Longitudinal characterization of the metabolome of dairy cows transitioning from one lactation to the next: Investigations in blood serum

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ABSTRACT

The objective of this study was to characterize changes in the serum metabolome and various indicators of oxidative balance in dairy cows starting 2 wk before dry-off and continuing until wk 16 of lactation. Twelve Holstein dairy cows (body weight 745 ± 71 kg, body condition score 3.43 ± 0.66; mean ± SD) were housed in a tiestall barn from 10 wk before to 16 wk after parturition. Cows were dried off 6 wk before the expected calving date (mean dry period length = 42 d). From 8 wk before calving to 16 wk after calving, blood samples were taken weekly to study redox metabolism by determining antioxidant capacity, measured as the ferric-reducing ability of plasma, reactive oxidative metabolites, oxidative stress index, oxidative damage of lipids, measured as thiobarbituric acid reactive substances, and glutathione peroxidase activity. According to these results, dairy cows had the lowest serum antioxidant capacity and greater levels of oxidative stress during the dry-off period and the early postpartum period. For metabolomics, a subset of serum samples including wk −7 (before dry-off), −5 (after dry-off), −1, 1, 5, 10, and 15 relative to calving were used. A targeted metabolomics approach was performed using liquid chromatography and flow injection with electrospray ionization triple quadrupole mass spectrometry using the MxP Quant 500 kit (Biocrates Life Sciences AG). A total of 240 metabolites in serum were used in the final data analysis. Principal component analysis revealed a clear separation by days of sampling, indicating a remarkable shift in metabolic phenotype between the dry period and late and early lactation. Changes in many non-lipid metabolites associated with one-carbon metabolism, the tricarboxylic acid cycle, the urea cycle, and AA catabolism were observed in the study, with changes in AA serum concentrations likely related to factors such as energy and nitrogen balance, digestive efficiency, and changing diets. The study confirmed an extensive remodeling of the serum lipidome in peripartum dairy cows, highlighting the importance of changes in acylcarnitine (acylCN), phosphatidylcholines (PC), and triacylglycerols (TG), as they play a crucial role in lipid metabolism. Results showed that short-chain acylCN increased after dry-off and decreased thereafter, whereas lipid-derived acylCN increased around parturition, suggesting that more fatty acids could enter mitochondria. Phospholipids and sphingolipids in serum showed changes during lactation. In particular, concentrations of sphingomyelins, PC, and lysoPC decreased around calving but increased in mid- and late lactation. In contrast, concentrations of TG remained consistently low after parturition. The serum concentrations of bile acids fluctuated during the dry period and lactation, with glycocholic acid, cholic acid, glycodeoxycholic acid, and taurocholic acid showing the greatest concentrations. These changes are likely due to the interplay of diet, liver function, and the ability of the gut microbiota to convert primary to secondary bile acids. Overall, these descriptive results may aid in hypothesis generation and in the design and interpretation of future metabolite-based studies in dairy cows. Furthermore, they contribute to our understanding of the physiological ranges in serum metabolites relative to the lactation cycle of the dairy cow. Key words: blood, metabolomics, dry-off, lactation stages

INTRODUCTION

It is well established that dairy cows undergo complex metabolic adaptations during the transition from late gestation to early lactation, requiring a change in nutrient supply to meet the demands of calving and the onset of milk production (Drackley et al., 2005; Pascottini
et al., 2020). Much less is known about the metabolic changes of the transition from late lactation into the dry period. However, it is becoming increasingly clear that this transition is as important and challenging as calving (Daniel et al., 2022). The dry period typically comprises 6 to 8 wk and implies a regenerative involution with the removal of senescent mammary epithelial cells and renewal of mammary cell progenitors to maximize milk production in the ensuing lactation (Kuhn et al., 2005; Kok et al., 2021). At that time, energy and protein are repartitioned from the mammary gland to the fetus; after calving, the mammary gland regains priority over other maternal tissues that undergo further adaptations to support lactation (Bauman and Currie, 1980; Drackley, 1999). In particular, adipose tissue and skeletal muscle are mobilized to meet the elevated energy and AA demands. Both lipolysis and muscle protein breakdown are processes that are amplified by the insufficient feed intake and concomitant increasing milk yield in early lactation (Drackley, 1999). Lipolysis leads to increased concentrations of fatty acids (FA) in the blood, which may exceed the oxidative capacity of the liver and thus lead to fatty liver and ketogenesis (Adewuyi et al., 2005; Overton et al., 2017). Moreover, increased circulating FA and ketone body concentrations may alter the inflammatory response of transition cows (Bernabucci et al., 2005; Sordillo and Raphael, 2013). Increased FA also increases the production of reactive oxygen species during β-oxidation (Schönfeld and Wojtczak, 2008).

Metabolomic approaches enable comprehensive investigations of the physiological response associated with the metabolic challenges of early lactation, including inflammation and oxidative stress, and may also be the starting point for identifying biomarkers associated with disease (Kenéz et al., 2016; Zhang et al., 2017a; Derno et al., 2019). Previous studies have examined the blood metabolome of dairy cows during the transition from late pregnancy to lactation using both targeted (Humer et al., 2016; Kenéz et al., 2016; Yang et al., 2020) and untargeted (Sun et al., 2014; Luo et al., 2019; Caixeta and Omontese, 2021) mass spectrometry-based metabolomics. Although these studies included samples collected during the dry period, the metabolic changes associated with the process of drying-off itself have generally been insufficiently studied, and the corresponding metabolomes remain undescribed.

Therefore, the objective of the present study was to extend the observation period to 8 wk before expected calving, thus comprising dry-off at wk 6 antepartum and continuing sampling until wk 16 postpartum. Furthermore, the targeted metabolomic analyses were extended to capture a broader range of metabolites, approximately twice as many as in previous studies. The study also evaluated indicators of oxidative status and lactoferrin (Lf), an inflammatory marker, to investigate possible relationships between metabolic profiles, oxidative status, and inflammation.

**MATERIALS AND METHODS**

This study was conducted between September 6, 2019, and April 22, 2020, at the Dairy Research Facility of Trouw Nutrition Research & Development (Kempenhof, Boxmeer, the Netherlands). All procedures described in this article strictly followed the Dutch Law on Experimental Animals, which is aligned with the corresponding European Directive ETS123 (Council of Europe 1985 and the 86/609/EEC Directive) and were approved by the Central Authority for Scientific Procedures on Animals (CCD, the Netherlands). Other outcomes from this study, such as DMI, milk production, total-tract nutrient digestibility, energy balance, and N balance, have been reported earlier by Daniel et al. (2022), and trace element balances were also reported by Daniel et al. (2023).

