoxicity and accumulation of triglycerides (TG) in the liver emerge, leading to a condition known as fatty liver disease (FLD) and ketosis (high circulating ketone bodies; Gröhn et al., 1999). Moreover, these diseases also develop with mastitis, metritis, milk fever, and displaced abomasum (Duffiel et al., 2009). Biomarkers of GIT permeability (i.e., LPS and LPS-binding protein) have also been noted in ketotic cows (Abuajamieh et al., 2016). High grain feeding is a common strategy to increase energy density in postpartum diets; however, starch fermentation can cause acidosis not only in the rumen but also in the hindgut. The latter can be accompanied with epithelial damage and develop with liver damage and systemic inflammation (Plaizer et al., 2018).

Vital processes, such as lipid, nucleotide, and protein synthesis, and maintenance of redox status occur in the one-carbon metabolism. The complex unison of the folate and the methionine cycle coordinates one-carbon metabolism in the dairy cow (Figure 1; McFadden et al., 2020). Specifically, the provision of the universal methyl donor *S*-adenosylmethionine (SAM) to support phosphatidylcholine (PC) synthesis via the PEMT pathway is crucial to maintain very-low density lipoprotein synthesis and subsequent secretion of TG within VLDL to prevent FLD (Pinotti et al., 2002). In turn, the transmethylation pathway hydrolyses *S*-adenosylhomocysteine (SAH) to homocysteine (HCY) to maintain the synthesis of antioxidants (i.e., taurine and glutathione; Figure 1).

We, animal researchers and nutritionists, need to develop nutritional strategies to modulate dairy cow health and metabolism. Feeding organic acids (citric and sorbic acids; OA) and pure botanicals (thymol and vanillin; PB) represents a promising strategy to reduce the use of antibiotic in livestock production systems (Rossi et al., 2020). These compounds have unique antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory properties, which when combined, have potential to improve gastrointestinal health by controlling bacterial pathogen population and enhancing barrier function (Tugnoli et al., 2020). Dietary supplementation OA/PB has been shown to improve animal performance by enhancing gastrointestinal health in swine and poultry species (Hassan et al., 2020, Grilli et al., 2015b). In heat-stressed dairy calves, we have observed that dietary OA/PB partially restored dry matter intake (DMI; Fontoura et al., 2023). Our group has also observed that OA/PB feeding not only restores DMI and production performance of heat-stressed dairy cows but also total-tract permeability (Fontoura et al., 2022). In this thesis, Chapter 1 evaluates the effects of heat stress and dietary supplementation of OA/PB on hepatic one-carbon metabolism of Holstein dairy cows.

Fatty acids (FA), especially those involved in the one-carbon metabolism, should also be considered to improve animals' diets. Very-long-chain n-3 (VLC n-3) FA are well-known to be bioactive molecules with beneficial effects for dairy cows, such as improved milk production, health, and reproductive performance (Mattos et al., 2000; Elis et al., 2016; Moallem, 2018). Specifically, eicosapentanoic (EPA, 20:5) and docosahexanoic acids (DHA, 22:6) are building blocks for the synthesis of eicosanoids with anti-inflammatory, anti-aggregatory, and vasodilatory properties (Patterson et al., 2012). These FA are also a preferable substrate for PC synthesis via the PEMT (DeLong et al., 199). In this thesis, Chapter 2 and 3 are focused in the bioavailability of microencapsulated sources containing VLC n-3 FA, their incorporation in the phospholipid fraction, and the synergy between choline and VLC n-3 to

improve PC synthesis. Finally, a long-chain FA that is worth to be studied as it has positive effects in modulating metabolism and inflammation (Sales-Campos et al., 2016; Tsimidou and Papoti et al., 2010), and FA digestibility (de Souza et a., 2018), is oleic acid (*cis*-9 18:1). The final chapter summarizes the effects of feeding different sources of VLC n-3 FA and oleic acid, in production performance and response to an immune challenge.

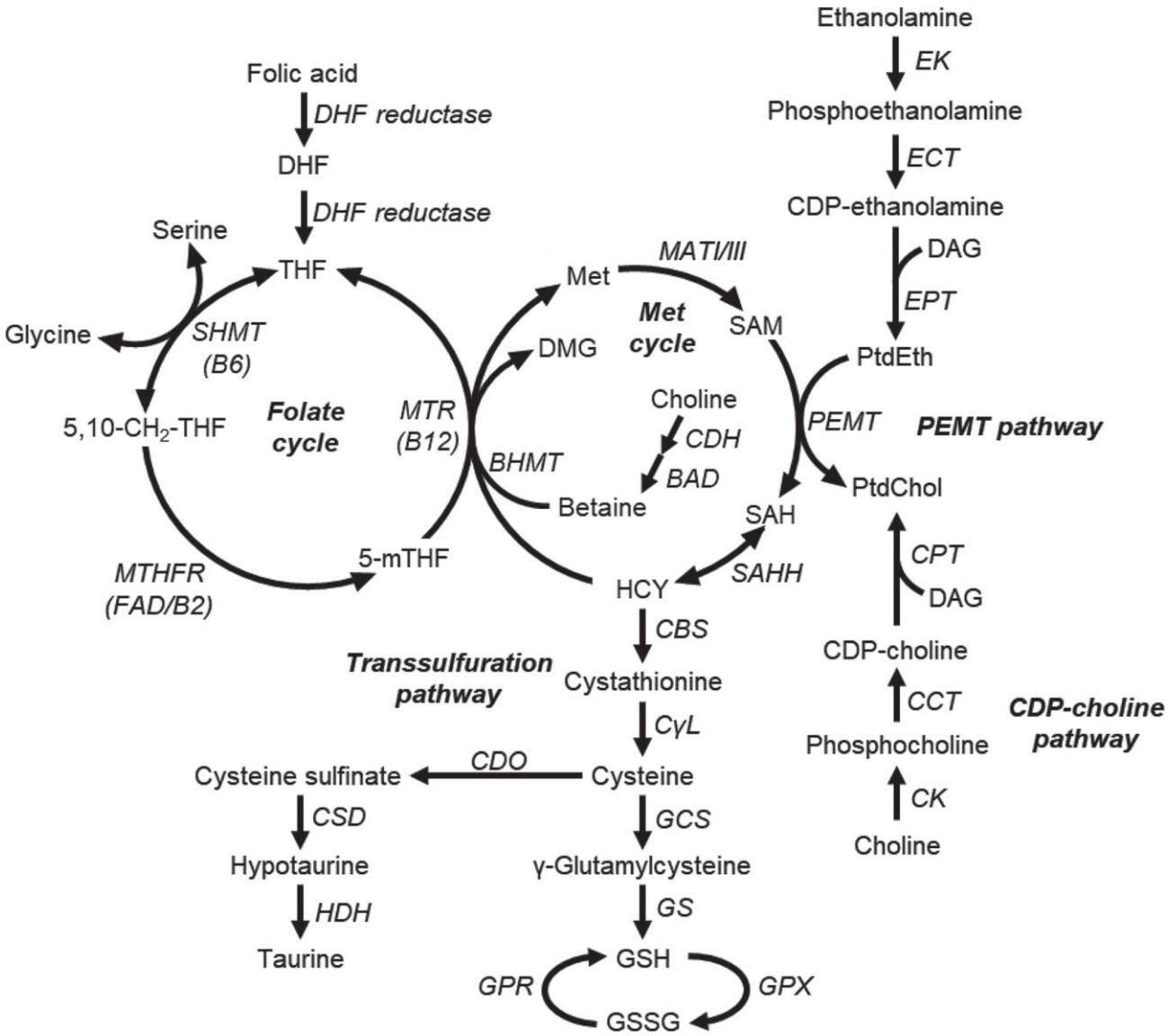

Figure 1. One-carbon metabolism, the transsulfuration pathway, and phosphatidylcholine (PtdChol) synthesis (McFadden et al., 2020).

BAD = betaine aldehyde dehydrogenase; BHMT = betaine-homocysteine methyltransferase; CBS = cystathionine β-synthase; CCT = CTP: phosphocholine cytidylyltransferase; CDH = choline dehydrogenase; CDO = cysteine dioxygenase; CDP = cytidine diphosphate; CγL = cystathionine γ-lyase; CK = choline kinase; CPT = cholinephosphotransferase; CSD = cysteine sulfinic acid decarboxylase; DAG = diacylglycerol; DHF = dihydrofolate; DMG = dimethylglycine; ECT = CTP: phosphoethanolamine cytidylyltransferase; EK = ethanolamine kinase; EPT = ethanolaminephosphotransferase; FAD = flavin adenine dinucleotide; GCS = γ-glutamylcysteine synthase; GPR = glutathione-disulfide reductase; GPX = glutathione peroxidase; GS = glutathione synthetase; GSH = reduced glutathione; GSSG = oxidized glutathione; HCY = homocysteine; HDH = hypotaurine dehydrogenase; MATI/III = methionine adenosyltransferase I/III (liver specific isoenzymes); 5-mTHF = 5-methyltetrahydrofolate; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; PEMT = phosphatidylethanolamine N-methyltransferase (other types of methyltransferases may utilize SAM; only PEMT is shown for simplicity); PtdEth = phosphatidylethanolamine; SAH = S-adenosylhomocysteine; SAM = S-adenosylmethionine (3 SAM are required to convert PtdEth to PtdChol); SHMT = serine hydroxymethyltransferase; THF = tetrahydrofolate. Associated B vitamin cofactors are denoted in parentheses.

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CHAPTER 2. OBJECTIVES

1. Determine the effects of heat stress and dietary supplementation of organic acids and pure botanicals on the one-carbon metabolism in Holstein dairy cows.

2. Determine the bioavailability of rumen-protected fish oil sources in different plasma and milk lipid fractions in Holstein dairy cows.

3. Determine the ability of the co-supplementation of rumen-protected fish oil and choline to support the synthesis of plasma phosphatidylcholines in Holstein dairy cows.

4. Determine the effects of feeding rumen-protected fish oil, algae oil, and oleic acid in production performance and response to an immune challenge in Holstein dairy cows.

CHAPTER 3. Effects of heat stress and dietary organic acids and botanicals on hepatic metabolomics and transcriptomics

Introduction

Reductions in milk protein content and yield motivated to investigate the impact of heat exposure on protein metabolism in dairy cows (Gao et al., 2017). McGuire et al. (1989) confirmed that heat stress (HS) reduces the intestine absorptive capacity of amino acids (AA), and this is probably explained by the loss in intestinal integrity (Koch et al., 2019). Our group has demonstrated that heat-stressed lactating dairy cows develop with an increased total-tract gut permeability (Fontoura et al., 2022). This condition leads to leakage of bacteria and their endotoxin (e.g., lipopolysaccharide [LPS]) into the bloodstream, which in turn triggers an immune response. This is associated with hepatic removal and utilization of AA to produce acute phase and heat-shock proteins (Rius et al., 2019). In addition, the activation of the immune system increases glucose consumption (Kvidera et al., 2017). It is well known that heat-stressed dairy cows have reduced feed intake, which partially explains the lowered production responses (Baumgard and Rhoads, 2013). Despite this hypophagia, increasing levels of circulating insulin concentrations are common in heat-stressed cows (Wheelock et al., 2010; Fontoura et al., 2022). Insulin is a hormone that inhibits lipolysis and might induce muscle protein breakdown to support gluconeogenesis. Urea is the end-product of AA catabolism and in heat-stressed cows, robust increases in plasma levels of urea-nitrogen have been a repeatedly observed response (Figure 2). Ruminants' protein metabolism allows to recycle urea in the rumen; however, excessive circulating urea can cause toxicity (Whitehair, 1989). Urea can damage cells by disrupting the osmotic balance and therefore, require osmoprotective responses to counteract it.

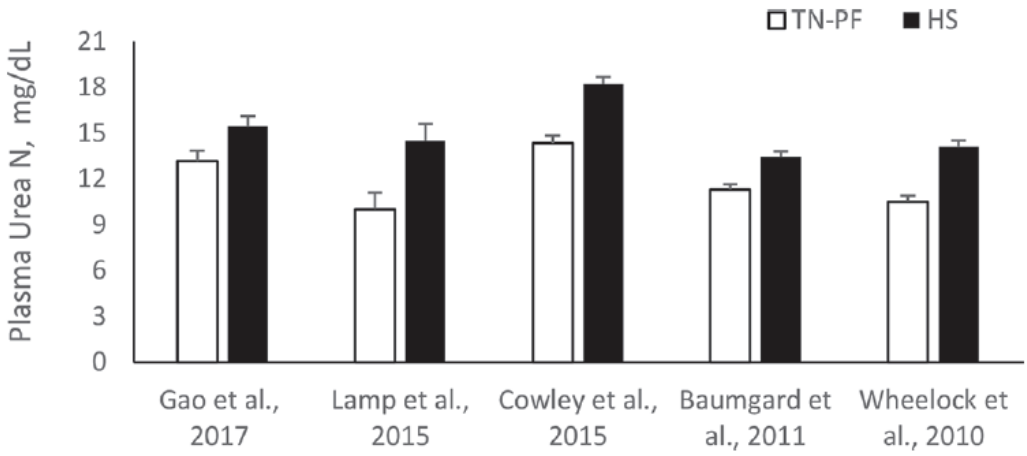


Figure 2. Plasma urea N concentration in heat stressed dairy cows across different studies; HS = heat stress; TN-PF = thermoneutral conditions pair-fed to HS. (Ríus et al., 2017).

Research from human and rodent species tells us that under hyperosmotic conditions, liver and kidney cells accumulate methylamine osmolytes such as betaine or glycerophosphocholine (GPC; Burg, 1995 and Burg et al., 2007). The abundance of betaine transporters increases under osmotic stress (Kempson et al., 2014). In response to changing levels of NaCl and urea, Burg and Gallazzini (2009) identified a reduction in the activity of glycerophosphocholine phosphodiesterase (GPC-pd), the enzyme that degrades GPC to choline, and as a result they observed an intracellular accumulation of GPC (Figure 3). The literature reports higher accumulations of GPC rather than betaine, and presumably it is due to a lower metabolic cost. The inhibition of an enzyme doesn't require extra energy whereas betaine transporters are against gradient concentration (Burg and Peters, 1998). GPC is synthesized from the degradation of phosphatidylcholine (PC) and broken down into choline and α-glycerophosphate (Figure 3). The inhibition of GPC-pd can reduce choline recovery and negatively affect the cytidine diphosphate (CDP-choline) pathway to support PC synthesis (Fernández-Murray and McMaster, 2005; Okazaki et al., 2018). Choline also has a one-carbon unit (i.e methyl group), which can be used in the one carbon metabolism. Choline can enter the methionine cycle through the oxidation into betaine. The methionine cycle is coupled to the folate cycle to drive the synthesis of S-adenosyl methionine (SAM; the Universal Methyl

Donor). SAM can then provide methyl groups to be used for DNA synthesis, PC synthesis via the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway, or to maintain the redox status through the transsulfuration pathway (Figure 1; McFadden et al., 2020).

