Dynamic fecal microenvironment properties enable predictions and understanding of peripartum blood oxidative status and nonesterified fatty acids in dairy cows

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ABSTRACT

The transition period in dairy cows is a critical stage and peripartum oxidative status, negative energy balance (NEB), and inflammation are highly prevalent. Fecal microbial metabolism is closely associated with blood oxidative status and nonesterified fatty acids (NEFA) levels. Here, we investigated dynamic changes in total oxidative status markers and NEFA in blood, fecal microbiome, and metabolome of 30 dairy cows during transition (−21, −7, +7, +21 d relative to calving). Then the Bayesian network and 9 machine-learning algorithms were applied to dismantle their relationship. Our results show that the oxidative status indicator (OSI) of −21, −7, +7 d was higher than +21 d. The plasma concentration of NEFA peaked on +7 d. For fecal microenvironment, a decline in bacterial α diversity was observed at postpartum and in bacterial interactions at +7 d. Conversely, microbial metabolites involved in carbohydrate, lipid, and energy metabolism increased on +7 d. A correlation analysis revealed that 11 and 10 microbial metabolites contributed to OSI and NEFA variations, respectively (arc strength >0.5). The support vector machine (SVM) radial model showed the highest average predictive accuracy (100% and 88.9% in the test and external data sets) for OSI using 1 metabolite and 3 microbiota. The SVM radial model also showed the highest average diagnostic accuracy (100% and 91% in the test and external data sets) for NEFA with 2 metabolites and 3 microbiota. Our results reveal a relationship between variation in the fecal microenvironment and indicators of oxidative status, NEB, and inflammation, which provide a theoretical basis for the prevention and precise regulation of peripartum oxidative status and NEB.

Key words: host microbiota interaction, machine learning, peripartum oxidative status diagnosis, predictive warning, series analysis

INTRODUCTION

The transition period refers to the 3 wk before and after parturition and is a critical stage for dairy cows (Horst et al., 2021). The peripartum oxidative status, negative energy balance (NEB), and inflammation have been previously identified as major risk factors for high disease incidences in dairy cows during the transition period (Kerwin et al., 2022b). Generally, dairy cows experience a multifactorial and complicated condition consisting of oxidative status, NEB, and additional symptoms (Perrone et al., 2020). Peripartum challenge arises from profound shifts in digestion, endocrine physiology, energy metabolism, reproduction, and lactation (Marei et al., 2022; Vossebeld et al., 2022). Under the combined effects of multiple sources, indicators of oxidative status, NEB, and inflammation show specific longitudinal variants and high individual differences (Sheldon et al., 2019), which prevent full mechanistic exploration and early predictive warnings (Pascottini et al., 2020).

Fecal microbiota have been identified indicative of reliable prediction and regulation of host metabolic challenge (Feng et al., 2022). As individual “fingerprints,” fecal microbiota vary between animals but remain stable within a single host, enabling identification of complex and variable host states (Chen et al., 2021). Precise predictions and the role of microbiota have been investigated in multiple large-scale cohorts in humans (Asnicar et al., 2021; Kurilshikov et al., 2021). In peripartum dairy cows, fecal microbiota have been reported to mediate inflammation and oxidative status in the liver, mammary gland, and uterus (Jeon et al., 2017; Hu et al., 2020; Pacifico et al., 2021), and thus they exert important physiological functions for...
reproduction as well as energy metabolism and lactation. Lipid metabolism is highly relevant for NEB and oxidative status in transition dairy cows, and hindgut microbiota could regulate hepatic lipid metabolism by influencing bile acid metabolism (Pacifico et al., 2021).

The mammary gland, as an organ with high metabolic intensity in transition dairy cows, is susceptible to inflammation and oxidative stress under the influence of microorganisms. Hu et al. (2020) found that fecal microbiota alter the blood-milk barrier, resulting in an invasion of neutrophiles and in the exacerbation of inflammation within mammary gland. As a major source of peripartum inflammation, a uterus infection is caused by intestinal pathogens such as Bacteroides, Porphyromonas, Fusobacterium, Prevotella, and Helcococcus (Jeon et al., 2017). Although previous work has shown that hindgut microbiota are involved in metabolic responses of the above mentioned organs, the exact functional role of fecal bacteria for regulating systemic oxidative status, inflammation, and NEB in transitioning dairy cows remains unclear.