**Animals, Diets, and Sampling**

Twelve Holstein Friesian cows took part in this experiment (Figure 1). At the beginning of the study, 6 cows were in their first lactation and 6 cows were between their second up to their fifth lactation (3.2 ± 1.2; mean ± SD). Cows were moved from a freestall to a tiestall with individual feed troughs and constant access to water at approximately 10 wk before expected calving. After 2 wk of adaptation to the housing and diet, sampling started. Cows were dried off 6 wk before expected calving, by abruptly stopping milking, and all cows were treated with a teat sealant (OrbeSeal, Zoetis). At dry-off the diet was changed from lactation to a dry cow diet (the ingredients and chemical composition analysis of the experimental diets shown in Supplemental Table S1; https://doi.org/10.6084/m9.figshare.23896431.v1; Ghaffari, 2023). In brief, the TMR (DM basis) for lactating cows contained 41.5% grass silage, 29.1% corn silage, and 29.4% mash compound feed; the TMR for dry cows contained 50.4% corn silage, 34.3% barley straw, and 15.3% mash compound feed. The animals received the TMR ad libitum. Daily TMR intake was calculated by subtracting the leftover TMR (each day between 0930 and 1000 h) from the TMR offered the previous day. Fresh TMR was immediately weighed and allocated to individual feed bins after the refusals were removed.

Blood samples were collected as reported previously (Daniel et al., 2022). From wk 8 before calving to wk 16 after calving, blood samples were taken weekly (Figure
1) each Monday at 1200 h from the tail vein in 9-mL tubes containing a clot activator (Vacutest Serum red cap, Vacutest Kima). The tubes were centrifuged at 1,500 × g for 10 min at 20°C. The serum aliquots were then stored in 1.5 mL cryotubes at −80°C.

**Indicators of Oxidative Status and Measurement of Lactoferrin**

Reactive oxidative metabolites (ROM) were measured using the derivatives of ROM (dROM) test according to Alberti et al. (2000) with modifications (Regenhard et al., 2014) using N,N-diethyl-p-phenylenediamine as chromogenic substrate. The dROM values are expressed as micrograms of H₂O₂ equivalents per liter. The mean intra-assay coefficient of variation (CV) was 4.5%, and the interassay CV was 8.5%. The antioxidant capacity was measured as the ferric-reducing ability of plasma (FRAP) according to Benzie and Strain (1996) with minor modifications; concentrations are given as micromoles of Fe³⁺ per liter. The mean intra-assay CV was 3.5%, and the mean interassay CV was 3.7%. An oxidative stress index (OSI) was calculated as follows: dROM/FRAP × 100 (Urh et al., 2019). Oxidative damage of lipids was assessed as thiobarbituric acid reactive substances (TBARS) using malondialdehyde as standard (Gutteridge and Quinlan, 1983); the concentrations obtained in the samples are given as nanomolar malondialdehyde. The activity of the antioxidant defense enzyme glutathione peroxidase (GSH-Px) was measured as described by Paglia and Valentine (1967); the activity units were related to the total protein content, assessed via the Bradford assay (Bradford, 1976), and are given as units (U) per gram of protein (1 U catalyzes the oxidation of 1.0 μmOL of reduced glutathione to oxidized glutathione per minute at pH 7.0 at 25°C). The mean intra-assay CV was 4.9%, and the mean interassay CV was 6.8%. The Lf concentration (μg/mL) was measured with an in-house-developed ELISA as described by Schmitz et al. (2004); the mean intra-assay CV was 2.0%, and the mean interassay CV was 5.0%.

**Metabolomics Analysis and Data Processing**

The MxP Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria) was used to determine metabolite concentrations as per the manufacturer’s protocol.
Serum concentrations of metabolites were calculated in the data table, and metabolites with more than 5% LOD were treated as missing values, blanks were left calculated by each laboratory as LOD. Values below analyzed immediately before running the samples was the procedure, 3 times a signal in the blank extracted and per metabolite. Based on Biocrates standard operating procedure, the median values of all PBS samples on the plate were used to calculate the limits of detection (LOD). Blank PBS samples (3 replicates) on 96 wells were used for metabolite profile analysis. The inserts were spotted with internal standards, and a predefined number of samples was added to the inserts. A phenyl isothiocyanate solution was then added to derivatize some analytes (e.g., AA). After derivatization, the target analytes were extracted with an organic solvent and then diluted.

Data were quantified using Sciex Analyst MS software (Analyst 1.7.2 Build 8287) and imported into Biocrates MetIDQTM software (Oxygen-DB110-3023) for analyte identification, calculation of concentrations, and compilation of data. For the LC-ESI-MS/MS assay, metabolites were quantified by stable isotope dilution and 7-point calibration curves. For the FI-ESI-MS assay, metabolite concentrations were calculated using a 1-point calibration of the internal standard and are also isotope-corrected. Metabolites were quantified according to the manufacturer’s protocol using MetIDQ Boron software (version 5-4-8-DB100-Boron-2607; Biocrates Life Sciences) for targeted metabolomic data processing and management. Blank PBS samples (3 replicates) were used to calculate the limits of detection (LOD). The median values of all PBS samples on the plate were calculated as a proxy for the background noise per metabolite. Based on Biocrates standard operating procedure, 3 times a signal in the blank extracted and analyzed immediately before running the samples was calculated by each laboratory as LOD. Values below LOD were treated as missing values, blanks were left in the data table, and metabolites with more than 5% missing values were excluded from the data analysis. Serum concentrations of metabolites were calculated in micromoles per liter and normalized for internal quality control samples. The MxP Quant 500 kit allows quantitative analysis of various metabolites belonging to 12 biochemical classes of lipids and another 14 classes of small molecules (Figure 1).

Statistical Analysis

The sample size for this study, which includes 12 cows, was selected considering the logistical and resource constraints of our tiestall facility and with the goal of closely monitoring individual nutrient balance during key physiological phases. No standard method currently exists for estimating sample size in metabolic phenotyping, as it relies on effect size estimation. The latter is not accessible in holistic hypothesis-free approaches. The sample size we used for the current cohort, studied as a group over time, allowed for a detailed analysis of nutrient balance but may have limited the statistical power of our metabolomics study.

Multivariate Analyses

Statistical analysis of the serum metabolite data was performed using the web-based metabolomic data processing tool MetaboAnalyst 5.0 (Pang et al., 2021; see http://www.metaboanalyst.ca for detailed methodology). To hedge the integrity of the collected data, the .csv file was carefully checked in advance to ensure that the data matrix consisted of numeric units only and that standard formatting protocols were followed. As an integral part of the data filtering process, variables were subjected to an interquantile range assessment to eliminate outliers and provide a refined data set for further analysis. To ensure the accuracy and reliability of the statistical analysis performed, a strict criterion was implemented to exclude all variables with more than 5% missing values (Pang et al., 2021). To address issues related to heteroskedasticity and skewness, the data were subjected to a generalized log-transformation (base 10) along with Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). The data were then processed using the MetaboAnalyst 5.0 tool, which allowed us to perform a variety of multivariate data analyses including adequate consideration of the repeated design. These analyses included principal component analysis (PCA), random forest classification, informative heat map generation, and hierarchical clustering (Pang et al., 2021). Random forest classification, a powerful machine learning algorithm in our analysis, created an ensemble of 500 decision trees using 7 key predictors. This methodology, combined with bootstrapping and random selection of predictors, helped us identify the top 15 metabolites.
in different classes (AA, acylcarnitine [acyICN], phospholipids, an so on) that showed remarkable changes during the sampling period.