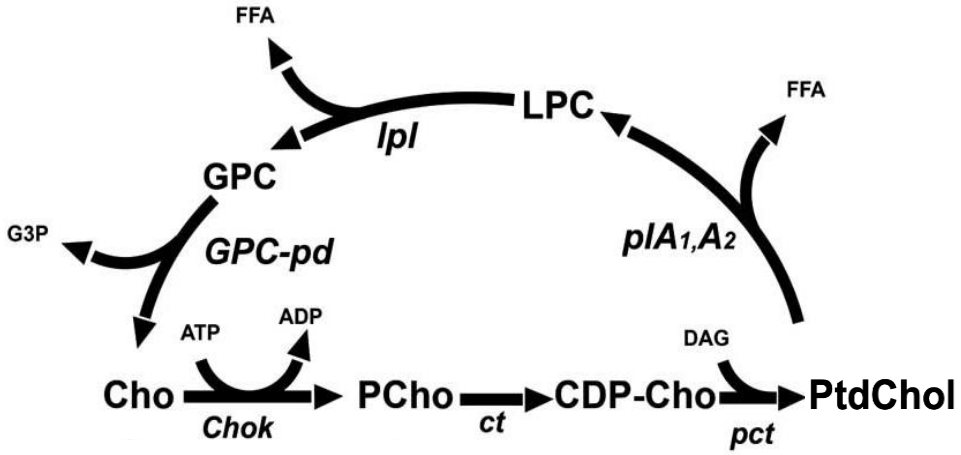


Figure 3. Phosphatidylcholine cycle; synthesis (CDP-choline or Kennedy Pathway) and degradation. CDP-Cho = cytidine diphosphate choline; Cho = choline; Chok = choline kinase; ct = cytidylyltransferase; DAG = diacylglycerol; FFA = free fatty acid; G3P = sn-glycerol-3-phosphate; GPC = glycerophosphocholine; LPC = lysophosphatidylcholine; PA = phosphatidate; PCho = phosphocholine; pct = phosphocholine transferase; plc = phospholipase C; pld = phospholipase D; plA1 = phospholipase A1; plA2 = phospholipase A2; lpl = lysophospholipase; pd = glycerophosphocholine phosphodiesterase. Adapted from Iorio et al., 2010.

Nutritional strategies need to be developed targeting at the mitigation of heat stress effects and gut-liver axis consequences that may involve disturbances in the one-carbon metabolism. Dietary supplementation of organic acid and pure botanicals (OA/PB) has been shown to improve animal performance by enhancing gastrointestinal health in swine and poultry species (Hassan et al., 2020, Grilli et al., 2015b). Supplementation of OA/PB was also investigated in dairy calves experiencing moderate heat stress (Fontoura et al., 2022), and it was observed a partial restore of dry matter intake (DMI). We conducted a recent study at Cornell University where we investigated the effects of heat stress conditions and dietary OA/PB supplementation in lactating Holstein dairy cows (Fontoura et al., 2022). In this study, OA/PB supplementation tended to elevate DMI and restore milk yield and energy-corrected milk. OA/PB was able to have a higher protein yield and lower milk and plasma urea, showing

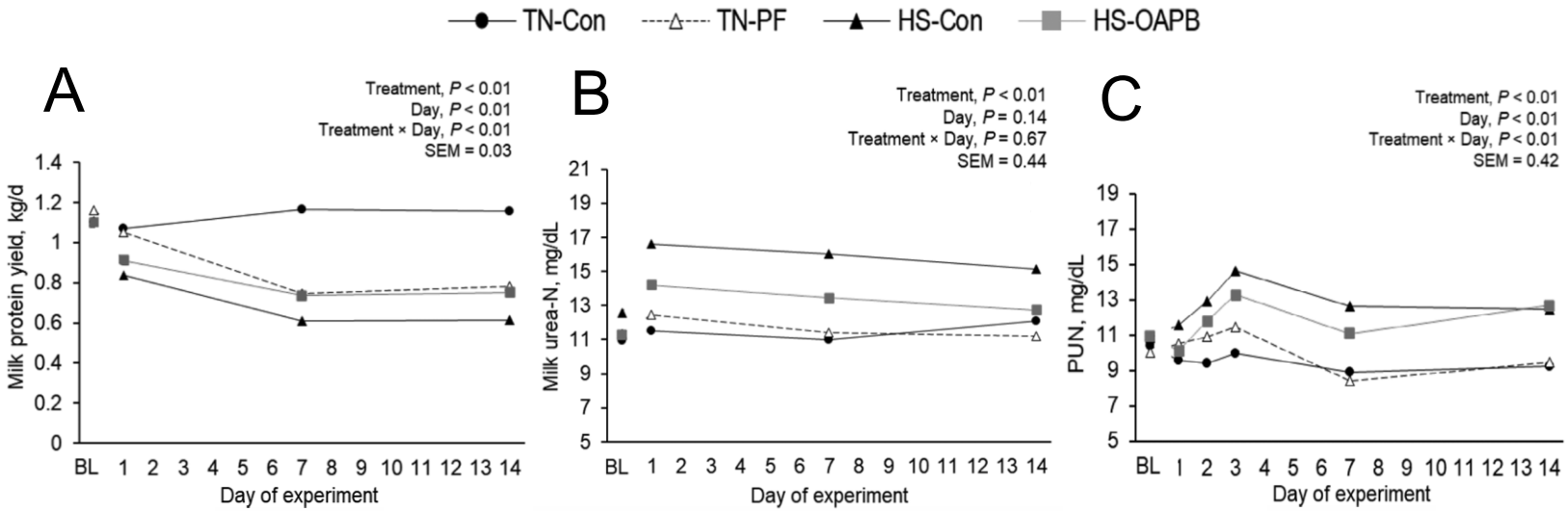
that it was able to improve N incorporation in the milk (Table 1; Figure 4). Guo et al. (2021) identified that heat stress develops with oxidative stress and increased mitochondrial dysfunction in the mammary gland, which may further contribute to the reductions in protein synthesis (Belhadj Slimen et al., 2014). Reduced glutathione (GSH) plays a critical role in protecting macromolecules from reactive oxygen and nitrogen species (Zhang and Forman, 2012). Supplementation of OA/PB also showed a modest but real improvement in total-tract gut permeability and an improved intestinal health supported by a reduced concentration of plasma LPS-binding protein (LBP), compared to their HS control counterparts (Fontoura et al., 2022). Lower circulating LBP could be indicative of a lessened immune activation (i.e release of acute phase proteins). For the present follow-up analyses from the Fontoura et al. (2022) trial we decided to analyze liver metabolites and transcriptomics related to the one-carbon metabolism, PC cycle, and inflammatory signaling, and plasma biomarkers of oxidative stress and inflammation (GSH and serum-amyloide A [SAA]). We hypothesized that (1) HS will develop with accumulation of glycerophosphocholine (GPC) in the liver and that dietary OA/PB will prevent it, and that (2) HS will experience oxidative stress and inflammation and OA/PB will ease its repercussions.

Table 1. Effects of heat stress and dietary organic acid and pure botanical supplementation on milk protein yield, milk urea-N, and plasma urea-N (Fontoura et al., 2022).

Variable	Treatment[1]					*P*-values[2]			
	TN-Con	TN-PF	HS-Con	HS-OAPB	SEM	Treatment	HS-Con vs. TN-Con	HS-Con vs. TN-PF	HS-Con vs. HS-OAPB
Milk protein yield, kg/d	1.13	0.86	0.69	0.80	0.03	<0.01	<0.01	<0.01	0.01
Milk urea-N, mg/dL	139	207	129	162	20.1	<0.01	0.64	<0.01	0.16
Plasma urea-N, mg/dL	9.4	10.1	12.8	11.8	0.42	<0.01	<0.01	<0.01	0.08

[1]Forty-six pregnant multiparous and lactating Holstein cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, n = 12), heat stress with no supplementation (HS-Con, n = 12), thermoneutral conditions pair-fed to HS-Con (TN-PF, n = 12), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus® R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, n = 10). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix.
[2]Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.

Figure 4. Effects of heat stress and dietary organic acid and pure botanical supplementation on milk protein yield, milk urea-N, and plasma urea-N (Fontoura et al., 2022).



Material and methods

Plasma and liver samples collected from the trial Fontoura et al. (2022) were used for these analyses. At Cornell University Large Animal Research and Teaching Unit, forty-six Holstein cows (208 ± 4.65 d in milk [mean ± SD], 3.0 ± 0.42 lactations, 122 ± 4.92 d pregnant) were enrolled in a study with a completely randomized design. Following a 7 d acclimation in thermoneutrality (temperature-humidity index [THI] 68), cows were assigned to 1 of 4 groups: thermoneutral conditions (TN-Con, n = 12), HS conditions (HS-Con, n = 12; diurnal THI 74 to 82), TN conditions pair-fed to match HS- Con (TN-PF, n = 12), or HS fed OA/PB (HS-OAPB, n = 10; 75 mg/kg of body weight; 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; AviPlus® R; Vetagro S.p.A) for 14 d. Cows were milked twice daily and fed a corn-silage based total mixed ration top-dressed without (triglyceride only) or with OA/PB.

Blood for plasma separation was collected in the morning (preprandial) on d −1 (baseline sample), 3, 7, and 14 by coccygeal venipuncture into an evacuated blood tube, which contained potassium EDTA as an anticoagulant when plasma was collected. Plasma SAA and GSH concentrations were determined using enzymatic methods and commercially available kits (SAA #EKX-UQWZTI-96; Nordic Biosite AB and, GSH #UNES00017; Assay Genie).

Liver biopsies were performed on d 6 of acclimation (baseline) and d 13 of environmental conditioning. After the hair was clipped, biopsy sites were sanitized with iodine scrub and anesthetized with a 12 mL of lidocaine HCl (Vedco Inc., Saint Joseph, MO) delivered subcutaneously. For liver biopsies, the 11th intercostal space was prepared by making a 0.5-cm incision through the skin, and a fabricated trocar was used to collect approximately 1 g of liver tissue (Hughes, 1962). Following the collection of tissue, biopsy sites were stapled and sprayed with antiseptic. Tissue was snap-frozen in dry ice and stored at −80°C until analyses.

Liver choline, betaine, dimethylglycine (DMG), methionine, SAM, S-adenosyl homocysteine (SAH), phosphocholine (Pchol), PC, lysophosphatidylcholine (LPC), GPC and, sphingomyelin (SM) concentrations were quantified using liquid chromatography coupled with mass spectrometry following the methods described by Jiang et al. (2012). For transcriptomics analyses, liver total RNA was isolated from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA), Phasemaker Tubes (Invitrogen), and RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions with minor modifications. Specifically, tissue samples were homogenized in TRIzol and lysates were transferred to Phasemaker Tubes with chloroform for 20 min rotation at 4°C. The aqueous phase was then transferred to the gDNA elimination column for 1 min rotation at room temperature. The eluted RNA was washed with cold 70% ethanol, transferred to the RNeasy mini spin column, and washed with buffer RPE and RW1. Finally, RNAs were eluted in RNAse-free water and concentrations were determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA quality control, library construction, and RNA sequencing were performed through the service provided by Novogene Inc. (Sacramento, CA, USA). Briefly, RNA quality was evaluated on Bioanalyzer 2100 (Agilent). Next, mRNA was enriched from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers followed by the second strand cDNA synthesis. The library was ready after a series of subsequent steps, including end pair, A-tailing, adapter ligation, size selection, amplification, and purification. Finally, the library was checked with Qubit and real-time PCR for qualification and evaluated on a bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms in paired-end mode (2x150bp). The adaptor removal and quality control of the raw sequencing reads were carried out using fastp (v0.23.2) as described by Chen et al. (2018). Reads with a percentage of low-quality base (quality score < 20)> 40% were removed. Reads with length < 30 bp or with too much Ns (>

5%) were also removed in this study. The cow reference genome (ARS-UCD 1.3) was downloaded from National Center of Biotechnology Information (2008) database, and the alignment of clean reads were performed with STAR (v2.7.9a; Dobin et al., 2013) allowing no more than 3 mismatches. The raw read counts for each gene were extracted using featureCounts (v2.0.3; Liao et al., 2014), and the gene expression level was normalized by transcripts per million (TPM) using IsoEM2 (Chen et al., 2018).

Genes selected for transcript profiling (Table 2) in liver tissue were those associated with inflammatory signaling and mediators (AOAH, MYD88, SAA3, and NFKB1), the transsulfuration pathway and antioxidant system (CBS, CDO1, GPX1, and GSR), the transmethylation pathway (AHCY, BHMT, CHD, MAT1A, and MTR), and phosphatidylcholine degradation (GDPD5). Plasma data were analyzed using a mixed model including random effect of cow, fixed effects of treatment, time, and their interaction and lactation, days in milk and baseline values included as covariates. Liver metabolites were analyzed using a general linear mixed model that included random effect of cow, fixed effects of treatment, time, and their interaction and lactation, days in milk and baseline values included as covariates. For the purpose of this thesis, the hepatic normalized gene expression value (TPM), was analyzed using t-test was used to calculate the difference between treatments. This data will have to be reanalyzed under the proper mixed model before peer-reviewed publication. Planned contrasts included HS-Con vs. TN-Con, HS-Con vs. TN-PF, and HS-Con vs. HS-OAPB. Main effects were declared significant at $P \leq 0.05$ and trending towards significance at $0.05 < P \leq 0.15$. Plasma and liver metabolites statistics were performed using the statistical software JMP Pro v. 16.2 (SAS Institute Inc., Cary, NC). Gene analyses were conducted using ggplot R package (Wickham, 2016).

Table 2. Symbol, name, and biological function of target genes.

Symbol	Name	Biological function
Inflammatory signaling and mediators		
AOAH	Acyloxyacyl hydrolase	Hydrolysis of acyloxylacyl-linked fatty acyl chains from bacterial lipopolysaccharides (i.e endotoxin detoxification).
MYD88	Myeloid differentiation primary response gene 88	Inflammatory signaling between toll-like receptor 4 and interleukin-1.
SAA3	Serum amyloid A 3	Acute phase protein.
NFKB1	Nuclear factor kappa B	Primary transcription factor for immune response.
Oxidative stress and transulfuration pathway		
CBS	Cystathionine-beta-synthase	Synthesize of cystathionine from homocysteine.
CDO1	Cysteine dioxygenase type 1	Cysteine regulator and initiator of Taurine pathway.
GPX1	Glutathione peroxidase 1	Reduction of organic hydroperoxides and hydrogen peroxide by glutathione, and thereby protect cells against oxidative damage.
GSR	Glutathione-disulfide reductase	Central enzyme of cellular antioxidant defense; reduces oxidized glutathione disulfide (GSSG) to the sulfhydryl form GSH.
Transmethylation pathway		
AHCY	S-adenosyl-L-homocysteine hydrolase	Hydrolysis of SAH to adenosine and homocysteine (HCY).
BHMT	Betaine-homocysteine S-methyltransferase	Conversion of betaine and homocysteine to DMG and methionine.
CHDH	Choline dehydrogenase	Oxidation of choline to betaine.
MAT1A	Methionine adenosyltransferase 1A	Formation of SAM from methionine and ATP.
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	Final step in methionine biosynthesis, transferring the methyl group of methyltetrahydrofolate to homocysteine.
Phosphatidylcholine degradation		
GDPD5	Glycerophosphodiester Phosphodiesterase Domain Containing 5	Degradation of GPC to choline.

Results

Plasma SAA and GSH concentrations increased in HS-CON cows, compared to TN-CON (P < 0.05), TN-PF (P < 0.01 and P < 0.08, respectively), and HS-OAPB (*P* < 0.01 and *P* < 0.08, respectively) with greater concentrations observed at d 7 of the experiment (Figure 5).

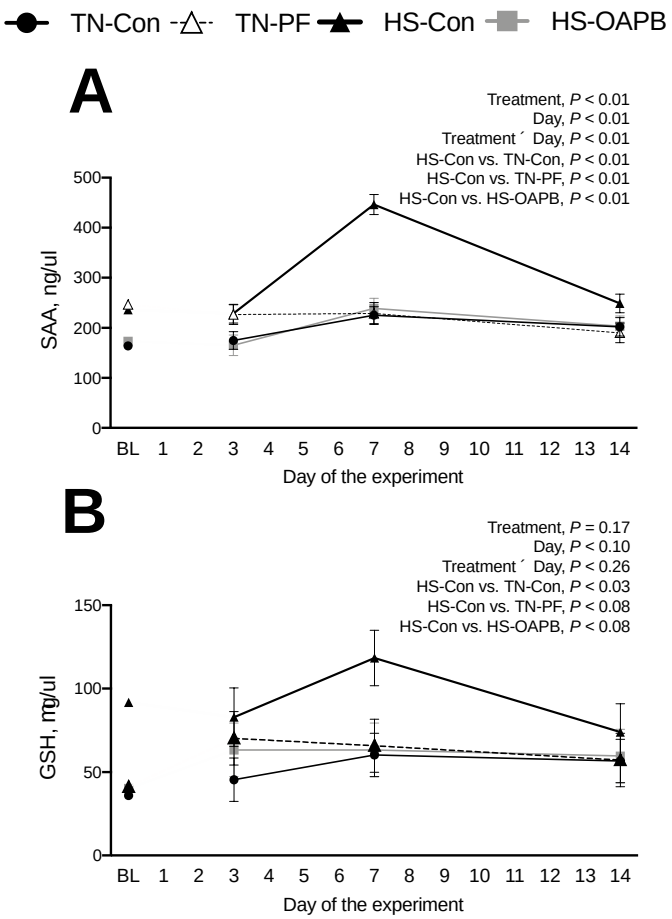


Figure 5. Effects of environmental conditioning and dietary organic acid and pure botanical supplementation on plasma (A) SAA, and (B) GSH of pregnant multiparous lactating Holstein cows.

Liver choline and choline metabolite results are depicted in Table 3. Choline concentrations were reduced in HS-Con compared to TN-Con (*P* = 0.02) and TN-PF (*P* = 0.05). We did not see changes in methionine or DMG but instead, betaine was increased in TN-PF group compared to HS-Con (*P* < 0.01). SAM tended to decrease in HS-Con compared to TN-Con (*P* < 0.10), which could be a consequence of the lower choline concentration. Although no differences were detected in SAH, HS-Con had a lower ratio SAM:SAH compared to TN-Con (*P* = 0.05) and HS-OAPB was able to restore it (*P* = 0.06). This ratio is the marker that indicates the remethylation capacity of the liver. No

changes were observed in hepatic Pchol, LPC, or SM concentrations. However, HS-Con increased PC compared to TN-PF ($P < 0.01$). In agreement with our hypothesis, HS-Con accumulated greater amounts of GPC compared to thermoneutrality ($P < 0.01$) and OAPB feeding was able to significantly prevent this accumulation ($P = 0.02$). Similar results were obtained for the GPC:choline ratio (negatively correlated to the activity of the GPC-pd), where HS-Con had greater values compared to thermoneutrality ($P < 0.01$) and HS-OAPB tended to lower the ratio ($P < 0.14$).

Table 3. Effects of heat stress and dietary organic acid and pure botanical supplementation on hepatic choline and choline metabolites.

Metabolite, umol/g[3]	Treatment[1]					*P*-values[2]			
	TN-Con	TN-PF	HS-Con	HS-OAPB	SEM	Treatment	HS-Con vs. TN-Con	HS-Con vs. TN-PF	HS-Con vs. HS-OAPB
Choline	308	294	234	246	27.7	0.04	0.02	0.05	0.70
Betaine	139	207	129	162	20.1	<0.01	0.64	<0.01	0.16
DMG	2.32	2.84	1.92	2.10	0.65	0.53	0.55	0.15	0.80
Methionine	70.3	74.8	75.6	69.9	7.64	0.87	0.53	0.91	0.50
SAM	38.5	32.6	30.7	36.2	4.2	0.36	0.10	0.68	0.24
SAH	11.9	11.8	12.0	11.1	0.97	0.80	0.93	0.87	0.43
SAM:SAH[4]	3.40	2.99	2.63	3.36	0.32	0.20	0.05	0.32	0.06
Pchol	1139	980	949	1009	199	0.80	0.37	0.87	0.79
PC	20406	19347	21458	21131	667	0.03	0.16	<0.01	0.67
LPC	639.9	633.5	663.9	653.8	29.2	0.69	0.40	0.28	0.73
GPC	4779	3847	7193	5997	437	<0.01	<0.01	<0.01	0.02
SM	1703	1864	1824	1775	114	0.61	0.34	0.73	0.70
GPC:choline[5]	19.12	13.9	33.6	28.2	3.20	<0.01	<0.01	<0.01	0.14

[1]Forty-six pregnant multiparous and lactating Holstein cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, n = 12), heat stress with no supplementation (HS-Con, n = 12), thermoneutral conditions pair-fed to HS-Con (TN-PF, n = 12), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus® R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, n = 10). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix.

[2]Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.

[3]Dimethylglycine (DMG), S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH), phosphocholine (Pchol), phosphatidylcholine (PC), lysophosphocholine (LPC), glycerophosphorylcholine (GPC), sphingomyelin (SM).

[4]Remethylation capacity; calculated as the ratio of SAM and SAH.

[5]Glycerophosphocholine phosphodiesterase (GPC-pd) activity (negatively correlated); calculated as the ratio of GPC and choline.

Hepatic gene expression is depicted in Table 4. Expression of MYD88, NFKB1, and SAA3, all of them playing a key role in regulating the inflammatory response, were upregulated by HS-Con compared to TN-Con ($P < 0.01$), TN-PF ($P \leq 0.01$), and HS-OAPB ($P \leq 0.12$). No changes were observed for AOAH gene, which encodes the enzyme that catalyzes the hydrolysis of acyloxylacyl-linked fatty acyl chains from bacterial LPS. Conversion of homocysteine to cystathionine, the first step in the transsulfuration pathway, regulated by CBS, tended to be lower in HS-OAPB ($P \leq 0.7$), and TN-Con ($P = 0.15$) compared to HS-Con. Expression of CDO1, a critical regulator of cellular cysteine concentrations, was upregulated in HS-Con cows compared to all treatments ($P \leq 0.05$). The expression of GPX1, the enzyme that uses GSH to reduce organic hydroperoxides and hydrogen peroxides, was upregulated in HS-OAPB relative to HS-Con ($P < 0.01$). No changes were observed in GSR expression, the enzyme that reduces oxidized glutathione disulfide (GSSG) to the sulfhydryl form GSH. Expression of AHCY, encoded to hydrolyze SAH to adenosine and homocysteine (HCY), and BHMT, which converts betaine and HCY to DMG, were upregulated in HS-Con relative to TN-Con ($P = 0.01$), TN-PF ($P \leq 0.03$), and HS-OAPB ($P \leq 0.08$). Oxidation of choline to betaine, regulated CHDH, increased in HS-Con compared to TN-PF cows ($P = 0.02$). MAT1A gene, which catalyzes the production of SAM from methionine and ATP was downregulated in TN-PF ($P < 0.01$) compared to HS-Con. The final step in methionine biosynthesis that transfers a methyl group of methyltetrahydrofolate to HCY, catalyzed by MTR, was upregulated in HS-Con compared to TN-Con ($P = 0.03$). GPDD5, the gene that encodes for GPC-pd; degradation of GPC to choline, was upregulated in HS-Con compared to TN-Con ($P < 0.01$), TN-PF ($P < 0.01$), and HS-OAPB ($P < 0.05$).

Table 4. Effects of heat stress and dietary organic acid and pure botanical supplementation on hepatic gene expression.

Gene, transcripts per million	Treatment[1]					*P*-values[2]			
	TN-Con	TN-PF	HS-Con	HS-OAPB	SEM	Treatment	HS-Con vs. TN-Con	HS-Con vs. TN-PF	HS-Con vs. HS-OAPB
Inflammatory signaling and mediators									
AOAH	1.91	1.67	1.61	1.58	0.33	0.81	0.43	0.87	0.94
MYD88	8.94	8.90	11.42	10.29	0.65	<0.01	<0.01	<0.01	0.08
SAA3	-3.19	53.26	173.19	96.65	40.83	<0.01	<0.01	0.01	0.13
NFKB1	3.60	3.89	4.68	4.12	0.25	0.01	<0.01	0.01	0.06
Oxidative stress and transsulfuration pathway									
CBS	33.6	29.3	30.5	26.7	1.9	0.02	0.15	0.55	0. 07
CDO1	284	184.9	352	285	30.5	<0.01	0.05	<0.01	0.05
GPX1	239	189.5	224	312	24.1	<0.01	0.57	0.16	<0.01
GSR	21.9	20.5	21.7	19.3	1.6	0.52	0.91	0.55	0.25
Transmethylation pathway									
AHCY	1059	1089	1408	1179	109	0.04	0.01	0.01	0.08
BHMT	282	303	400	257	37.01	0.02	0.01	0.03	<0.01
CHDH	11.8	7.75	10.80	9.30	1.14	0.02	0.45	0.02	0.24
MAT1A	189	134	198	178	13.32	<0.01	0.56	<0.01	0.23
MTR	4.08	4.92	5.33	4.48	0.49	0.14	0.03	0.43	0.12
Phosphatidylcholine degradation									
GDPD5	1.27	1.29	2.29	1.62	0.29	0.01	<0.01	<0.01	0.04

[1]Forty-six pregnant multiparous and lactating Holstein cows were randomly assigned to 1 of 4 treatments at enrollment: unsupplemented thermoneutral conditions (TN-Con, n = 12), heat stress with no supplementation (HS-Con, n = 12), thermoneutral conditions pair-fed to HS-Con (TN-PF, n = 12), and HS supplemented with organic acids and pure botanicals (OA/PB; 75 mg/kg of BW; AviPlus® R; contains 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; Vetagro S.p.A.; HS-OAPB, n = 10). Control cows (not supplemented with OA/PB) received a matching dose of the lipid matrix.

[2]Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.

Discussion

Our results indicate that heat stress alters hepatic one-carbon metabolism, and oxidative stress and inflammatory-related pathways, and that OA/PB supplementation can prevent these alterations. The accumulation of GPC in the liver of HS-Con cows explains the lower concentrations in choline and SAM, and therefore the reduced remethylation capacity. GDPD5 expression was upregulated in HS-Con cows, suggesting that by negative feedback, gene transcription is stimulated and inhibition of GPC-pd might be happening in protein translation or post-translation steps. This observation comes in parallel with the impaired N metabolism and provides more evidence to support the idea that high circulating urea affects choline recycling in the liver. Although choline concentrations were decreased, Pchol or PC levels were not affected in HS-Con. Okazaki et al. (2018) instead, silencing GDPD5 reduced intracellular Pchol in mouse adipocites. Heat stress upregulated the expression of inflammatory signaling genes, which were accompanied with high circulating SAA. Instead, endotoxin detoxification (AOAH) was downregulated, contrary to what we expected from the circulating LBP results in Fontoura et al. (2022). Interestingly, heat stress upregulated the methionine cycle (CHDH, BHMT, MTR, and MAT1). We speculate that this could be a negative feedback mechanism; low choline and SAM concentrations and the need to maintain methyl donor supply. Expression of CBS, the first reaction of the transsulfuration pathway, was downregulated in HS-OAPB compared to HS-Con. This is in accordance with the greater methylation capacity, which provides more SAH available to be hydrolyzed to HCY. Instead, HS-Con had greater plasma GSH concentration compared to HS-OAPB as well as greater CDO1 expression, enzyme involved in the regulation of taurine synthesis, relative to HS-OAPB and their counterparts in thermoneutrality. We do not have reactive oxygen species data but it is known that heat stressed dairy cows develop with oxidative stress (Guo et al., 2021). For now, our results indicate that heat stress stimulates the synthesis of antioxidants and that OA/PB feeding lessens the requirement of these molecules.

Ongoing hepatic metabolomic and transcriptomic analyses will allow having a wider vision of the effects of heat stress and dietary OA/PB supplementation in the one-carbon metabolism.

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CHAPTER 4. Temporal changes in plasma and milk fatty acids and plasma phospholipid concentrations in response to an esophageal bolus of rumen-protected fish oil in lactating Holstein dairy cows

Introduction

Very-long-chain n-3 (VLC n-3) fatty acids (FA) are polyunsaturated lipids commonly found in dietary sources such as fish and marine algae. In humans, VLC n-3 FA composed of 20 or 22 carbons are regarded as essential for maintaining normal physiological function and the prevention of cardiovascular disease, inflammation, and cancer (Simopoulus, 2002; Wang et al., 2006; Lavie et al., 2009; Yashodhara et al., 2009). Specifically, eicosapentanoic (**EPA**, 20:5) and docosahexanoic acids (**DHA**, 22:6) are building blocks for the synthesis of eicosanoids with anti-inflammatory, anti-aggregatory, and vasodilatory properties (Patterson et al., 2012). These FA also have insulin-sensitizing properties (Gingras et al., 2007; Capel et al., 2015). In this context, much attention has been given to the enrichment of cow's milk in VLC n-3 FA (Castañeda-Gutiérrez et al., 2007; Bernal-Santos et al., 2010; Moran et al., 2017) as a means to enhance the supply of these FA for human diets containing dairy (Ursin, 2003; Gebauer et al., 2006; Danaei et al., 2009).

Dietary VLC n-3 FA may also improve milk production, health, and reproductive performance in dairy cattle (Mattos et al., 2000; Elis et al., 2016; Moallem, 2018). The bioavailability of dietary VLC n-3 FA for metabolic use is limited by (1) their typical low concentration in traditional feedstuffs (i.e., α-linolenic acid [**ALA**; 18:3] is the most common n-3 FA) and (2) their extensive ruminal microbial biohydrogenation to unsaturated intermediates and saturated FA (Palmquist, 2006; Castañeda-Gutiérrez et al., 2007; Jenkins et al., 2008). Moreover, the conversion of ALA to EPA and DHA occurs with poor efficiency (Hagemeister, 1991; Pawlosky et al., 2001; Whelan and Rust, 2006). Consequently, dietary strategies that provide intact VLC n-3 FA for postruminal absorption are of interest in dairy cattle nutrition. Calcium-salts of PUFA are a traditional means of rumen protection but are still prone to rumen biohydrogenation (Palmquist, 2006; Castañeda-Gutiérrez et al., 2007; Leduc et al., 2017). Our objective was to quantify changes in circulating n-3 FA, polyunsaturated

phospholipids, and milk n-3 FA in response to an esophageal bolus of microencapsulated fish oil with triglyceride or starch in lactating dairy cows. We focused on phospholipids because VLC n-3 FA are readily incorporated into this fraction of plasma lipids, with a limited transfer into triglycerides (Lengi and Corl, 2012; Myers et al., 2019).

Material and methods

All procedures were carried out in compliance with Cornell University's Institutional Animal Care and Use Committee (protocol #2017-0110; Ithaca, NY). At the Cornell University Vet Teaching Dairy (Ithaca, NY), 6 mid-lactation, multiparous, pregnant Holstein dairy cows (mean ± SD: 155 ± 19 DIM; 3.0 ± 0.5 BCS; 3.2 ± 1.1 lactations; 644 ± 23 kg BW) were acclimated to tie-stalls for 10 d. Cows were then randomly assigned to treatments in a study with a replicated 3 × 3 Latin Square design. Treatments were provided as a single esophageal bolus in a gelatin capsule and included fish oil microencapsulated with palm oil triglycerides (TAG; 36% fish oil; Vetagro S.p.A., Reggio Emilia, Italy), fish oil encapsulated with modified starch (STR; 45% fish oil; Salmate®, The Ballard Group Inc., Cincinnati, OH), or unsupplemented control (i.e, an empty gelatin capsule; CON). The provision of either fish oil product delivered 10 g equivalent to the sum of 20:5n-3 and 22:6n-3 and contained less than 1% of 22:5n-3. Cows were fed a conventional TMR composed of corn silage and a mixed legume haylage to meet or exceed nutrient requirements (NRC, 2001), and *ad libitum* access to water was provided. Cows were milked at 0630, 1230, and 2100 h and milk yields were recorded daily. A 7-d washout period was utilized between each bolus delivery to avoid carryover effects between experimental periods.