Univariate Analysis

Descriptive statistics and correlations between serum metabolites, using Spearman’s rank method, were conducted using the statistical software JASP version 0.14 (JASP Team, 2019). Changes in serum metabolite concentrations and blood oxidative parameters were examined with linear mixed-effects models that included repeated measures and ANOVA. In this model structure, individual cows were treated as a random effect and sampling time as a fixed effect. This analysis was facilitated by the Statsmodels library (version 0.12.2; https://www.statsmodels.org) in Python (version 3.9.5; https://www.python.org). Before proceeding with model fitting, we used the libraries SciPy (version 1.6.3; https://www.scipy.org) and Matplotlib (version 3.4.2; https://matplotlib.org) in Python to check the assumptions of normality and homoscedasticity. In cases where these assumptions were not met, the NumPy library (version 1.21.2; https://numpy.org/doc/1.25/release/1.21.2-notes.html) was used for the necessary data transformations, including logarithmic transformations to ensure a more symmetric and homoscedastic distribution. After transformation, specific models were fitted for each metabolite, followed by derivation of $P$-values. These $P$-values were used to evaluate the significance of the temporal variations in metabolite concentrations. Given the large number of assays performed, the resulting $P$-values were adjusted using the Benjamini-Hochberg procedure to control for false discovery rate (Benjamini and Hochberg, 1995). The threshold of significance was set at $P \leq 0.05$; trends were declared at $0.05 < P < 0.10$.

RESULTS

Oxidative Status

Mean serum dROM concentrations ranged from 1.03 to 1.88 μg H$_2$O$_2$-equivalents/L and increased immediately after dry-off and gradually increased after calving, with peak values observed after dry-off (wk 5–6 antepartum) and after calving (wk 2–6 postpartum; Figure 2A). The range of average FRAP values was between 205 and 295 μM Fe$^{2+}$/L, with a decrease after dry-off toward a nadir 1 wk before calving, followed by increasing values during lactation (Figure 2B). The OSI (dROM/FRAP) results indicate high oxidative stress during the first 3 wk of the dry period and a further increase toward calving. These elevated levels were maintained until wk 8 postpartum, followed by lower values (Figure 2C). The mean serum GSH-Px activity ranged from 0.17 to 0.27 U/mL, with higher activity observed in wk 10 to 14 of lactation than in the dry period (Figure 2D). Mean TBARS concentrations ranged from 183 to 315 nM and decreased after dry-off, with a nadir 1 wk before parturition, but increased from wk 1 to wk 7 after parturition and remained at this level in the following week with greater fluctuations and variation than before (Figure 2E). Mean concentrations of Lf ranged from 0.35 to 1.13 μg/mL, increased after dry-off, and then decreased over time from peak values at wk 4 antepartum to lowest values at wk 6 to 14 postpartum (Figure 2F).

Serum Metabolic Profiling

The score plots obtained via PCA showed a distinct separation of data based on sampling days (Figure 3) and the lactation cycle. The first principal component explained 45.9% of the total variation, and the first 2 principal components together explained 63.4% of the total variation. In chronological order, the PCA values of the different collection days showed a clear shift in serum metabolites from wk −7 to 15 relative to calving.

Descriptive statistics, encompassing measures such as the mean, standard deviation, minimum, and maximum for metabolites in the serum of dairy cows are presented in Supplemental Table S2 (https://doi.org/10.6084/m9.figshare.23896431.v1; Ghaffari, 2023), and their corresponding abbreviations are provided in Supplemental Table S3 (https://doi.org/10.6084/m9 .figshare.23896431.v1; Ghaffari, 2023). Among a total of 240 metabolites, hexoses including glucose, lactic acid, cholesteryl esters (CE 18:2 and CE 18:3), Gly, Gln, Ala, Val, phosphatidylcholine (PC) aa C36:2, PC aa C34:2, Arg, Ile, Leu, hippuric acid, and p-cresol sulfate (p-cresol-SO$_4$) were the most abundant metabolites in serum (Supplemental Figure S1; https://doi .org/10.6084/m9.figshare.23896431.v1; Ghaffari, 2023). Supplemental Figure S2 (https://doi.org/10.6084/m9 .figshare.23896431.v1; Ghaffari, 2023) illustrates longitudinal changes in the serum concentrations of glucose and lactic acid in dairy cows.

The serum metabolites of the different classes of compounds (AA, AA-related, biogenic amines, and others) that are not lipids are presented as heat maps (Figure 4) to show how they changed during the dry period and lactation. The effect of sampling time was significant for all AA except for Ala, Ile, Leu, Val, Phe, and Tyr, as shown in Figure 4A. For Arg ($P = 0.09$), Gln ($P = 0.09$), Pro ($P = 0.09$), and Thr ($P = 0.07$; Figure 4A), we noted a trend toward significance of the effects of sampling time. Among the AA shown in Figure 4A,
Arg, Asp, His, Gln, Met, Glu, Cys, and Lys had greater concentrations during the dry period than in lactation, whereas Gly, Ser, Asn, Thr, Trp, and Pro had lesser concentrations during the dry period than at mid-lactation (wk 10 and 15), or at the end of the previous lactation. According to the random forest analysis, Gly, Glu, Cys, and Asn were the most changed AA during the sampling period (Figure 4B). Serum Cys concentrations increased after dry-off and decreased after calving and did not reach the levels observed during dry period by the end of the observation period. Serum Gly showed a decrease before parturition, followed by an increase after calving and a subsequent decrease. The serum concentrations of Ala decreased gradually from wk 7 antepartum to wk 5 postpartum but increased significantly at wk 10 and 15 postpartum.

Figure 4C shows the heat map of AA-related metabolites, biogenic amines, and further small metabolites from other non-lipid compound classes such as carboxylic acids, cresols, indoles, and choline. The effect of sampling time was significant for all metabolites in this heat map (Figure 4C) except for α-aminoadipic acid, anserine, trans-4-hydroxyproline, taurine, p-cresol-SO₄, 3-indoleacetic acid, and indoxyl sulfate. For Orn ($P = 0.08$) and phenyl-acetyl-glycine ($P = 0.06$), we detected a trend toward significance of the
effects of sampling time. The heat map identified 3 distinct clusters. The first cluster showed lower serum concentrations of aconitic acid, 3-indole-propionic acid, carnosine, \( \beta \)-aminobutyric acid, 5-amino valeric acid, \( \alpha \)-aminobutyric acid, proline betaine, citrulline, \( \beta \)-alanine (\( \beta \)-Ala), trimethylamin N-oxide (TMAO), kynurenine, and methionine sulfoxide during the dry period. Conversely, higher serum concentrations of these metabolites were observed during lactation at wk +5, +10, and +15 relative to calving. In the second cluster, the serum concentrations of phenyl-acetyl-glycine, choline, Orn, serotonin, p-cresol-SO\(_4\), and Cys were greater in the dry period and lesser in lactation, especially in the first week after calving. In the last cluster, lower serum concentrations of asymmetric dimethylarginine (ADMA), homoarginine, homocysteine (HCys), betaine, sarcosine, 1-methylhistidine (1-Met-His), 3-methylhistidine, creatinine, and symmetrical dimethylarginine (SDMA) were observed in mid- and late lactation. The most changed metabolites in these compound classes during the sampling period, as determined by random forest analysis, were homoarginine, 5-amino valeric acid, 1-Met-His, Cys, ADMA, and \( \beta \)-aminobutyric acid (Figure 4D).