Jugular catheters (Micro-Renathane Implantation Tubing, 2.03 mm o.d. × 1.02 mm i.d.; Braintree Scientific Inc., Braintree, MA) were placed 24 h before the bolus administration and flushed with heparinized saline every 7 h to maintain patency, as previously described (Mathews et al., 2015). Blood samples were collected in EDTA-containing tubes (Covidien, Mansfield, MA) at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24 and 36 h, relative to bolus administration via jugular catheter. For separation of EDTA-containing plasma, blood samples were placed on ice for 30 min followed by centrifugation at 3,400 × *g* for 15 min at 4°C. Plasma was stored at −80°C until analysis. Milk was collected at 6 consecutive milkings starting before

the bolus administration. Two milk aliquots were collected, one was transferred into a sealed tube containing 2-bromo-2-nitropropane-1,3-diol and stored at 4ºC for milk composition analysis within 3 d of collection, and a second one was placed in a conical falcon tube and subsequently stored at -20ºC for milk FA analyses. Total mixed ration samples were analyzed for nutrient composition by near-infrared spectroscopy (Cumberland Valley Analytical Services, Cumberland, MD; AOAC International, 1995, method 989.03; Thompson et al., 1995).

Extracted plasma was analyzed for FA concentrations using gas chromatography as previously described (Rico et al., 2021a). Changes in plasma lysophosphatidylcholines (LPC) and phosphatidylcholines (PC) at 0, 4, 10 and 16 h were measured using liquid chromatography-mass spectrometry as described previously (Rico et al., 2021b) with modifications. A 4000 QTRAP® LC-MS/MS System (Sciex; Framingham, MA) was used and extracts were ionized using electrospray ionization in positive mode. Analytes were identified in multiple reaction monitoring mode, which is specific to each analyte, where the transition of precursor ([M+H]+) to fragment (loss of choline-184.1) of each analyte were detected. For quantitative measurements, the identified LPC and PC area-under-the-curve responses were normalized to their corresponding PC and LPC deuterium-heavy isotope labeled internal standard responses in each analytical run.

Milk samples were analyzed for fat, true protein, and lactose concentrations using Fourier transform infrared spectroscopy and SCC by flow cytometry (Dairy One, Ithaca, NY). Milk FA were extracted according to the method developed by Feng et al. (2004) with modifications. Briefly, 38 mL of milk were centrifuged at 17,800 × g for 30 min at 4°C. The fat-cake layer was transferred to a 1.5-mL microtube and stored at -80ºC until lipid extraction. Total lipid of fat cake (320 ± 10 mg) was extracted using n-hexane/Isopropanol solution (3:2,

vlo/vol) as described by Lock et al. (2013). Gas-liquid chromatography was performed on a GC system-8890 (Agilent Technologies, USA) equipped with a flame-ionization detector (FID), autosampler, a split/splitless injector and a CP-Sil 88 column (100 m × 0.25mm internal diameter, 0.20-µm film thickness; Agilent Technologies, USA). Hydrogen was used as the carrier gas at a flow rate of 1 mL/min and for the FID at 40 mL/min and nitrogen makeup gas at 30 mL/min, and the injector and detector temperature was kept at 250°C. The oven temperature program was as follows: initial temperature at 80°C and held for 1 min and then raised to 215°C at a rate of 2 °C/min, and held for 21.5 min (Duplessis et al., 2022). For each GC analysis, 1 µL of sample was injected and a 1:100 split ratio was used. Individual peaks were identified using reference standards (GLC reference standard 463, GLC reference standard 481-B, and octadecadienoic mixture (# UC-59 M, Nu-Chek Prep Inc.) short-chain fatty acid methyl ester was corrected for mass discrepancy using response factors published by Ulberth and Schrammel (1995). Fatty acid concentration was determined yield on a mass basis using the molecular weight of each FA while correcting for glycerol as described by Schauff et al. (1992).

All plasma and milk data were analyzed as repeated measures over time relative to start of treatment under the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) under the following model:

$$Y_{ijklm} = \mu + C_i + P_j + T_k + H_l + pVar_m + e_{ijklm}$$

Where Y_{ijklm} = dependent variable, µ = overall mean, C_i = random effect of cow (i = 1 to 6), P_j = fixed effect of sampling period (j = 1 to 3), T_k = fixed effect of treatment (k = 1 to 3), H_l = fixed effects of time (l = 1 to 36), $pVar_m$= baseline values for each variable used as a covariates, and e_{ijklm} = residual error. The least squares means comparisons were performed using pre-planned non-orthogonal contrasts of interest (CON vs. TAG and STR, and TAG vs. STR).

Significance was declared as a $P \leq 0.05$. Tendencies were declared when $P > 0.05$ and ≤ 0.15. Covariance structure evaluations, as well as model quality and residual distribution assessments, were performed as previously described (Rico et al., 2021b).

Results

The nutrient composition of the TMR included 29.9% aNDFom, 39.9% NFC, 27.6% starch, 16.5% CP, 4.9% EE, and 7.5% ash. No differences ($P > 0.10$) were detected among treatments for dry matter intake (26.6 ± 0.63 kg/d), milk yield (39.02 ± 1.7 kg/d), content and yields of milk fat (3.83 ± 0.3 % and 1.46 ± 0.11 kg/d), protein (2.91 ± 0.04 % and 1.13 ± 0.03 kg/d), and lactose (4.9 ± 0.03 % and 1.89 ± 0.08 kg/d) on the day following the bolus administration.

We did not detect any significant effects of treatment or treatment × time for plasma 18:3 n-3 ($P = 0.34$ and $P = 0.15$), 20:3n-3 ($P = 0.57$ and $P = 0.70$), 22:3n-3 ($P = 0.41$ and $P = 0.37$), or 22:5n-3 ($P = 0.46$ and $P = 0.30$; Figure 6B). However, treatment × time effects or tendencies were detected in 20:5 n-3 ($P = 0.05$; Figure 6A), 22:6 n-3 ($P = 0.07$; Figure 6C), 20:5 + 22:6 n-3 ($P < 0.01$; Figure 6F) and plasma total n-3 ($P = 0.02$; Figure 6E). Both TAG and STR tended to increase plasma concentrations of total polyunsaturated LPC ($P = 0.07$) and significantly increased LPC-20:5, −22:5 and −22:6 ($P < 0.01$; Figure 7) with treatment × time interaction ($P < 0.05$) observed at 10 and 16 h relative to bolus administration. We did not detect differences in plasma total polyunsaturated PC; however, we did detect significant increases in PC-38:5 and PC−38:6 ($P < 0.01$), at 10 and 16 h ($P < 0.01$). Plasma PC-40:5 and PC-40:6 concentrations were also greater in STR cows, relative to TAG cows ($P < 0.05$; Figure 7). We did not detect 22:6 n-3 in milk FA analysis. No significant changes were observed for milk 20:5 n-3 ($P = 0.68$ and $P = 0.39$; Figure 8A) and 22:5 n-3 ($P = 0.23$ and $P = 0.26$; Figure 11B). Instead, a treatment × time interaction ($P < 0.05$) was detected for the sum of n-3 in milk (Figure 8C), with greater concentrations observed at 6 h in STR and at 30 h in TAG cows relative to control. Total 18:1 tended to be higher for both STR and TAG cows compared to CON ($P = 0.11$ and $P = 0.74$; Figure 8D).

Figure 6. Temporal changes in plasma (A) 20:5 *c*5, *c*8, *c*11, *c*14, *c*17, (B) 22:5 *c*5, *c*8, *c*11, *c*14, *c*17, (C) 22:6 *c*4, *c*7, *c*10, *c*13, *c*16, *c*19, (D) 20:5 *c*5, *c*8, *c*11, *c*14, *c*17 + 22:6 *c*4, *c*7, *c*10, *c*13, *c*16, *c*19 and (E) total n-3 concentrations (% of total FA) in six mid-lactation Holstein dairy cows provided with a single esophageal bolus of fish oil encapsulated in triglyceride (TAG; closed grey circle) or starch (STR; closed black circle), or unsupplemented control (CON; open white circle). Total n-3 FA = 18:3n-3 + 18:4n-3 + 20:3n-3 + 20:4n-3 + 20:5n-3 + 22:3n-3 + 22:5n-3 + 22:6n-3 18:3 *c*9, *c*12, *c*15., + 18:4 *c*6, *c*9, *c*12, *c*15., + 20:3 *c*11, *c*14, *c*17., + 20:4 *c*8, *c*11, *c*14, *c*17., + 20:5 *c*5, *c*8, *c*11, *c*14, *c*17., 22:3 *c*13, *c*16, *c*19., + 22:5 *c*7, *c*10, *c*13, *c*16, *c*19., + 22:6 *c*4, *c*7, *c*10, *c*13, *c*16, *c*19. Data are presented as LSM ± SEM. Main effect of treatment; $P < 0.05$ denotes significance, $P < 0.15$ denotes tendency.

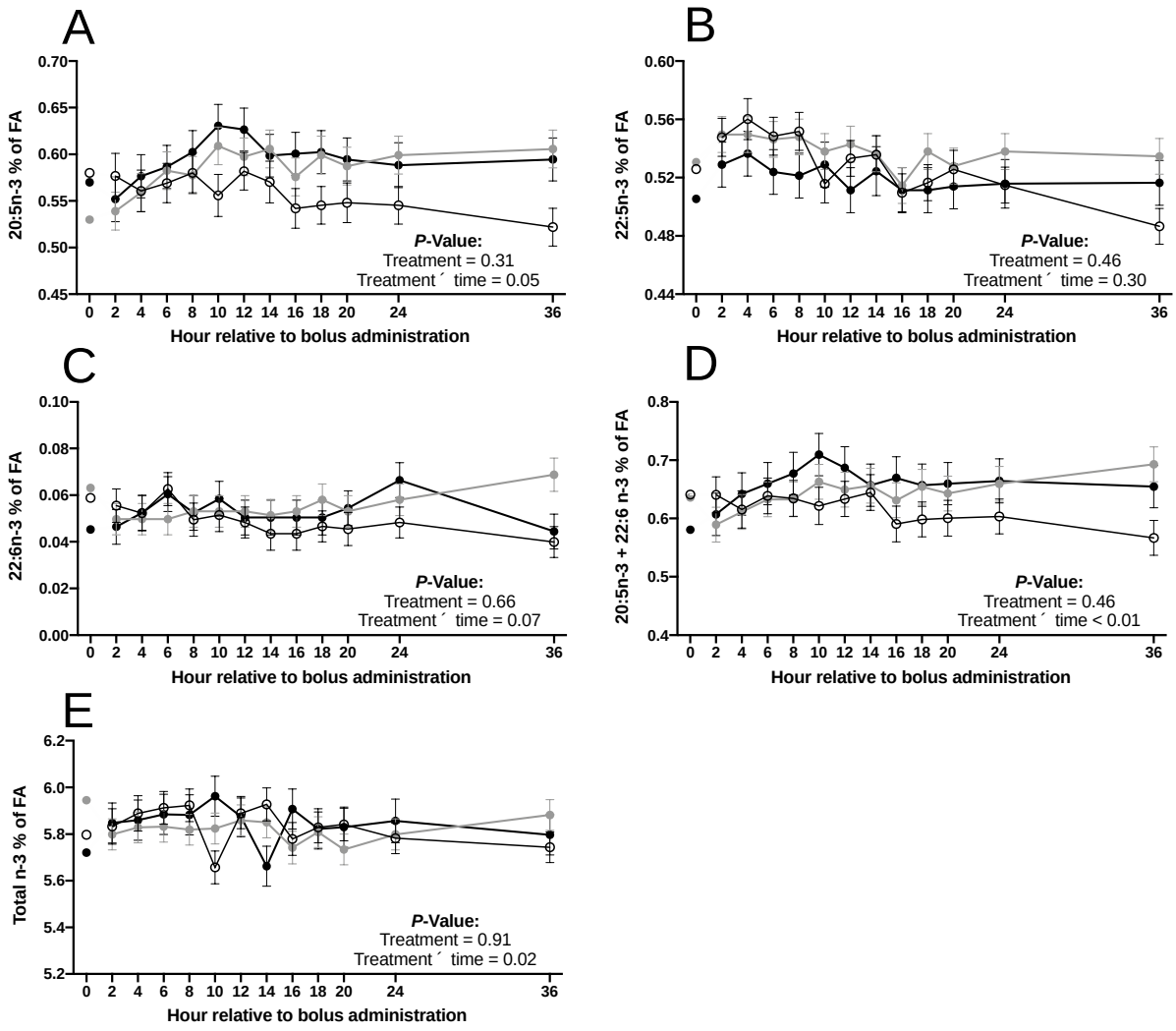


Figure 7. Temporal changes in plasma (A) total polyunsaturated LPC, (B) LPC-20:5 (C) LPC-22:5, (D) LPC-22:6, (E) PC-38:5, (F) PC-38:6, (G) PC-40:5, (H) PC-40:6 concentrations in six mid-lactation Holstein dairy cows provided with a single esophageal bolus of fish oil encapsulated in triglyceride (TAG; closed grey bar) or starch (STR; closed black bar), or unsupplemented control (CON; white bar). Total polyunsaturated LPC = LPC-18:2 + LPC-18:3 + LPC-20:3 + LPC-20:4 + LPC-20:5 + LPC-22:5 + LPC-22:6 + LPC-24:5. Data are presented as LSM ± SEM. Main effect of treatment; $P < 0.05$ denotes significance, $P > 0.05$ and ≤ 0.15 denotes tendency. *, denotes difference from control, $P < 0.05$; † denotes difference between TAG and STR, $P < 0.05$.

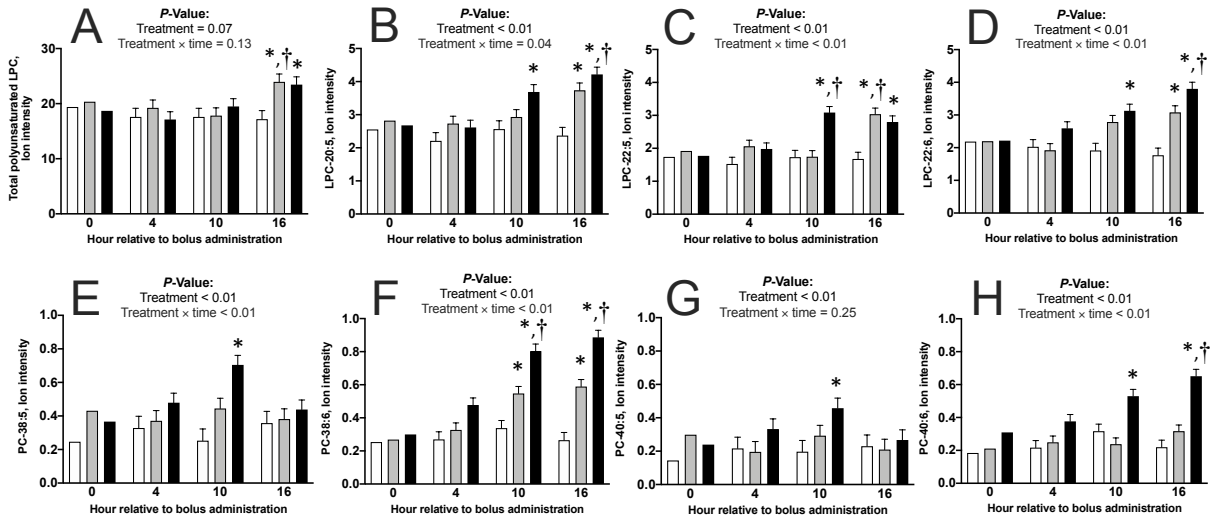
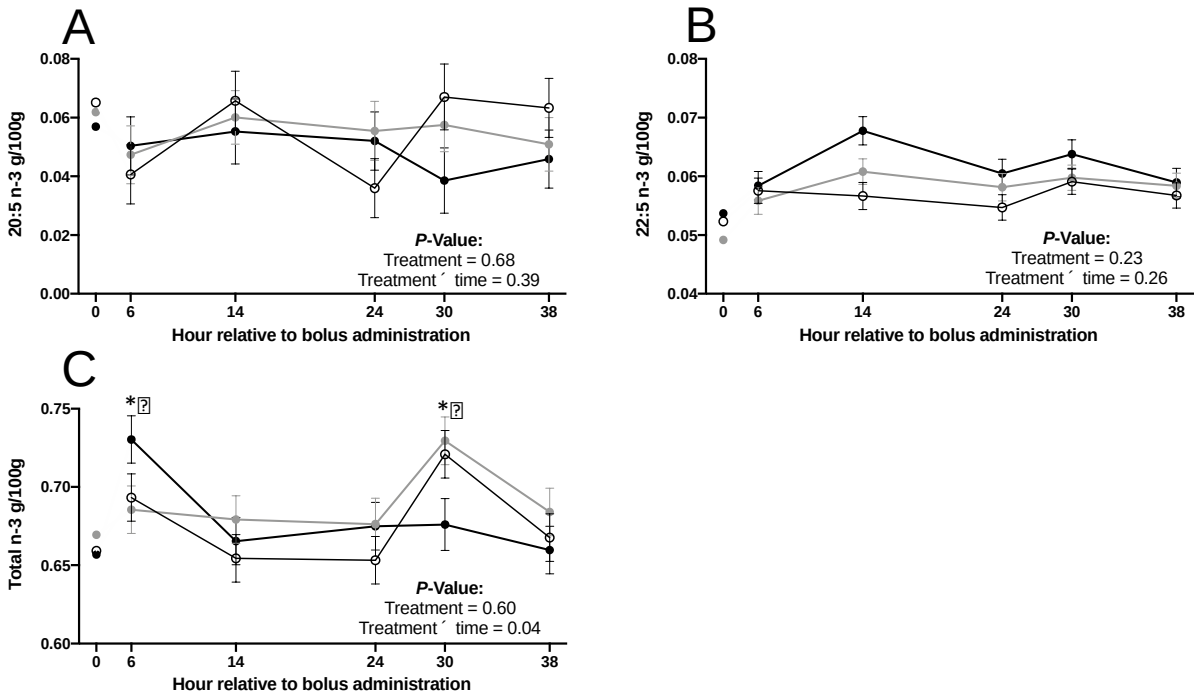