The acylCN results are shown as a heat map in Figure 5A, which reveals 2 main clusters. It is noteworthy that some of the short- and medium-chain acylCN had greater concentrations before dry-off and decreased during early and mid-lactation. The effect of sampling time was significant for all acylCN (Figure 5A) except for propionylpropenoylcarnitine (C3:1), hexenoylcarnitine (C6:1), decanoylcarnitine (C10), \( \text{trans} \)-2-decenoylcarnitine (C10:1), myristoylcarnitine (C14), tetradecenoylcarnitine (C14:1), palmitoylcarnitine (C16:0), and 3-hydroxyhexadecadinooylcarnitine (C16:2-OH). We observed a trend (\( P = 0.08 \)) toward significance in the effects of sampling time for tetradecadinooylcarnitine (C14:2). The most changed acylCN were carnitine (C0), tiglylcarnitine (C5:1), octadecenoyl-carnitine (C18:1), and octadecanoylcarnitine (C18:0; Figure 5B), as revealed by random forest analysis.

Figure 6A shows the changes in the PC concentration over time, with most PC showing lower concentrations around calving (wk −1 and +1) and higher concentrations before dry-off and in mid- and late lactation. The effect of sampling time was significant for all PC (Figure 6A) except for lysoPC a (C17:0 and C20:3), PC aa (C34:4, C36:6, C38:1, C38:3, C38:4, C40:2, C42:1,
Figure 4. Heatmap and major metabolites that changed during the sampling period as a result of random forest classification for serum amino acids (A, B) and biogenic amines and other small molecules (C, D). As the heatmap shows, the colors indicate the concentration of serum metabolites (mean centered by the range of each variable). α-AAA = α-aminoadipic acid, AABA = α-aminobutyric acid, t4-OH-Pro = trans-4-hydroxyproline, Ac-Orn = acetylornithine, ADMA = asymmetric dimethylarginine, Met-SO = methionine sulfoxide, Orn = ornithine, 1-Met-His = 1-methylhistidine, 5-AVA = 5-aminovaleic acid, 3-Met-His = 3-methylhistidine, BABA = β-aminobutyric acid, PAG = phenylacetylglucine, Cit = citrulline, PheAlaBetaine = phenylalanine betaine, ProBetaine = proline betaine, DOPA = dihydroxyphenylalanine, SDMA = symmetric dimethylarginine, HArg = homoarginine, HCys = homocysteine, TrpBetaine = tryptophan betaine, β-Ala = β-alanine, GABA = γ-aminobutyric acid, PEA = phenylethylamine, AconAcid = aconitic acid, HipAcid = hippuric acid, p-Cresol-SO4 = p-cresol sulfate, TMAO = trimethylamine N-oxide, 3-IAA = 3-indoleacetic acid, Ind-SO4 = indoxyl sulfate, 3-IPA = 3-indole-propionic acid. Asterisks denote significant variations due to sampling time (*P ≤ 0.05), hashtag symbols indicate a trend toward significance (#P < 0.10), and ns represents nonsignificant results.
C42:4, C42:6), and PC ae (C30:2, C34:0, C36:0, C36:1, C36:2, C38:1, C38:2, C38:3, C38:4, C38:5, C38:6, C40:3, C40:4, C40:5). We observed a trend toward significance in the effects of sampling time for PC: lysoPC a C20:4 (P = 0.06), PC aa C32:0 (P = 0.07), PC aa C38:5 (P = 0.09), PC ae C40:6 (P = 0.06), PC ae C32:2 (P = 0.06), and PC ae C40:2 (P = 0.07). According to random forest analysis, the most changed PC during the sampling period were PC aa C34:3, PC aa C40:3, lysoPC a C14:0, PC aa C42:5, and PC aa C40:4 (Figure 6B).

As shown in Figure 7A, significant alterations were observed in ceramides (CER), sphingomyelins (SM), CE, and triacylglycerols (TG) during both the dry period and lactation. However, specific species, including CE (14:0, 15:0, 16:0, 16:1, 17:0, 17:1, 18:2, 18:3, 18:0, and 20:5), SM-hydroxy (OH; C14:1, C16:1, C22:2, and C24:1), SM (C16:1, C24:1, and C26:0), and TG (18:0_38:7), did not exhibit significant alterations. Nevertheless, we found a trend (P = 0.06) for certain PC species, namely SM (C16:0, C18:0, and C18:1) and TG (20:0_34:1), in response to sampling time. Based on random forest analysis (Figure 7B), TG (C16:0_35:1, C14:0_36:1, and C18:0_32:1), SM (C24:0), and CE (C18:3) were the most altered metabolites in this class.

In the current study, 13 of 14 detectable bile acids were found, with glycocholic acid (GCA) and cholic acid (CA) being the most abundant (39% and 23%, respectively). Glycodeoxycholic acid (GDCA), taurocho-
lic acid (TCA), and taurodeoxycholic acid (TDCA) were also detected (13%, 11%, and 6%, respectively), whereas the other 8 bile acids had concentrations ≤3% (Figure 8A). Figure 8B shows a simplified diagram of bile acid metabolism in the liver and intestine. Bile acids are divided into 2 main clusters with different subclusters, as shown by the hierarchical cluster analysis in Figure 8C. The first cluster contains mainly taurine-conjugated bile acids such as TCA, taurochenodeoxycholic acid, TDCA, tauroliothocholic acid, and tauromurocholic acid, which increased during the dry period and decreased after calving (Figure 8D). Two Gly-conjugated bile acids (glycolithocholic acid and glycolithocholic acid sulfate) were also contained in this cluster, but the initial increase after dry-off was followed by a further decrease toward calving. The other main cluster contained primary (CA), and secondary (deoxycholic acid; DCA) bile acids with greater concentrations during early to mid-lactation as well as GCA, GDCA, glycochenodeoxycholic acid, and glycoursoxycholic acid with peak concentrations 1 wk after calving (Figure 8D). Notably, the sampling time was
significant only for CA, DCA, glycochenodeoxycholic acid, glycolithocholic acid sulfate, and taurolithocholic acid (Figure 8D).

DISCUSSION

Feed intake, milk yield, whole-tract nutrient digestibility, energy balance, and N balance from cows in this study were reported in a previous publication (Daniel et al., 2022). The current publication focused on determining the blood oxidative status and serum metabolome using a targeted metabolomic approach to better understand the metabolic and oxidative adaptations that occur along the lactation cycle. This study uniquely covers multiple phases, including dry-off, calving, and early to late lactation. To our knowledge, this fills a gap in the literature by providing a comprehensive analysis of multiple metabolites.

Blood Oxidative Status

Transition cows undergo different degrees of oxidative stress due to imbalance between antioxidants and pro-oxidants (Miller et al., 1993; Bernabucci et al., 2005; Sordillo and Aitken, 2009). This study found that during the dry period, FRAP levels decreased while
dROM levels increased, resulting in higher OSi levels. This increase was likely related not only to the ceased output of antioxidants via milk but also to the lesser supply of antioxidants such as vitamin E in the dry cow diet as compared with the lactation diet. During lactation, levels of FRAP increased and peaked around 10 wk, whereas dROM and OSi levels decreased. The loss of antioxidants via milk was compensated by the greater supply with the lactation diet. The increase in dROM after dry-off might also be related to the involvement and remodeling processes in the mammary gland for which increased mRNA abundance of genes related to oxidative stress (GPX1 and SOD2) has been demonstrated (Piantoni et al., 2010). However, the activity of GSH-Px in serum in the present study remained rather stable during the transition from lactation to dry-off and increased slightly after calving to decrease again after the third week of lactation. The most abundant blood lipids are CE (Varman and Schultz, 1968; Moore and Christie, 1979). In this study, CE (specifically, CE 18:2 and 18:3) were ranked as the third and fourth most abundant metabolites overall and were identified as the most abundant lipids. Oxidized CE have been found to account for between 11% and 92% of the CE-PUFA pool in human atherosclerotic plaques (Ravandi et al., 2014). Considering the decreased CE concentrations during the dry period (Varman and Schultz, 1968), it is possible that the decrease in TBARS levels during the dry period could be attributable to a reduction in lipid substrate availability for oxidation, rather than a decrease in oxidative capacity at this time. However, it is important to note that further research may be needed to fully understand the relationship between TBARS and CE.
Figure 8 (Continued). (A) Pie chart illustrating the distribution of serum bile acids identified in this metabolomics analysis. (B) Schematic representation of the enterohepatic circulation of bile acids. (C) Hierarchical clustering of oxidative parameters, and (D) changes of primary and secondary bile acids during the experimental period (n = 12 cows). Data are presented as means ± SEM. CA = cholic acid, GCA = glycocholic acid, TCDCA = taurochenodeoxycholic acid, TLCA = tauroliothocholic acid, TMCA = tauromurocholic acid, GLCA = glycolithocholic acid, GLCAS = glycolithocholic acid sulfate, GCDCA = glycodeoxycholic acid, GUDCA = glycoursodeoxycholic acid, CDCA = chenodeoxycholic acid, DCA = deoxycholic acid, LCA = lithocholic acid, UDCA = ursodeoxycholic acid, TDCA = taurodeoxycholic acid, TCA = taurocholic acid, GDCA = glycodeoxycholic acid.
In the present study, the serum concentration of Lf was found to increase after dry-off and decrease toward calving without increasing further. Lactoferrin is a glycoprotein that binds iron and has bacteriostatic, antioxidant, and anti-inflammatory properties (Yang et al., 2000; Zhang et al., 2021). It is considered a specific marker of neutrophil turnover (Birgens, 1985) and is a major component of biologically important mucosal fluids and neutrophil granules (Alexander et al., 2012). The observed increase in serum Lf after dry-off is very likely a response to mammary gland involution, during which Lf and other milk components are released into the remaining secretions of the mammary gland (Welty et al., 1976; Nickerson, 1989; Stelwagen et al., 2009). Alternatively, the increase in Lf could be related to the adaptive responses of the gastrointestinal tract to a change in diet, as Lf expression has been demonstrated in the intestine of mice and increased during *Escherichia coli* infection (Liang et al., 2020). The extent to which local release of Lf into the intestinal lumen affects circulating concentrations is not well understood, as studies have been largely limited to severe clinical inflammatory bowel disease. To our knowledge, no previous studies have documented changes in circulating Lf from the dry-off period to mid-lactation. The present results highlight the complexity of oxidative stress during the dry period and early lactation in dairy cows and show the different patterns of the various indicators of oxidative status in the transition from one lactation to the subsequent one.

**Serum Metabolite Profiling**

In this study, a targeted metabolomics approach was used to investigate changes in AA, biogenic amines, and metabolic intermediates in dairy cows during the transition from dry period to lactation. Metabolites associated with several metabolic pathways, including the tricarboxylic acid cycle, the urea cycle, lipid metabolism, and the degradation of AA and FA were identified and quantified. Metabolic profiles of cows in established and late lactation at wk 5, 10, and 15 and during the dry period (wk −5 and −1) overlapped, whereas cows in early lactation (wk +1) had a separate cluster due to changes in metabolic patterns at calving, as shown by PCA analysis. This pattern change is consistent with the findings of Kenéz et al. (2016) in their study of longitudinal changes in blood metabolome profile, except for dry-off, which was not described in their study. The complex interplay of multiple organ-level and interorgan mechanisms complicates the interpretation of the blood metabolome profile (Schwab and Broderick, 2017; Ghaffari et al., 2019; Martineau et al., 2019).

**Small Non-Lipid Metabolites**

In this metabolomics study, small non-lipid metabolites were analyzed, including alkaloids, amino oxides, AA, AA-related compounds, bile acids, biogenic amines, cresols, and indoles and their derivatives. Of the 226 serum metabolites affected by time, hexoses (mainly glucose) and lactic acid were the most abundant. The non-lipid metabolites were mostly associated with one-carbon metabolism, the tricarboxylic acid cycle, the urea cycle, and AA degradation.

The metabolomics profiling of dairy cows in this study unveiled changes in AA, biogenic amines, and small molecules concentrations during the dry period and lactation. The AA status in the blood relies on several factors, including intestine uptake, muscle protein turnover, hepatic AA uptake, metabolic rate, and milk protein synthesis (Maeda et al., 2012; Samman et al., 2014; Sadri et al., 2023). The liver extracts high amounts of His, Met, Phe, and Trp but low amounts of Lys and branched-chain AA (BCAA; Hanigan, 2005; Lapierre et al., 2005). Uptake of EAA by the mammary gland is crucial for milk protein production (Nichols et al., 2022). The current study investigated the concentrations of group 1 EAA (His, Met, Trp, and Phe + Tyr) and group 2 EAA (BCAA [Ile, Leu, and Val], Arg, Lys, and Thr) in dairy cows during the dry period and lactation. The transfer of group 1 EAA into milk occurs in a 1:1 ratio, whereas group 2 EAA show excessive uptake in the mammary gland compared with their incorporation into milk protein (Mepham, 1982; Lapierre et al., 2012). Excess group 2 EAA are metabolized intramammally for nonessential AA synthesis and intermediates of glycolysis and the tricarboxylic acid cycle (Bequette et al., 1996; Mabjeesh et al., 2000; Lapierre et al., 2009). The results of this study showed that certain AA, including His and Met in group 1 and Val, Arg, and Lys in group 2, had higher concentrations during the dry period but lower levels during lactation. In contrast, other AA in group 1, such as Trp, Phe, and Tyr, and group 2, including Leu, Ile, and Thr, displayed lower concentrations during the dry period but higher levels during lactation. The dynamic and tightly regulated nature of AA metabolism in the body, coupled with differences in the specific roles and functions of individual amino acids, may explain why not all AA within each group show the same trend in dairy cows during the dry period and lactation.