Figure 8. Temporal changes in milk (A) 20:5 *c*5, *c*8, *c*11, *c*14, *c*17, (B) 22:5 *c*7, *c*10, *c*13, *c*16, *c*19, (C) total n-3, and (D) total 18:1 (g/ 100g of FA) in six mid-lactation Holstein dairy cows provided with a single esophageal bolus of fish oil encapsulated in triglyceride (TAG; closed grey circle) or starch (STR; closed black circle), or unsupplemented control (CON; open white circle). Total n-3 = 20:3 *c*11, *c*14, *c*17, + 20:5 *c*5, *c*8, *c*11, *c*14, *c*17, + 22:5 *c*7, *c*10, *c*13, *c*16, *c*19. Data are presented as LSM ± SEM. Main effect of treatment; $P < 0.05$ denotes significance, $P > 0.05$ and ≤ 0.15 denotes tendency. *, denotes difference from control, $P < 0.05$; † denotes difference between TAG and STR, $P < 0.05$.



Discussion

Minimizing ruminal hydrogenation of dietary VLC n-3 FA to enhance their post-ruminal supply and absorption is a major challenge in dairy cow nutrition (Stamey Lanier and Corl, 2015). Commercially available calcium-salts of FA have been traditionally used to increase dietary energy density while minimizing the negative impacts of dietary PUFA on ruminal microorganisms, nutrient degradation, and DMI (Jenkins and Palmquist, 1984; Palmquist 2006; Maia et al., 2007). Unfortunately, their ability to protect essential n-3 PUFA from biohydrogenation appears to be negligible (Castañeda-Gutiérrez et al., 2007; Leduc et al., 2017), which has prompted the development of alternative technologies that may overcome this limitation. Palm oil triglyceride and modified starch fish oil supplements used in this study were effective in increasing the concentrations of phospholipids enriched with n-3 acyl chains. These outcomes suggest that the provided VLC n-3 FA were protected to some degree from microbial biohydrogenation in the rumen. Moreover, the incorporation of these FA into plasma indicates that the fish oil supplements provided post-ruminally bioavailable VLC n-3 PUFA, which in turn are expected to be readily absorbed in the small intestine (Tou et al., 2011; Boerman et al., 2015; Rico et al., 2021). Increases in some *cis* and *trans* 18:1 FA could indicate a degree of ruminal activity as fish oil is known to accumulate these FA in the rumen (Shingfield et al., 2003; Loor et al., 2005).

The tenuous response in circulating EPA and DHA, observed with a simultaneous robust increase in LPC containing these FA, and PC with 5 or 6 double bonds, is consistent with the idea that PUFA incorporation within phospholipids is preferred. Stamey et al. (2012) confirmed that feeding RP algal oil increased the incorporation of DHA in phospholipids but not in other fractions including triglycerides. We have also shown that the abomasal infusion of fish oil increases DHA enrichment in phospholipids in dairy cows (Myers et al., 2019; Rico et al., 2019). Such outcomes are deemed desirable when we consider that circulating

phospholipids containing PUFA like DHA and EPA are low in transition cows and a key feature of hepatic lipid deposition (Rico et al., 2021b). Increasing endogenous VLC n-3 FA supply is believed to enhance the synthesis of very low-density lipoproteins and aide in triglyceride secretion from liver (McFadden et al., 2020).

We conclude that fish oil protected by palm oil triglycerides or modified starch is an approach to increase circulating n-3 FA and phospholipids containing these FA. Future studies should define the bioavailability of n-3 FA in fish oil protected by triglycerides or starch, relative to each other but also calcium salts of fish oil. Additionally, studies are needed to characterize the effects of these feeding technologies on milk production, health, and reproduction in dairy cattle.

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CHAPTER 5. Temporal changes in plasma polyunsaturated phospholipids and choline metabolites concentrations in response to an esophageal bolus of rumen-protected fish oil in early-lactation Holstein dairy cows fed rumen-protected choline

Introduction

Postpartum hepatic steatosis or fatty liver disease (FLD) in dairy cattle remains a significant nutrition and management challenge, leading to decreased health, fertility, and lactation performance (Wensing et al., 1997; Bobe et al., 2004). Accumulated lipids, particularly triacylglycerol (TAG), may reduce gluconeogenesis and lead to inflammation (Rukkwamsuk et al., 1999; Sordillo et al., 2009). Very low-density lipoproteins (VLDL) are involved in the transportation of TAG from the liver to other tissues in the body. However, the dairy cow has a reduced capacity to export TAG within VLDL from liver, relative to non-ruminants (Pullen et al., 1990). Although not a measure of VLDL secretion, Davis et al. (2018) and Saed Samii et al. (2018a) confirmed dramatic reductions in circulating plasma TAG and TAG-rich lipoproteins in dairy cows transitioning from gestation to lactation. Cholesterol and phospholipids are essential structural components of VLDL (Puppione, 1978).

Phosphatidylcholine (PC) is the most abundant glycerophospholipid of VLDL surface monolayers. PC synthesis occurs through the cytidine diphosphate (**CDP**-choline) pathway (i.e., Kennedy pathway) and the phosphatidylethanolamine *N*-methyltransferase (**PEMT**) pathway (McFaddden et al., 2020). Feeding rumen-protected choline (RPC) is a very common dietary strategy in peripartum dairy cows (Arshad et al., 2020) as it to supports PC synthesis (Myers et al., 2019). The CDP-choline pathway utilizes choline as the key precursor while the PEMT pathway relies on the transmethylation cycle and the prerequisite methyl donor *S*-adenosylmethionine (SAM) to methylate phosphatidylethanolamine (PE; Figure 1). It is generally recognized that PEMT is a liver-specific enzyme (Vance and Ridgway, 1988). Choline can contribute to the methyl group pool through oxidization to betaine (Zeisel, 1990); betaine is then catalyzed by betaine-homocysteine *S*-methyltransferase (BHMT) and transfers one methyl group to homocysteine (HCY) to regenerate methionine by methionine synthase (MTR) and produce dimethylglycine (DMG); methionine is finally converted into SAM by

methionine adenosyltransferase (MAT; Figure 1). In primary bovine hepatocytes, choline supplementation upregulated the expression of genes involved in transmethylation (BHMT, MTR, methylenetetrahydrofolate reductase), indicating that choline supplementation was contributing to methionine remethylation (Chandler and White, 2017). As discussed by McFadden et al. (2020), methionine, betaine and folic acid are other methyl donnors supplemented to dairy cows because of their involvement in the one-carbon metabolism, especially methionine which has shown to be a preferable substrate for the PEMT pathway (Zhou et al., 2017 and 2018).

Although much research has gone into investigating the vital role of choline in PC, we must keep in mind the entire structure and the possible functional associations of the two fatty acyl chains found in their composition. Saed Samii et al. (2017, 2018) revealed that hepatic levels of highly unsaturated phosphatidylcholine (PC) are reduced in transition cows with high lipid content in the liver. In non-ruminants, evidence suggests that PEMT prefers PE enriched in very long chain (VLC) n-3 FA such as eicosapentaenoic acid (C20:5n-3; EPA) or docosahexaenoic acid (C22:6n-3; DHA). Instead, the CDP-choline pathway prefers diacylglycerol that contains saturated fatty acids and monounsaturated fatty acids, such as palmitic (C16:0; PA) and oleic acids (*cis*-9 c18:1; OA; DeLong et al., 1999). These FA are usually mobilized from adipose tissue in transition cows, leading to increased hepatic triglyceride deposits (Contreras et al., 2010). In lactating cows, Myers et al. (2019) abomasally infused algae oil rich in DHA and enhanced the incorporation of DHA and other polyunsaturated FA (PUFA) in PC, relative to cows infused PA. Circulating lysophophatidylcholines (LPC; a form derived from PC turnover) and PC containing PUFA like DHA and EPA are low in cows transitioning from gestation to lactation and a key feature of hepatic lipid deposition (Rico et al., 2021).

In our prior work, Rico et al. (2023; unpublished; Chapter 4), we fed an esophageal bolus of rumen-protected (RP) fish oil rich in EPA and DHA and observed increases in plasma concentrations of VLC n-3 FA, LPC containing n-3 FA and PC with 5 or 6 double bonds, confirming the idea that PUFA incorporation within the phospholipid fraction is preferred (Stamey et al., 2012). Fish oil also contains trimethylamine N-Oxide (TMAO; Lombardo et al., 2021), an osmolyte molecule associated with the progression of cardiovascular disease, nonalcoholic fatty liver disease, type 2 diabetes, and chronic kidney disease (Chen et al., 2016; Missailidis et al., 2016; Heianza et al., 2017; Tan et al., 2019; León-Mimila et al., 2021). In dairy cows, abomasal infusion of choline chloride, dietary supplementation of lecithin, which contains PC, or supplementation of RPC, increased plasma TMAO concentrations (Myers et al., 2019; Wang et al., 2021, and France et al., 2022). Choline can be degraded to trimethylamine (TMA) in the rumen or intestines by bacterial trimethylamine lyase; TMA is then absorbed and rapidly further oxidized in the liver to form TMAO (Neill et al., 1978 and Zeisel et al., 1983), and thus, limit dietary choline bioavailability. Although TMAO is a health concern in humans, the intravenous infusion of TMAO in early lactation dairy cows did not impair health (Myers et al., 2021). PUFA supply in dairy cows is limited because of rumen biohydrogenation, feeding fish oil in a rumen-protected form is a means to provide EPA and DHA post-ruminally. Therefore, co-supplementation of RP choline (RPC) and RP fish oil in transition cows may be a means to maximize PEMT activity, PC synthesis and thus support the secretion of TAG within VLDL. Our objective was to quantify changes in circulating polyunsaturated LPC and PC, and choline metabolites in response to an esophageal bolus of RP fish oil in lactating dairy cows supplemented with RPC.

Material and methods

At the Università Cattolica del Sacro Cuore Research Dairy Farm (Piacenza, Italy), eighteen early-lactation, multiparous, Holstein dairy cows (3.6 ± 2 lactations; 2.7 ± 0.3 BCS) were used in this study. Cows were part of a larger study that evaluated the effects of feeding RPC from -21 d prepartum to 35 d postpartum. In a complete randomized design, cows were allocated to 1 of 2 groups (n = 9/group); control (unsupplemented TMR; CON), or TMR supplemented with 60 g/d of RPC (lipid microencapsulated choline chloride [CC] 25%; Ruprocol®, Vetagro S.p.A., Reggio Emilia, Italy; CHOL). The RPC supplement was applied once per day and mixed into the TMR. Cows were kept in a common pen, had free access to water, and were individually fed using the Calan Broadbent feeding system (American Calan Inc., Northwood, NH). TMR was offered once daily at 0900 h, and 10% expected orts were collected individually and weighed daily. The diet was composed of corn silage and a mixed legume haylage according to the nutrient requirements of dairy cattle (NRC, 2001). The nutrient composition of the TMR included 32.2% aNDFom, 16.5% CP, 3.4% EE, and 6.7% ash. Cows were milked at 0600, and 1600 h and milk yield and components were recorded daily. Production and blood results from this study are reported elsewhere (Sáinz de la Maza-Escolà et al., 2022).

At d 27 ± 4 (mean ± SD) postpartum all cows were provided an esophageal bolus in a gelatin capsule containing 100 g of lipid microencapsulated fish oil (36% fish oil; Prototype 6, Vetagro S.p.A., Reggio Emilia, Italy). The fish oil prototype was manufactured by a patented microencapsulation technique, which protects nutrients and other active compounds from ruminal degradation yet releases them for absorption in the small intestine. This prototype was previously tested by Rico et al. (2023; unpublished; Chapter 4). The hydrogenated palm oil matrix used in this prototype is similar to the matrix used in other Vetagro products. The provision of the fish oil bolus delivered 10.2 g equivalent to the sum of EPA (6.8 %) and DHA

(4.4 %) and contained less than 1% of docosapentaenoic acid (22:5 n-3; DPA). Blood samples were collected in EDTA-containing tubes (Covidien, Mansfield, MA) at h 0, 10, and 24, relative to bolus administration via jugular venipuncture. For separation of EDTA-containing plasma, blood samples were placed on ice for 30 min followed by centrifugation at 3,400 × g for 15 min at 4°C. Plasma was stored at −20°C until analysis. Total mixed ration samples were analyzed for nutrient composition using standard procedures (AOAC International, 2012). Milk fat, protein and lactose of each cow was analyzed at each milking by an in-line automatic milk components analyzer (Afimilk Ltd. Kibbutz Afikim, Israel).