Furthermore, this study investigated changes in serum metabolites associated with the folate and Met cycle of one-carbon metabolism, including Met, HCys, betaine, choline, and Ser, during the dry period and lactation. One-carbon metabolism consists of several
metabolic pathways, which are essential for cellular functions and provide one-carbon units (methyl groups) for the methylation of DNA, creatine, phospholipids, polyamines, and AA homeostasis (Clare et al., 2019; McFadden et al., 2020). Dairy cows require more Met during the transition from pregnancy to lactation to support fetal growth in late pregnancy and milk protein synthesis early in lactation, but their supply may be lower than the need due to inadequate DMI (McFadden et al., 2020). In the current study, the greater serum concentrations of Met during the dry period compared with the lactation also suggest greater Met demand during lactation. Lactating dairy cows tend to have a negative balance of methyl groups due to the high excretion of methylated compounds in milk and the breakdown of dietary methyl donors in the rumen, which can be alleviated by supplying rumen-protected Met during the first 2 wk after parturition (Pinotti et al., 2002; Dalbach et al., 2011). In the current study, the greater concentrations of Ser during the early lactation compared with the dry period also suggest potentially greater metabolic activity via the trans-sulfuration pathway (Kalhan and Marczewski, 2012). The sulfur of HCys derived from the Met cycle can be transferred to Ser by the action of cystathionine-β-synthase to form cystathionine and is further converted to Cys, which is the only way to generate Cys in vivo (Shbodio et al., 2019). The transition from pregnancy to lactation can lead to an inadequate supply of methyl donors in dairy cows, which can contribute to oxidative stress and inflammation (McFadden et al., 2020). This inadequate supply may also be related to the important intermediate of the one-carbon cycle HCys, which has been shown to increase in response to oxidative stress (widner et al., 2002). Notably, our study revealed higher levels of HCys during the dry period than during the lactation period. Thus, further research is needed to determine the necessary methyl donor requirements in lactating dairy cows and to address potential deficiencies.

In this study, serum concentrations of Glu, Gln, His, Arg, ADMA, SDMA, and Orn were higher during the dry period and lower during lactation. Glutamine, a derivative of Glu, serves as the major fuel for rapidly replicating tissues such as the gastrointestinal tract and immune system, and comprises 24 to 35% of bovine casein, along with Glu-derived Pro (Windmueller and Spaeth, 1974; Lacey and Wilmore, 1990; Newsholme and Parry-Billings, 1990; Creamer, 2003). Glutamine has been suggested to limit milk protein synthesis, especially in early lactation when the energy balance is negative (Meijer et al., 1995). Based on our results, the levels of Gln in the blood showed a trend toward significance concerning the sampling time. We suggest that the decrease in serum Gln levels in the cows studied may not be due solely to their energy status. This is supported by the fact that all cows achieved positive energy balance within 11 wk of calving despite the observed decline in Gln levels. The decrease in serum Gln concentration could be due to the increased immune system requirement or intestinal demand resulting from the increased DMI in our study. Glutamine is known to be an important fuel source for intestinal tissue (Windmueller and Spaeth, 1974) and a precursor for nucleotides during periods of increased cell turnover (Lacey and Wilmore, 1990).

In the current study, Gly was identified as the most altered AA using random forest analysis. Blood Gly concentration was lower prepartum, increased from 267.4 μmol/L to 565.9 μmol/L after calving, and decreased thereafter. Previous studies have shown that Gly concentrations increase after parturition (Zhou et al., 2016a,b). The increased Gly concentrations after parturition could be due to the increased breakdown of muscle protein (Doepel et al., 2002). During the periparturient period, oxidative degradation of choline could lead to the production of Gly and methyl groups for the synthesis of Met from HCys (Wu et al., 2013), which is consistent with the negative correlation (\( \rho = -0.49, P < 0.01; \) Supplemental Figure S3, https://doi.org/10.6084/m9.figshare.23896431.v1; Ghaffari, 2023) between Gly and choline levels in serum observed in this study.

Biogenic amines are a group of nitrogenous compounds formed in plants, microbes, and animals in various metabolic pathways through the decarboxylation of AA and amination and transamination of aldehydes and ketones (Medina et al., 2003). The endogenous metabolism of Trp is mainly (95%) via the kynurenine pathway involving Trp 2,3-dioxygenase and indoleamine 2,3-dioxygenase (Hüther et al., 2016), and only 1 to 2% enters the serotonin pathway (Bender, 1983). In addition, a small portion (4–6%) of ingested Trp is degraded by intestinal bacteria, primarily generating indole and other related compounds (Gao et al., 2018). The serum kynurenine concentrations measured in this study confirmed previously published results in dairy cows showing a significant increase during the postpartum period (Ghaffari et al., 2019). Although increased kynurenine concentrations at stable Trp levels suggest reduced serotonin synthesis, our study showed that the time courses of Trp, kynurenine, and serotonin concentrations were largely comparable. The only significant correlation, using Spearman’s rank method, was identified between Trp and kynurenine (\( \rho = 0.38; P < 0.01 \)), as shown in Supplemental Figure S4 (https://doi.org/10.6084/m9.figshare.23896431.v1; Ghaffari, 2023). The ratio of kynurenine to Trp in serum, an indicator of indoleamine 2,3-dioxygenase activity and inflammation...
(Schröcksnadel et al., 2006), did not change significantly over time, unlike kynurenine and Trp alone in this study.

Carnosine is an endogenous dipeptide (β-Ala and L-His) synthesized mainly in muscle (Boldyrev et al., 2013). The increase of the carnosine concentrations in the serum of dairy cow during the postpartum period has also been shown in other studies (Huber et al., 2016; Zhang et al., 2017b; Ghaffari et al., 2019) and was paralleled by a decrease in His and an increase of β-Ala. Carnosine is degraded to the dipeptide anserine, which in turn is metabolized to 1-Met-His and β-Ala (Boldyrev et al., 2013). One of the AA-related metabolites highly regulated by the body, 1-Met-His showed a decrease after calving (wk +5, +10, and +15), which might indicate reduced degradation of carnosine and anserine.

The urea cycle is closely related to the tricarboxylic acid cycle because one of its nitrogen atoms is obtained through the transamination of oxaloacetate to aspartate and returned to this cycle as fumarate (Shambaugh, 1977). Its main function is to convert ammonia to urea for ammonia detoxification (Li et al., 2019). In the rumen, ammonia is produced by microbial degradation of AA, dietary nonprotein nitrogen, and endogenous urea (Li et al., 2019). This ammonia can then be used by microbes as a nitrogen source for protein synthesis, be absorbed from the rumen, or move on from the rumen in the liquid fraction to subsequent sections of the gastrointestinal tract (Li et al., 2019). Serum urea nitrogen concentrations were higher during the dry period than during lactation (Daniel et al., 2022), indicating an oversupply of nitrogen during the dry period. Excessive dietary nitrogen intake leads to ineffective nitrogen utilization by dairy cows, resulting in environmental pollution (Castillo et al., 2000), of both air and surface waters. This conclusion is supported by the low metabolic nitrogen efficiency observed, which was less than 20%. The results suggest that lactation may lead to increased metabolic activity and depletion of urea cycle intermediates, as evidenced by the lower serum concentrations of Arg, SDMA, ADMA, and Orn during lactation as compared with the dry period.

In the current study, TMAO concentrations in the serum decreased after dry-off at wk −5 but increased during lactation (wk +5, +10, and +15 relative to calving). Dietary carnitine, choline, and PC can be degraded by the gut microbiota to trimethylamine (Koeth et al., 2014), which is efficiently absorbed by the intestine and then rapidly oxidized in the liver by flavin-containing monoxygenases (FMO1 and FMO3) to TMAO (Bennett et al., 2013). In lactating dairy cows, the abomasal infusion of choline chloride or dietary supplementation with lecithin increased plasma TMAO concentrations, suggesting that the postruminal degradation of choline to trimethylamine is probably similar to that in nonruminants (Myers et al., 2019; Wang et al., 2021).

In the current study, the choline concentration in serum increased 1.5-fold after dry-off at wk −5, whereas it decreased during lactation, probably reflecting the excretion pattern of choline in milk. The most common form of choline found in biological systems is PC, a phospholipid found in all cell membranes and lipoproteins that transports lipids throughout the circulatory system, which is essential for cellular function (Fagone and Jackowski, 2013). The methyl groups in choline make it an important component of acetylcholine, a neurotransmitter, as well as a substitute for Met in one-carbon metabolism (McFadden et al., 2020). In addition to feed, choline can also be synthesized endogenously, mostly in the liver, by phosphatidylethanolamine N-methyltransferase (Reo et al., 2002). Understanding the changes in choline concentration during different physiological stages of dairy cows may be useful in optimizing choline intake in their diets, which could help to improve milk production and quality.

**Lipid Metabolism**

Metabolomics analysis confirmed extensive remodeling of the serum lipidome in dairy cows during the transition from one lactation period to the next and highlighted the importance of changes in acylCN, phospholipids, and neutral lipids (e.g., TG). Acylcarnitines, PC, and SM were of interest in the current study because of their central roles in lipid metabolism. We observed time-dependent changes in serum concentrations of carnitine, of 11 short-chain (C2–C5), 10 medium-chain (C6–C12), and 7 long-chain (C14–C18) acylCN. Carnitine and its acyl esters (acylCN) are circulating metabolites that play a fundamental role in the carnitine shuttle, the mechanism that transports FA into and out of mitochondria for β-oxidation (Knottnerus et al., 2018). Short-chain acylCN are mainly synthesized from AA and FA, whereas medium- and long-chain acylCN are derived exclusively from β-oxidation (Makrecka-Kuka et al., 2017). As lipolysis increases around calving, mitochondrial β-oxidation may be overloaded, leading to the increase of long-chain acylCN (Kessel et al., 2008; Ghaffari et al., 2021). Incomplete oxidation of long-chain FA caused by impaired activity of carnitine palmitoyltransferase or depletion of intermediates of the tricarboxylic acid cycle can increase concentrations of long-chain acylCN (Flanagan et al., 2010; Murphy et al., 2012; Ramos-Roman et al., 2012).

Serum concentrations of carnitine increased 2-fold after dry-off (wk −5) and decreased during lactation among the cows of this study, likely reflecting the
excretion pattern of carnitine in milk and its uptake by peripheral tissues during previous and current lactations. Carnitine is synthesized mainly in the liver from Lys and Met (Krajcovicová-Kudláčková et al., 2000). This is consistent with previous studies (Yang et al., 2019; Ghaffari et al., 2020) showing that serum carnitine concentrations in dairy cows decrease from the dry period to lactation. Acetylcarnitine (C2), the most abundant acylcarnitine in serum, was increased after dry-off, decreased at wk −1, and ultimately lower concentrations were observed in mid-lactation compared with immediately postcalving. These changes probably reflect the excess formation of acetyl-CoA in the mitochondrial matrix relative to the flux into the tricarboxylic acid cycle after dry-off. In the current study, the short-chain acylCN (i.e., propionylcarnitine [C3], butyrylcarnitine [C4], and valerylcarnitine [C5]) appeared to be the major acylCN during dry-off. These acylCN are mainly derived from BCAA (Damberova et al., 2022). In contrast, most lipid-derived acylCN, such as C18, and C18:1 identified by the Volcano plot, increased around parturition, which is consistent with the increased lipolysis in response to surging energy demand for milk synthesis. It remains to be determined whether an increase in acylCN during the transition period predicts metabolic disturbances (e.g., ketosis) during early lactation.

Several lipids, including PC, lysoPC, CER, glycosylated CER, and SM, play important roles in membrane synthesis, immunity, and signal transduction (Morita and Ikeda, 2022). According to our results, the concentrations of several PC (including C34:3, C40:3, C42:5, and C40:4) in the serum of dairy cows decreased significantly after parturition and increased gradually until mid-lactation (wk +5, +10, and +15). Phosphatidylcholines are essential for the assembly and secretion of lipoproteins (Jonas, 1984; Higgins and Fieldsend, 1987). After calving, cows had increased concentrations of certain lysoPC species, including lysoPC a C16:0 and C18:2, compared with 1 wk before calving. In addition, concentrations of certain lysoPC, including lysoPC a C14:0, C16:0, C18:2, C17:0, C20:3, and C20:4 species, were higher in cows in mid-lactation compared with early lactation. LysoPC are formed through the cleavage of PC by phospholipase A2, the transfer of FA to free cholesterol by lecithin-cholesterol acyltransferase, and they increase in hypoxic conditions (Law et al., 2019). Different lysophospholipids vary depending on the length and saturation of their acyl chain (van der Veen et al., 2017). Altered serum PC concentrations have been associated with fatty liver disease in transition cows (Imhasly et al., 2015). Evidence suggests that saturated lysophospholipids exert proinflammatory effects and impair insulin signaling (Murugesan et al., 2003; Wang-Sattler et al., 2012). Kenéz et al. (2016) reported that glycerophospholipids and sphingolipids decrease before calving and increase thereafter; however, the signaling mechanisms of lysoPC in dairy cows remain unclear.