All plasma metabolites were measured using liquid chromatography tandem mass spectrometry (LC–MS/MS). Analyses were performed on a 1290 Infinity ultra-high-performance liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) coupled to a Q Trap 5500 linear ion trap triple quadrupole mass spectrometer (Sciex, Darmstadt, Germany) and equipped with an electrospray ionization (ESI) source in a positive mode. LPC and PC sample preparation was conducted according to the method developed by Dei Cas et al., 2020 with modifications. Briefly, plasma (10 µL) was diluted with water (90 µL) and added with a methanol/chloroform mixture (900 µL, 2:1, v/v) and internal standards (PC d7 and LPC d7 5 ug/mL in methanol, 10 µL). The samples were extracted for 10 min with an oscillator mixer. After centrifugation (15 min at 13400 rpm), an aliquot of the organic phase (20 µL) was withdrawn and evaporated under a stream of nitrogen. The residue was dissolved in 100 µL of methanol and injected (1 µL) in LC-MS/MS. Chromatographic separation was achieved on a reverse-phase Acquity® BEH C8 column (1.7 µm, 2.1 x 100 mm; Waters, MA, USA) equipped with pre-column using as mobile phases water + 0.2% formic acid + 2 mM ammonium formate (A) and methanol + 0.2% formic acid + 1 mM ammonium formate (B). The flow rate was 0.4 mL/min and the column temperature was set to 40 °C. The elution gradient (%B) was linear and set as follows: 0-1 min (70%), 1-14 min (70-99%), 14-17 min

(99%), 17-18 min (99-70%), held until 22 min. Plasma choline, methionine, betaine, DMG and TMAO were extracted according to the method developed by Steuer et al., 2016, with modifications. Briefly, plasma (10 µL) was diluted with water (90 µL) and added with acetonitrile (900 µL) and internal standards (choline-d9, methionine-d3, and TMAO-d9; 2 ug/mL in methanol; 10 µL). The samples were extracted for 10 min with an oscillator mixer. After centrifugation (10 min at 13400 rpm), an aliquot of the organic phase (25 µL) was diluted in 75 µL acetonitrile and injected (1 µl) in LC-MS/MS. Chromatographic separation was conducted in the instrument above-described equipped with pre-column using as mobile phases water + 0.1% formic acid (A) and acetonitrile 90% + 0.2% formic acid + 10 mM ammonium acetate (B). The flow rate was 0.35 mL/min and the column temperature was set to 40 °C. The elution gradient (%B) was linear and set as follows: 0-1 min (99%), 1-8 min (99-30%), 8-10 min (30%), 10-10.1 (30-99%), held until 3 min. All compounds were monitored in multiple reaction monitoring (MRM) mode. For quantitative measurements, the area of the signals of each compound was normalized to that corresponding deuterium isotopes used as internal standards in each analytical run.

Baseline plasma data were analyzed using a general mixed model including random effect of cow, and fixed effects of days in milk (DIM), parity, and treatment. All plasma timepoints data were analyzed as repeated measures over time relative to fish oil bolus administration under a mixed model including random effect of cow and fixed effects of DIM, parity, baseline measurement, and treatment, time and their interaction. The fixed effect of DIM and parity were tested but removed from the model because $P > 0.30$. Observations were deemed as outliers if Studentized residuals were > 3.0 or < −3.0. Tukey's post hoc test was used for multiple comparison correction of *P*-values for all pairwise comparisons of least squares means. Significance was declared as a $P \leq 0.05$. Tendencies were declared when $P > 0.05$ and

≤ 0.15. All statistics were performed using the statistical software JMP Pro v. 16.2 (SAS Institute Inc., Cary, NC).

Results

No differences ($P > 0.15$) were detected among treatments for dry matter intake (24.4 ± 2.1 kg/d; mean ± SD), milk yield (46.5 ± 5.8 kg/d), content and yields of milk fat (4.34 ± 0.3 % and 1.91 ± 0.26 kg/d), protein (3.25 ± 0.34 % and 1.44 ± 0.28 kg/d), and lactose (4.72 ± 0.29 % and 2.08 ± 0.35 kg/d) on the day following the bolus administration.

At h 0 (baseline; Table 5), plasma betaine concentrations were greater in CHOL, relative to CON ($P = 0.05$). Plasma DMG ($P = 0.13$) and PC-18:0/22:5 ($P = 0.10$) tended to be higher in CHOL compared to control group. However, choline, methionine, TMAO and the rest of PC and LPC concentrations were not modified by treatment before the administration of the fish oil bolus.

Plasma changes after single rumen-protected fish oil bolus administration are depicted in Table 6. No differences were detected in plasma choline and betaine (Figure 9). Methionine concentration tended to decrease in CHOL cows compared to CON (Treatment, $P = 0.07$; Figure 9). CHOL significantly increased DMG compared to CON (Treatment, $P = 0.03$; Figure 9) with time effect ($P = 0.03$) mostly observed at 10 h relative to bolus administration. Both CHOL and CON cows increased TMAO with the peak concentration at h 10 (Time, $P < 0.01$; Figure 9). We did not detect any significant effects for plasma LPC-22:5, LPC-22:6, and total LPC (Figure 10). However, CHOL increased LPC-20:5 compared to CON (Treatment, $P = 0.03$; Figure 10). We observed increases in plasma PC-16:0/20:5, PC-18:0/20:5, and total PC concentrations in CHOL cows compared CON ($P < 0.05$; Figure 11) with treatment × time interaction ($P = 0.06$, $P = 0.01$, and $P = 0.04$, respectively; Figure 11) observed at h 24 relative to bolus administration. CHOL had greater concentrations in plasma PC-16:0/22:6 compared to CON (Treatment, $P = 0.01$; Figure 11) with time effect ($P = 0.01$) observed at h 24 relative to bolus administration. Plasma PC-18:0/22:6 tended to be higher for CHOL compared to CON

(P = 0.07; Figure 11). No changes were detected in plasma PC-16:0/22:5 and PC-18:0/22:5 (Figure 11).

Table 5. Baseline plasma choline metabolites and polyunsaturated LPC and PC concentrations in early-lactation Holstein cows that were supplemented with rumen-protected choline (RPC) or not before the provision of a single esophageal bolus of encapsulated fish oil.

Metabolite, µg/mL	Treatment[1]			*P*-value[2]
	CON	CHOL	SEM	Treatment
Choline metabolites				
Choline	2.81	2.98	0.16	0.41
Methionine	6.11	6.85	0.35	0.16
Betaine	5.09	6.14	0.36	0.05
DMG[3]	3.12	3.80	0.32	0.13
TMAO[4]	7.57	8.99	1.81	0.57
Polyunsaturated LPC[5]				
LPC-20:5	0.24	0.26	0.02	0.49
LPC-22:5	0.68	0.55	0.10	0.31
LPC-22:6	0.07	0.09	0.01	0.41
Total LPC[6]	1.00	0.96	0.07	0.69
Polyunsaturated PC[7]				
PC-16:0/20:5	5.61	6.16	0.81	0.62
PC-18:0/20:5	8.19	6.13	1.27	0.24
PC-16:0/22:5	0.74	0.69	0.14	0.80
PC-18:0/22:5	3.22	3.85	0.25	0.10
PC-16:0/22:6	0.02	0.02	<0.01	0.39
PC-18:0/22:6	1.97	1.95	0.27	0.96
Total PC[8]	19.4	18.6	2.7	0.85

[1]Eighteen multiparous early-lactation cows were randomly assigned to 1 of 2 treatments at -21d prepartum: unsupplemented (CON. n = 9) or supplemented with 60g/d of RPC (lipid-microencapsulated choline chloride 25%; Ruprocol®, Vetagro S.p.A., Reggio Emilia. Italy; CHOL. n = 9) and provided with a single esophageal bolus of encapsulated fish oil (36% fish oil; Prototype 6. Vetagro S.p.A., Reggio Emilia. Italy) at d 27 ± 4 (mean ± SD) postpartum.
[2]Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.
[3]Dimethylglycine.
[4]Trimethylamine *N*-oxide.
[5]Lysophosphatidylcholine.
[6]Total LPC is the sum of LPC-20:5, LPC-22:5, and LPC-22:6.
[7]Phosphatidylcholine.
[8]Total PC is the sum of PC-16:0/20:5, PC-18:0/20:5, PC-16:0/22:5, PC-18:0/22:5, PC-16:0/22:6, and PC-18:0/22:6.

Table 6. Plasma choline metabolites and polyunsaturated LPC and PC concentrations in early-lactation Holstein cows that were supplemented with rumen-protected choline (RPC) or not and provided with a single esophageal bolus of encapsulated fish oil.

Metabolite. µg/mL	Treatment[1]			*P*-value[2]		
	CON	CHOL	SEM	Treatment	Time	Interaction
Choline metabolites						
Choline	2.50	2.60	0.09	0.45	0.40	0.76
Methionine	6.91	6.04	0.31	0.07	0.52	0.57
Betaine	5.42	5.62	0.49	0.80	0.25	0.66
DMG[3]	3.99	4.37	0.12	0.03	0.03	0.82
TMAO[4]	12.6	15.1	1.50	0.27	<0.01	0.98
Polyunsaturated LPC[5]						
LPC-20:5	0.26	0.31	0.01	0.03	0.76	0.29
LPC-22:5	0.66	0.65	0.02	0.78	0.71	0.80
LPC-22:6	0.10	0.09	0.01	0.57	0.43	0.66
Total LPC[6]	1.03	1.08	0.04	0.39	0.99	0.24
Polyunsaturated PC[7]						
PC-16:0/20:5	3.91	4.42	0.16	0.05	0.05	0.06
PC-18:0/20:5	6.70	7.50	0.24	0.03	0.15	0.01
PC-16:0/22:5	0.02	0.02	0.00	0.44	0.38	0.59
PC-18:0/22:5	7.80	8.02	0.28	0.59	0.63	0.16
PC-16:0/22:6	2.18	2.42	0.06	0.01	0.01	0.46
PC-18:0/22:6	0.80	0.88	0.03	0.07	0.13	0.36
Total PC[8]	21.6	23.6	0.65	0.04	0.16	0.04

[1]Eighteen multiparous early-lactation cows were randomly assigned to 1 of 2 treatments at -21d prepartum: unsupplemented (CON. n = 9) or supplemented with 60g/d of RPC (lipid-microencapsulated choline chloride 25%; Ruprocol®, Vetagro S.p.A., Reggio Emilia. Italy; CHOL. n = 9) and provided with a single esophageal bolus of encapsulated fish oil (36% fish oil; Prototype 6. Vetagro S.p.A., Reggio Emilia. Italy) at d 27 ± 4 (mean ± SD) postpartum.
[2]Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.
[3]Dimethylglycine.
[4]Trimethylamine *N*-oxide.
[5]Lysophosphatidylcholine.
[6]Total LPC is the sum of LPC-20:5, LPC-22:5, and LPC-22:6.
[7]Phosphatidylcholine.
[8]Total PC is the sum of PC-16:0/20:5, PC-18:0/20:5, PC-16:0/22:5, PC-18:0/22:5, PC-16:0/22:6, and PC-18:0/22:6.

Figure 9. Temporal changes in plasma (A) choline, (B) methionine, (C) betaine, (D) dimethylglycine (DMG), and (E) trimethylamine N-oxide (TMAO) concentrations (μg/mL) in eighteen early-lactation Holstein dairy cows supplemented with rumen-protected choline (CHOL; black bar) or unsupplemented (CON; white bar) and provided with a single esophageal bolus of encapsulated fish oil (36% fish oil; Prototype 6, Vetagro S.p.A., Reggio Emilia, Italy). Data are presented as LSM ± SEM. Different superscripts denotes significance ($P < 0.05$).

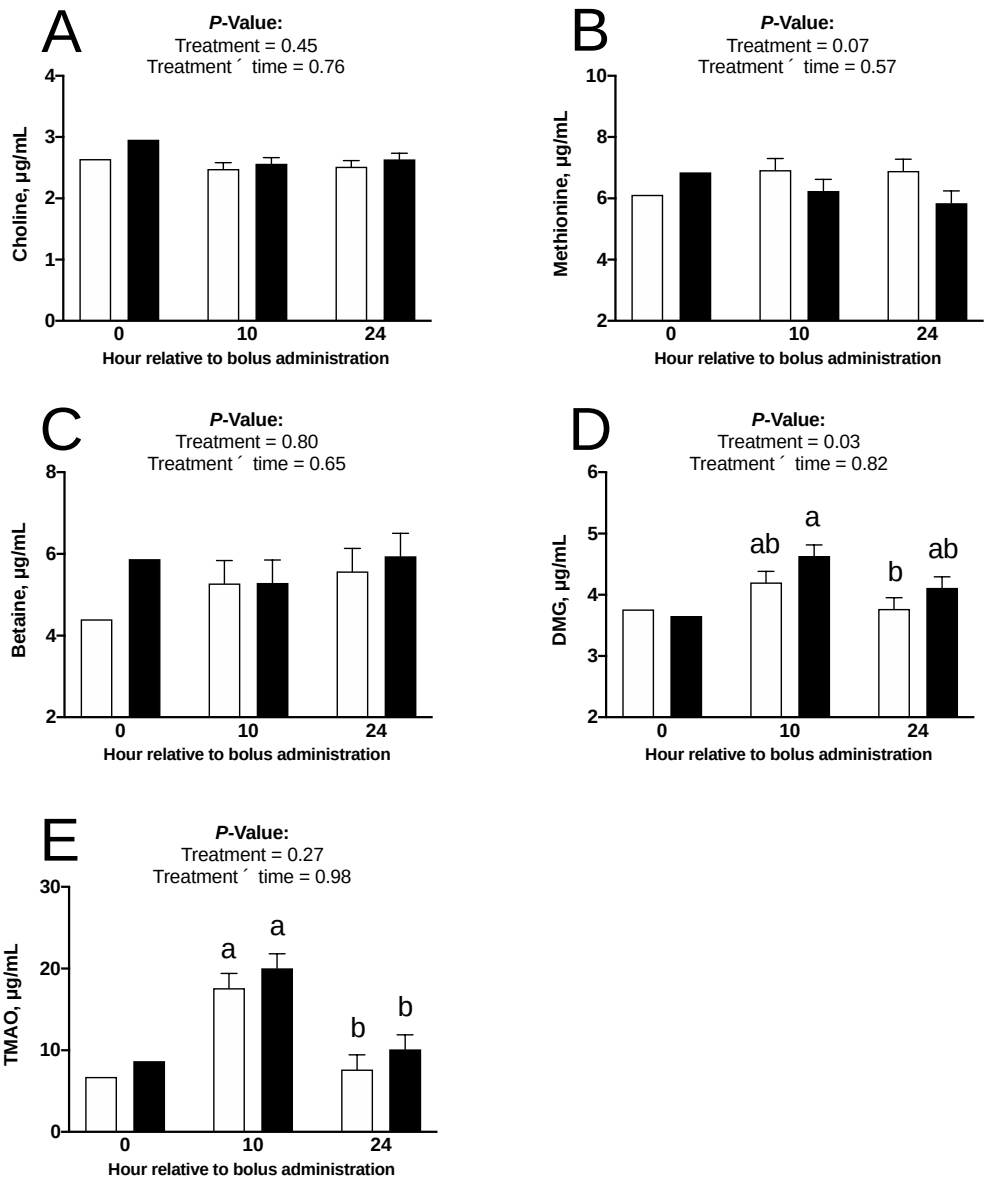


Figure 10. Temporal changes in plasma (A) LPC-20:5, (B) LPC-22:5, (C) LPC-22:6, (D) total polyunsaturated LPC concentrations (µg/mL) in eighteen early-lactation Holstein dairy cows supplemented with rumen-protected choline (CHOL; black bar) or unsupplemented (CON; white bar) and provided with a single esophageal bolus of encapsulated fish oil (36% fish oil; Prototype 6, Vetagro S.p.A., Reggio Emilia, Italy). Total polyunsaturated LPC = LPC-20:5 + LPC-22:5 + LPC-22:6. Data are presented as LSM ± SEM. Different superscript denotes significance ($P < 0.05$).