The most abundant sphingolipid species found in serum in our study were SM C16:0, SM C24:0, SM (OH) C22:1, SM C18:0, and SM C16:1, although the reason for this distribution remains unknown. Sphingolipids are synthesized differently from glycerophospholipids and involve the combination of an AA (mainly Ser) with a FA (mainly palmitate) activated by fatty acyl-CoA and catalyzed by serine palmitoyltransferase (Skotland and Sandvig, 2019; Green et al., 2021). Sphingolipids are a class of lipids with multiple functions, including apoptosis, cell cycle regulation, stress response, and inflammatory response (Adada et al., 2016). Our study revealed a significant decrease in serum concentrations of certain sphingolipids in dairy cows after parturition, followed by a gradual increase until mid- and late lactation. One possible explanation for this decrease in circulating sphingolipids is a reduction in DMI around calving. Alternatively, it could be due to inadequate intake of phosphocholine, which may suppress hepatic synthesis and subsequent export of SM via lipoproteins. Our results are consistent with those of Rico et al. (2017), who observed that concentrations of phosphocholine-containing SM such as SM C16:0, SM C22:0, and SM C24:0 were lowest in all cows at the time of parturition. High concentrations of SM in blood have been associated with lipolysis (Humer et al., 2016) and metabolic disorders (Dervishi et al., 2018). In periparturient dairy cows, plasma CER concentrations, particularly C24:0, were positively correlated with concentrations of FA and inversely correlated with insulin sensitivity (Rico et al., 2015). Kenéz et al. (2016) also observed a negative correlation between SM concentration and glucose and FA levels in blood. However, despite significant differences in lipomobilization in dairy cows, a recent metabolomics study by Schären et al. (2021) found no differences in SM blood concentrations between different metabotypes. Therefore, further research is needed to investigate the functions of sphingolipids in dairy cows.

In this study, serum concentrations of TG were found to increase during the dry period, whereas they decreased with lactation, with some TG, such as TG (C14:0_36:1), TG (C16:0_35:1), and TG (C18:0_32:1), showing greater changes and decreases during lactation. This could be mainly due to a lesser mammary uptake of TG from the blood, since preformed TG are a major source of milk fat for the mammary gland (Lock and Bauman, 2004). In our study, serum TG was negatively correlated with other lipids (acylCN, CER, phospho-
lipids, and sphingolipids), and a decrease in serum TG during lactation was associated with an increase in serum acylCN, CER, phospholipids, and sphingolipids during postpartum. Changes in circulating FA have been of great interest for many years in dairy transition research, as they are associated with fatty liver and impaired liver function (Starke et al., 2010). Increasing hepatic lipid accumulation (i.e., increased liver TG) may result in a decrease in circulating TG, indicating an impaired hepatic ability to export these lipids (Rico et al., 2021).

Among the important molecules that play an active role in cholesterol trafficking and homeostasis are CE formed by the esterification of cholesterol with long-chain FA (Tosi and Tugnoli, 2005). In the present study, CE (18:2) and CE (18:3) were among the 15 most abundant metabolites. Further, the serum concentrations of CE decreased sharply around calving and increased during mid- and late lactation, which is in line with a previous study (Kessler et al., 2014). In addition, the TG concentrations as well as lipoprotein-associated cholesterol fractions in plasma (very-low-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol) were dramatically reduced at the onset of lactation in dairy cows, despite the apparent increase in cholesterol synthesis (Kessler et al., 2014). The concentration of CE in blood depends on the activity of lecithin-cholesterol acyltransferase, which binds FA to cholesterol and forms CE (Tessari et al., 2020), and decreased activity of lecithin-cholesterol acyltransferase leads to a decrease in plasma CE. In dairy cows, the activity of lecithin-cholesterol acyltransferase decreases after calving (Van den Top et al., 2005). According to Bastida et al. (2020), dairy cows with hepatic lipidosis had lower CE concentrations than cows without hepatic FA infiltration. However, a decreased lecithin-cholesterol acyltransferase activity shortly after calving was also observed in healthy cows without fat infiltration in the liver (Pöösö et al., 2000).

**Bile Acids**

Bile acids (BA), which consist of a polar carboxylate side chain and a nonpolar steroid carbon skeleton, are synthesized in the liver from cholesterol, conjugated to either taurine or Gly, and are stored in the gallbladder for later release into the intestinal lumen (de Boer et al., 2018; Chen et al., 2021; Ciocan et al., 2022). Upon excretion, gut microbiota modify (dehydroxylation or deconjugation, or both) primary BA to form secondary BA (de Boer et al., 2018). Bile acids are then actively taken up from the distal ileum and colon, transported to the liver via portal venous blood, and about 95% are reabsorbed via the enterohepatic circulation (Chiang, 2013; de Boer et al., 2018). The concentration of most BA in serum changed during the dry period and lactation in the present study, with GCA, CA, GDCA, and TCA showing the highest concentrations. Also, CA and DCA were higher during lactation, whereas Gly-conjugated BA were elevated shortly after calving in this study. Taurine-conjugated BA had greater concentrations during the dry period than during lactation. Taurine-conjugated BA are more hydrophilic and have a lower acid dissociation constant (pKa) than glycine-conjugated BA (~1.5 for taurine conjugation, ~4.5 for glycine conjugation), which affects their ionization state under physiological and pathological conditions (Hofmann and Roda, 1984; Hofmann, 2009; Sanyal et al., 2021). Due to their greater water solubility and ionization state, taurine conjugates require active or facilitated transport across cell membranes and, unlike glycine conjugates, show limited passive diffusion (Kamp et al., 1993; Hofmann and Hagey, 2008). In this study, the concentrations of the most abundant taurine-conjugated BA (TCA and TDCA) were found to be inversely related to the concentration of free taurine during the dry period, suggesting that taurine was used for BA conjugation, resulting in lower levels of free taurine in the blood. However, we found no significant correlation between taurine, TCA, and TDCA.

The regulation of BA concentrations in cattle is a complex process influenced by several factors, including diet, gut microbiota, and liver health. Liu et al. (2020) found that BA concentrations were influenced by diet and gut bacterial balance, and Urbaneta and Casadesús (2017) showed that Clostridiales such as Ruminococcaceae can convert primary bile acids to secondary bile acids through 7α-dehydroxylation in Angus cattle. In line with these findings, the intestinal abundance of (unclassified) Ruminococcaceae has been reported to increase in dairy cows in response to calving (Tröscer-Mufotter et al., 2022). Increases in Lactobacillus, Clostridium, and Bifidobacterium may also make BA available for further biotransformation by other microbes in grain-fed cattle (Urbaneta and Casadesús, 2017). Liver function and health, such as fatty liver disease, may also influence the BA concentrations in blood (Gerspach et al., 2017). However, previous studies found no significant differences in total serum BA concentration in dairy cows with moderate and severe fatty liver (Garry et al., 1994; Rehage et al., 1999). We speculate that the changes in serum BA during the experimental period are due to changes in diet and adaptation of the hepatic BA synthesis during the dry period and lactation.

Finally, the choice of blood collection matrix and tubes is critical to the precision of metabolomic analysis.