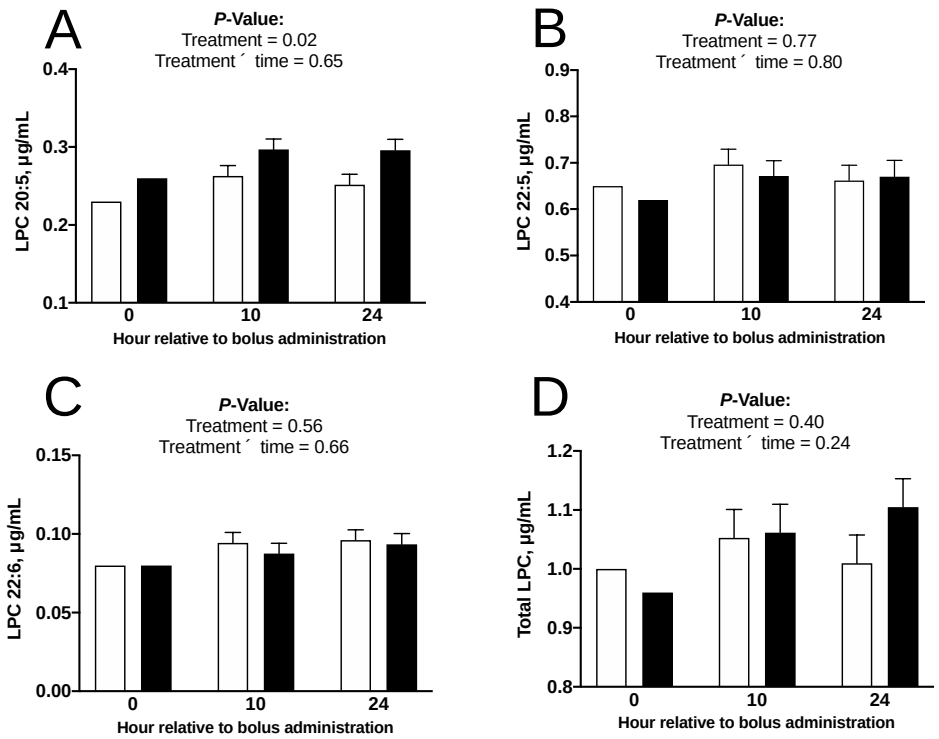
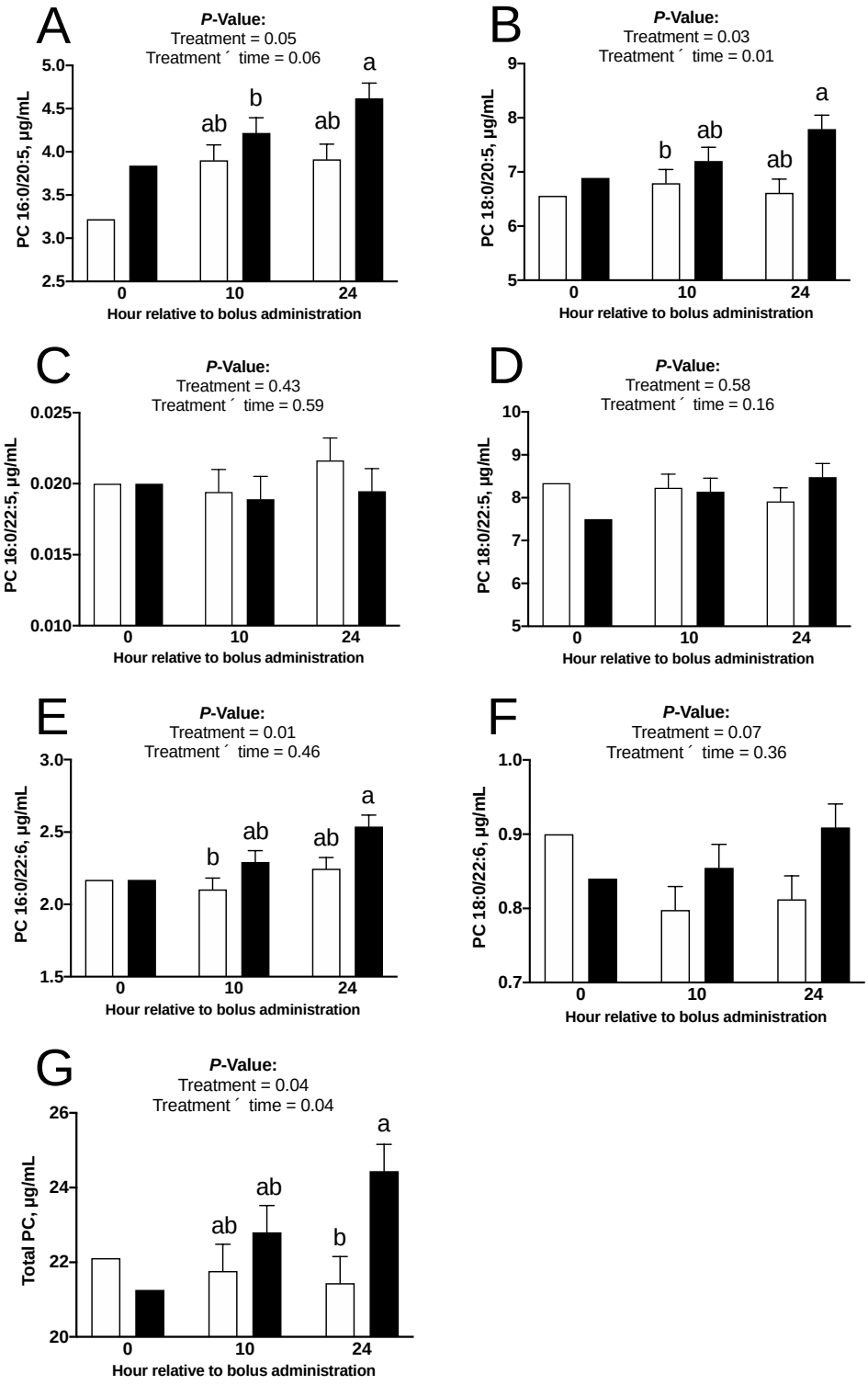


Figure 11. Temporal changes in plasma (A) PC 16:0/20:5, (B) PC 18:0/20:5, (C) PC 16:0/22:5, (D) PC 18:0/22:5, (E) PC 16:0/22:6, (F) PC 18:0/22:6, and (G) total polyunsaturated PC concentrations (μg/mL) in eighteen early-lactation Holstein dairy cows supplemented with rumen-protected choline (CHOL; black bar) or unsupplemented (CON; white bar) and provided with a single esophageal bolus of encapsulated fish oil (36% fish oil; Prototype 6, Vetagro S.p.A., Reggio Emilia, Italy). Total polyunsaturated PC = PC 16:0/20:5 + PC 18:0/20:5 + PC 16:0/22:5 + PC 18:0/22:5 + PC 16:0/22:6 + PC 18:0/22:6. Data are presented as LSM ± SEM. Different superscripts denotes significance ($P < 0.05$).



Discussion

The co-supplementation of dietary methyl donors and n-3 FA to promote hepatic PC synthesis is a great opportunity to enhance TAG export in postpartum dairy cows at risk for metabolic disease. Actual nutritional strategies do not consider FA nutrition for these purposes. Our data shows that the supplementation of RPC plus the provision of a RP fish oil bolus improves the synthesis of polyunsaturated PC in early lactation dairy cows. This combination also increased plasma concentration of DMG and tended to lower plasma methionine. Overall suggesting the activation of the transmethylation cycle to supply methyl groups to the PEMT pathway. These results provide evidence to consider FA nutrition to optimize PC synthesis in transition dairy cows.

Before the fish oil provision, RPC supplemented cows had greater plasma betaine concentrations, a common response when feeding RPC (de Veth et al., 2016). Regardless of whether cows were supplemented with RPC or not, fish oil increased circulating TMAO. Although this molecule is associated to cardiovascular diseases in humans (Tan et al. 2019; León-Mimila et al..2021), it does not impair health in early lactation dairy cows (Myers et al. 2021). Future studies should determine the ability of this feeding strategy to enhance hepatic TAG secretion within VLDL and to reduce fatty liver prevalence in dairy cattle.

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CHAPTER 6. Effects of feeding different microencapsulated sources of long and very-long chain unsaturated fatty acids on production performance and response to an immune challenge

Introduction

Fat supplementation is a common strategy to increase dietary energy density in dairy cows' diets (Rabiee et al., 2012). The effects of feeding fat may depend upon the fatty acid (FA) composition (Drackley et al., 2000). Fish and algae oil are common source of very-long-chain n-3 (VLC n-3) fatty acids (FA). Because of their anti-inflammatory and insulin-sensitizing effects (Patterson et al., 2012; Gingras et al., 2007; Capel et al., 2015), feeding these type of polyunsaturated fats in dairy cows can improve milk production, health, and reproductive performance (Mattos et al., 2000; Elis et al., 2016; Moallem, 2018). Olive oil is a source of unsaturated long-chain FA (mostly oleic acid; OA). Similarly to VLC-n3 FA, OA has also shown to have a modulatory effect in the prevention of cardiovascular diseases and inflammation in humans (Sales-Campos et al., 2016; Tsimidou and Papoti et al., 2010). In dairy cows, OA feeding improves FA digestibility as well as animal performance (de Souza et al., 2018). Current technologies that aim to prevent ruminal digestion of unsaturated FA are currently a limiting factor for the proper assessment of the effects of these FA in dairy cows (Castañeda-Gutiérrez et al., 2007). The objective of this study was to evaluate the effects of feeding microencapsulated fish oil, algae oil, and oleic acid on production performance and response to an immune challenge in Holstein dairy cows.

Material and methods

This study was designed to assess FA digestibility of VLC n-3 and OA microencapsulated products. Measurements of production variables and response to an immune challenge have been added afterwards; in which we have compared n-3 sources relative to each other (fish oil vs. algae oil) and relative to OA (n-3 vs. OA), considering OA as a positive control group only for this scenario. Digestibility methods and results of FA digestibility are not reported in this thesis.

All procedures were carried out in compliance with University of Maryland Institutional Animal Care and Use Committee (protocol # 1921622-1; College Park. MD). Nine mid-lactation multiparous Holstein dairy cows (mean ± SD: 82 ± 18 DIM; 3.7 ± 0.9 lactations) were acclimated to free stalls at Central Maryland Research and Education Center (Ellicott City. MD) for 5 d. Cows were then randomly assigned to treatments in a replicated 3 × 3 Latin Square design with 9 d experimental periods and a 7-d washout period was utilized to minimize carryover effects between experimental periods. Cows were kept in a common pen, had free access to water, and were individually fed using the Calan Broadbent feeding system (American Calan Inc. Northwood. NH). A total mix ration (TMR) composed of corn silage and alfalfa haylage was offered daily at 0830 h. The diet was formulated according to the nutrient requirements of dairy cattle (NRC. 2001). The nutrient composition of the TMR included 27.6% NDF, 45.3% NFC, 30.3% starch, 17.1% CP, 3.4% EE, and 7.5% ash. Daily amounts of TMR were adjusted to ensure a 10% feed refusal to achieve the *ad libitum* feed intake of the groups. Cows were milked at 0630, and 1530 h and milk yields were recorded daily. All products were provided by Vetagro S.p.A., Reggio Emilia, Italy and microencapsulated in a hydrogenated palm oil matrix similar to the matrix used in other Vetagro products. Treatments were top-dressed with the TMR and consisted of microencapsulated fish oil (FO; Prototype 6; 36% fish oil), algae oil (ALG; Prototype 39; 45% algae oil), and oleic acid (OA; Prototype 40; 20% oleic acid). The provision of the FO product delivered 25 g equivalent to the sum of EPA (9.4%) and DHA (5.9%). The ALG product delivered 25 g equivalent to the sum of EPA (0.2%) and DHA (23.2%).

The provision of the OA product delivered 40 g of oleic acid (*cis*9 18:1). We choose this dose based on the production and FA digestibility results of Prom et al. (2021) dose-response trial with abomasal infusions of oleic acid. The n-3 treatments (FO and ALG) received an extra dose of lipid matrix to match the same amount of fat provided by the OA product (226 g/d of total fat). Prototype 6 was previously tested by Rico et al. (2023; under review; Chapter 4).

Dry matter intake (DMI) and milk yields were recorded daily throughout the entire experiment. Body weights were recorded at d -1 and at d 9. Baseline measurements for DMI and milk yield were calculated as an average during the last 3 d of the acclimation/washout. Milk was collected at each milking during the last 2 d of acclimation/washout, during the last 3 d of the experimental period, and in the morning milking post-experiment.

At d 9 of the experimental period, all cows were subjected to an LPS challenge by intravenous infusion (30 ng/kg of BW of *Escherichia coli* O55:B5, Millipore Sigma) between 0700 and 0800 h. Lyophilized LPS was dissolved in sterile 0.9% NaCl. diluted to a stock solution of 1 mg/mL, and stored at −20°C. On the day of challenge, LPS stock solution was diluted at 200 μg/mL in sterile 0.9% NaCl to 0.3 μg/mL. At 0, 1, 2, 4, 6, 12 and 24 h relative to the start of LPS challenge, rectal temperatures and respiration rates were recorded. Rectal temperatures were measured using a large-animal digital rectal thermometer (model GLA M900; GLA Agricultural Electronics) and respiration rates were determined by counting flank movements for a 15-s period. then multiplying by 4 to obtain movements per minute.

Milk samples were analyzed for fat, true protein, and lactose concentrations using Fourier transform infrared spectroscopy and SCC by flow cytometry (Dairy Herd Improvement Association. Manheim. PA). Yields of 3.5% FCM, ECM, and milk components were calculated using milk yield and component concentrations for each milking, summed for a daily total, and averaged for each collection period. The efficiencies for milk yield, FCM (FCME), and ECM (ECME) were calculated

as the ratio of milk yield, FCM, or ECM in relation to DMI. Somatic cell score was calculated from SCC for statistical analysis using a logarithmic transformation: Log2 (SCC/100.000) + 3 (Ali and Shook. 1980).

Data related to DMI, milk yield, milk efficiency, rectal temperature, and respiration rates were analyzed as repeated measures over time relative to start of the experimental period or LPS challenge under a mixed model including random effect of cow and period, and fixed effects of days in milk (DIM), parity, baseline measurement, and treatment, time and their interaction. Cow was nested within treatment.

Data related to BW, milk components, 3.5% FCM and ECM efficiencies, and production responses to the LPS challenge were analyzed under a mixed model including random effect of cow and period, and fixed effects of days in milk (DIM), parity, baseline measurement. and treatment. The fixed effect of DIM and parity were tested but removed from the model when $P > 0.30$. Observations were deemed as outliers if Studentized residuals were > 3.0 or < −3.0. The least squares means comparisons were performed using preplanned nonorthogonal contrasts of interest (e.g., ALG vs. FO. ALG and FO [n-3] vs. OA) using unadjusted *P*-values. A T-test was used for multiple comparison correction of *P*-values for all pairwise comparisons of least squares means. Significance was declared as a $P \leq 0.05$. Tendencies were declared when $P > 0.05$ and ≤ 0.15. All statistics were performed using statistical software JMP Pro v. 16.2 (SAS Institute Inc., Cary. NC).

Results

Although 9 cows were initially enrolled in the experiment, during period 2, 1 cow (n = 1 for ALG) had to be partially removed from the study due to a mastitis infection during the first washout and was resolved in d 4. Data from this cow was removed from baseline measurements to d 3. Supplementation of OA tended to increase BW ($P = 0.14$; Table 7) compared to n-3, with a daily live weight variation of 1.86 kg ($P = 0.09$; Table 7). Although no treatment effect was detected for milk yield ($P = 0.66$), towards the end of the experimental period (d 7 and 8), ALG cows outperformed (Treatment × time, $P = 0.02$; Figure 12). Cows supplemented with OA tended to increase DMI ($P = 0.15$) and to lower feed efficiency ($P = 0.08$) compared to n-3, especially on the last day of experiment (Treatment × time, $P = 0.06$ and $P = 0.04$, respectively; Figure 12). FO supplementation tended to increase ECM ($P = 0.08$) compared to ALG. Incorporation of fat into milk (i.e., 3.5% FCM, milk fat content and yield) was significant or tended to be significantly superior in FO compared to ALG ($P < 0.02$) and in OA compared to n-3 cows ($P < 0.09$; Table 7). ALG cows decreased SCS compared to FO ($P < 0.01$) and OA ($P = 0.05$; Table 7). Lactose yield tended to increase in ALG compared to FO ($P < 0.11$; Table 7). No changes were detected in milk protein, MUN, and lactose content.

During the LPS challenge, rectal temperature in cows supplemented with ALG did not peak as high as in FO or OA (Treatment × time, $P < 0.01$; Figure 13A). When looking at the area under the curve (AUC) until h 6 relative to LPS challenge, rectal temperatures tended to be lower for ALG compared to FO ($P = 0.15$; Figure 13B), and for n-3 groups compared to OA ($P = 0.06$; Figure 13B). Similarly, respiration rates were lower in ALG compared to FO ($P = 0.03$; Figure 13C) or tended to have a smaller AUC ($P = 0.07$; Figure 13D). Groups supplemented with n-3 tended to have a lower respiration rate compared those with OA ($P = 0.08$; Figure 13C).

Production responses to the LPS challenge conducted at d 9 of the experiment can be found in Table 8. OA supplementation tended to suffer a greater reduction in milk yield compared to n-3 ($P < 0.12$) with no negative effects on milk components. Milk fat content was significantly higher for ALG treatment compared to FO ($P < 0.04$). No other changes were observed in milk components between ALG and FO. Cows supplemented with ALG tended to experience loss of appetite compared to FO (DMI; $P = 0.06$) and therefore, tended to be more efficient in terms of milk. ECM, and 3.5% FCM ($P < 0.11$).

Table 7. Production responses in lactating Holstein dairy cows supplemented with microencapsulated FA products containing n-3 or OA.

Variable	Treatment[1]				*P*-values[2]		
	ALG	FO	OA	SEM	Treatment	ALG vs. FO	n-3 vs. OA
Productive performance							
BW, kg	678	692	702	7.97	0.20	0.30	0.14
Live weight variation, kg/d	-1.61	-0.09	1.86	1.28	0.18	0.41	0.09
DMI, kg/d	26.7	26.8	27.3	0.74	0.34	0.89	0.15
Milk yield, kg/d	40.1	39.8	39.3	0.67	0.66	0.77	0.40
ECM, kg/d	41.5	43.5	43.3	1.64	0.16	0.08	0.34
3.5% FCM, kg/d	42.1	45.0	45.1	1.84	0.02	0.01	0.09
Milk composition, %							
Fat	3.86	4.15	4.35	0.09	<0.01	0.02	<0.01
Protein	3.09	3.15	3.16	0.04	0.57	0.41	0.50
Lactose	4.89	4.89	4.82	0.05	0.64	0.36	0.95
SCS	1.27	2.75	2.86	0.44	<0.01	<0.01	0.05
MUN, mg/dL	13.4	14.1	14.2	1.16	0.26	0.19	0.28
Milk solids, kg/d							
Fat	1.52	1.69	1.72	0.06	<0.01	<0.01	<0.01
Protein	1.22	1.24	1.23	0.06	0.87	0.60	0.97
Lactose	2.05	1.80	1.96	0.07	0.11	0.04	0.76
Feed efficiency ratio							
Milk[3]	1.52	1.47	1.45	0.04	0.15	0.38	0.08
ECM[4]	1.60	1.58	1.53	0.04	0.47	0.70	0.24
3.5% FCM[5]	1.63	1.62	1.57	0.04	0.58	0.88	0.30

[1]Nine multiparous and lactating Holstein cows were enrolled and randomly assigned to treatments in a 3 × 3 Latin square design with 8-d experimental periods: microencapsulated fish oil (FO; Prototype 6; 36% fish oil), algae oil (ALG; Prototype 39; 45% algae oil), and oleic acid (OA; Prototype 40; 20% oleic acid).
[2]Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.
[3]Milk efficiency was calculated as the ratio of milk yield and DMI.
[4]ECM efficiency was calculated as the ratio of ECM and DMI.
[5]3.5% FCM efficiency was calculated as the ratio of 3.5% FCM and DMI.

Figure 12. Production responses in lactating Holstein dairy cows supplemented with microencapsulated FA products containing n-3 or OA; (A) milk yield, (B) DMI, (8) milk efficiency, and (D) BW.

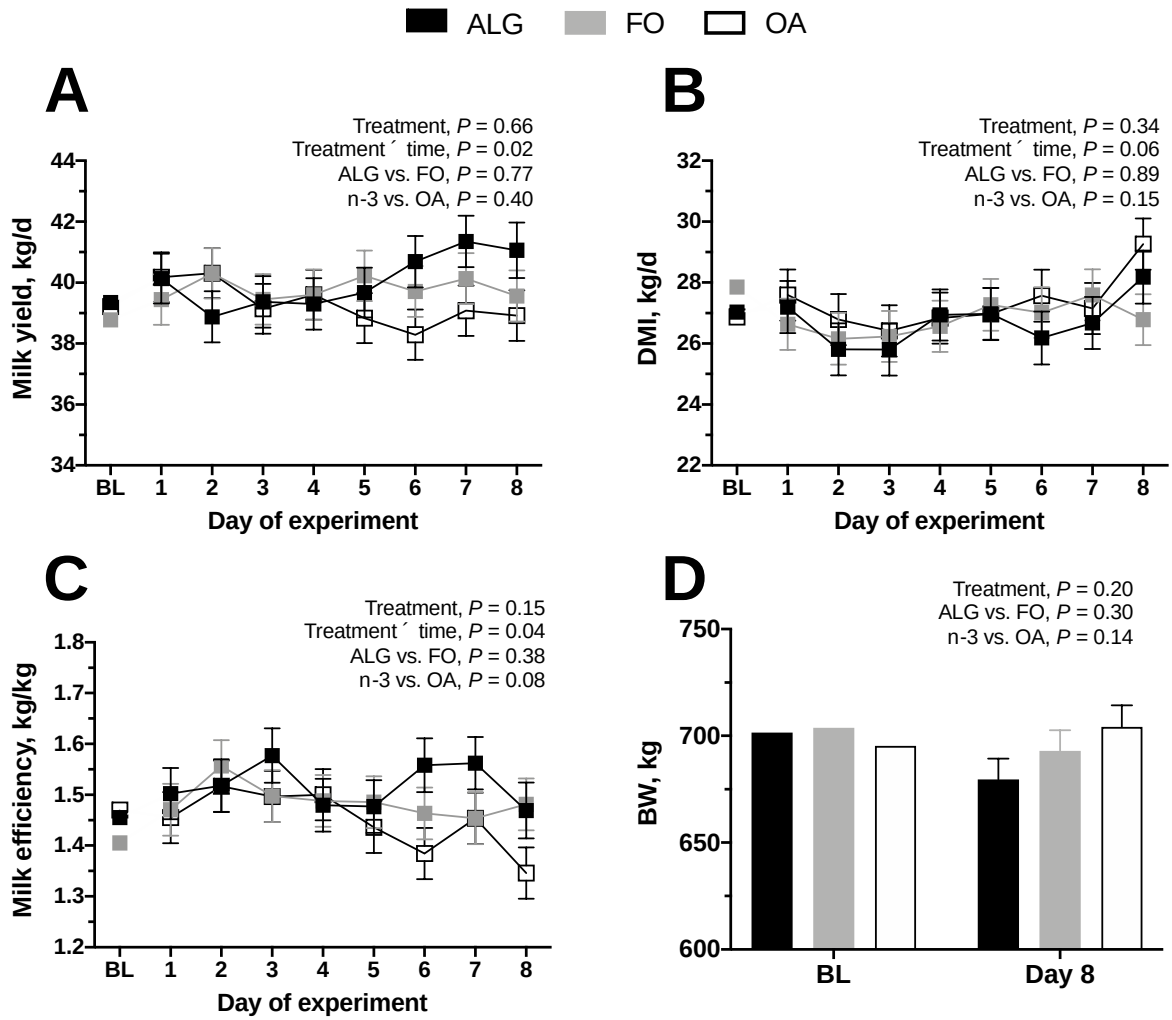

Figure 13. Rectal temperature and respiration rate in response to an LPS challenge in lactating Holstein dairy cows supplemented with microencapsulated FA products containing n-3 or OA; (A) rectal temperature, (B) rectal temperature; area under the curve (AUC) at h6, (8) respiration rate, and (D) respiration rate; AUC over 24 h.

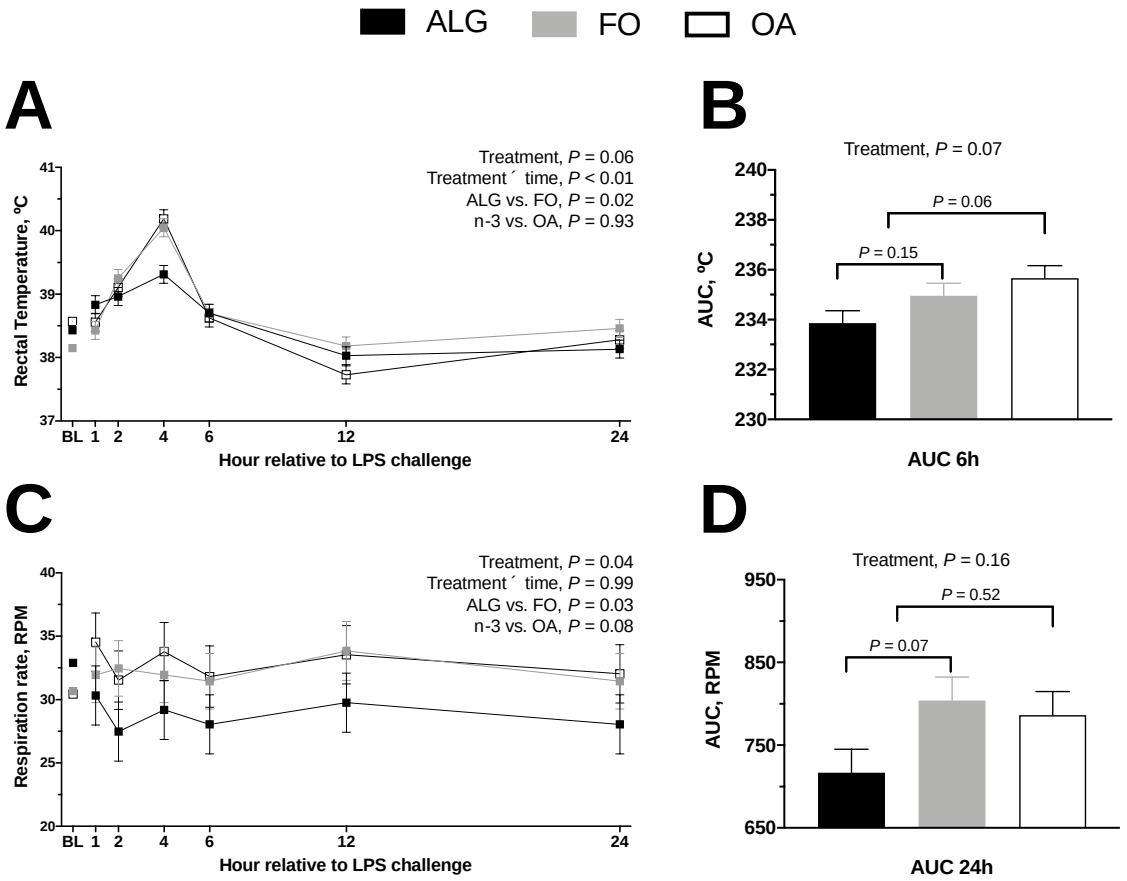

Table 8. Production responses to an LPS challenge in lactating Holstein dairy cows supplemented with microencapsulated FA products containing n-3 or OA.

Variable	Treatment[1]				*P*-values[2]		
	ALG	FO	OA	SEM	Treatment	ALG vs. FO	n-3 vs. OA
Productive performance							
Milk yield, kg/d	34.9	35.1	32.1	1.49	0.36	0.93	0.16
Milk yield reduction, %	-11.8	-11.4	-19.5	3.75	0.29	0.95	0.12
DMI, kg/d	26.3	24.0	24.3	1.14	0.14	0.06	0.38
DMI reduction, %	-7.96	-13.35	-13.0	5.56	0.42	0.24	0.53
ECM, kg/d	37.8	37.1	34.9	1.49	0.47	0.81	0.22
3.5% FCM, kg/d	38.7	37.8	35.7	1.53	0.48	0.73	0.23
Milk composition, %							
Fat	4.33	4.02	4.29	0.11	0.07	0.04	0.36
Protein	3.10	3.15	3.18	0.03	0.32	0.33	0.23
Lactose	4.77	4.78	4.78	0.02	0.90	0.69	0.83
SCS	1.52	1.38	1.37	0.26	0.92	0.74	0.82
MUN, mg/dL	15.3	15.2	15.6	0.30	0.64	0.86	0.39
Milk solids, kg/d							
Fat	1.38	1.41	1.36	0.06	0.86	0.75	0.68
Protein	1.04	1.11	1.02	0.05	0.40	0.33	0.37
Lactose	1.57	1.67	1.52	0.09	0.51	0.47	0.39
Feed efficiency							
Milk[3]	1.34	1.47	1.35	0.06	0.16	0.11	0.38
ECM[4]	1.44	1.55	1.46	0.10	0.16	0.09	0.45
3.5% FCM[5]	1.47	1.58	1.49	0.08	0.19	0.10	0.57

[1]Nine multiparous and lactating Holstein cows were enrolled and randomly assigned to treatments in a 3 × 3 Latin square design with 9-d experimental periods and challenged with LPS at d 9: microencapsulated fish oil (FO; Prototype 6; 36% fish oil), algae oil (ALG; Prototype 39; 45% algae oil), and oleic acid (OA; Prototype 40; 20% oleic acid).

[2]Statistical significance was considered when $P \leq 0.05$. Trend toward significance was considered when $0.05 < P \leq 0.15$.

[3]Milk efficiency was calculated as the ratio of milk yield and DMI.

[4]ECM efficiency was calculated as the ratio of ECM and DMI.

[5]3.5% FCM efficiency was calculated as the ratio of 3.5% FCM and DMI.

Discussion

Effects of feeding fat may depend upon the FA profile of the supplement (Drackley et al., 2000). Long chain FA are a common type of preformed FA (i.e., oleic acid), and a substrate for milk lipid synthesis in the mammary gland. If the mammary gland capacity is surpassed, these FA might be redirected to other tissues (i.e., adipose tissue; Glasser et al., 2008; Rico et al., 2017). In this study, OA supplementation has shown to be a means to increase milk fat and also increase BW, relative to n-3, suggesting that this source of FA can alter energy partitioning. Differences in production parameters between algae and fish oil are complex to be attributed to their FA profile since there is no evidence that DHA and EPA may have a different effect on milk and energy-corrected milk yield. However, the higher concentration of DHA in algae oil may explain why rectal temperatures were lower when cows were challenged with LPS. Weldon et al, (2007) observed that DHA is more effective than EPA in alleviating LPS-induced pro-inflammatory cytokine production in macrophages.

Upcoming analyses that will include FA digestibility, milk FA profile and, plasma inflammatory and metabolic markers in the LPS challenge that will provide more information to better understand the effects of these sources of FA on the immunometabolism of lactating dairy cows.

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CONCLUSIONS

1. Dairy cattle exposed to heat stress conditions develops with acute inflammation, oxidative stress and reduced methylation capacity.

2. In parallel with the improved N metabolism and reduced GIT permeability, heat-stressed cows supplemented with organic acids and pure botanicals had an enhanced hepatic methylation capacity and inflammatory and oxidative status.

3. Fish oil protected by palm oil triglycerides or modified starch is an approach to increase circulating n-3 fatty acids and phospholipids containing these fatty acids in lactating dairy cows.

4. The co-supplementation of dietary rumen-protected choline and fish oil containing n-3 fatty acids improves the synthesis of polyunsaturated phosphatidylcholines in early lactation dairy cows.

5. Feeding rumen-protected oleic acid modulates energy partitioning toward milk fat synthesis in lactating dairy cows.

6. Lactating dairy cows fed rumen-protected algae oil outperformed against an immune challenge compared to rumen-protected fish oil.