Except for microbiota, fecal metabolites are the ultimate microbiome targets in the host, and microbial metabolites might mirror microbial functions (Rohe et al., 2016). With increasing evidence for the relationship between fecal microbiota and host oxidative status, inflammation, researchers focused on exploring potential mediating metabolites secreted by fecal microbiota, such as aromatic AA, short fatty acids, vitamins, and bile acids, among others (Nicholson et al., 2012). Although intestinal metabolic landscapes were observed in transitioning dairy cows exposed to different diets and farm management conditions, precise metabolic changes regulating levels of host oxidative status, inflammation, and NEB still remain to be discovered. In addition, the gut epithelium of dairy cows is covered by a single epithelium layer, which makes it even more susceptible to lumen acidification and lipopolysaccharides than the stomach with a squamous epithelium (Petri et al., 2021). However, studies containing variation in metabolism and metabolites specifically secreted by fecal microbiota in dairy cows are still rare. Broad availability of metabolite tracing tools makes it possible to distinguish microbial metabolism and non-microbial metabolites (Yu et al., 2022). Moreover, advanced machine-learning models have proven relevant for screening variables and predicting host oxidative status and inflammation.

Here, we first explored stages variation of fecal microbial composition, functions, and related metabolites in transitioning dairy cows from 21 d before until 21 d after calving. Next, we investigated the potential correlation between temporal changes in the microenvironment and peripartum oxidative status indicator (OSI) and nonesterified fatty acids (NEFA), as obtained from multiple machine-learning algorithms. Our results provide a theoretical basis for noninvasive, accurate, and early warning of high levels of peripartum OSI and NEFA in dairy cows and give rise to the possibility of preventing and treating peripartum diseases accurately on a large scale.

**MATERIALS AND METHODS**

All experimental procedures used in this study were approved by the Animal Care Committee of Zhejiang University (Hangzhou, China) and conducted in accordance with the university’s guidelines for animal research. The experimental protocol was approved by the Animal Care Committee of Zhejiang University (Hangzhou, China).

**Sample Collection**

Briefly, 30 healthy dairy cows in the peripartum period were first selected from a herd of 2,000 dairy cows, with parity (mean = 1.93, SD = 1.00) and BCS on −21 d (mean = 3.21, SD = 0.41) and +21 d (mean = 2.76, SD = 0.4). Body condition was scored following the method described by Edmonson et al. (1989), using the average of a graded scale ranging (1 = thin, 5 = fat) at 3 time points (0600, 1400, and 2000 h), to reduce the error of subjective factors. Body weight was estimated on calving day (BW; 690 kg, SD = 50) and +21 d (BW; 630 kg, SD = 40). Milk yield was recorded after calving on +7 d (39 kg, SD = 11), +14 d (42 kg, SD = 6), +21 d (46 kg, SD = 6). All animals had the same diet, water, and environment. Ingredients of the basal diet before calving and after calving are listed in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). Alfalfa, steam-flaked corn, and sugar beet pulp were included as replacements of wheat bran, rice, and straw after calving. Moreover, corn silage (28% to 19.8%) and oat hay (19.9% to 7.05%) were declined, with the supplement of soybean meal (10.3% to 17.7%). Blood samples (5 mL) were collected from the coccyeal vein of cows on −21 d (−3 wk), −7 d (−1 wk), +7 d (+1 wk), and +21 d (+3 wk) relative to calving day, each time at 0600 h with ethylenediaminetetraacetic acid (EDTA) evacuated tubes. Blood samples were centrifuged at 3,000 × g for 15 min to collect plasma at 4°C. Rectal fecal samples were collected before the morning feeding at 0600 h using sterilized gloves and stored in 50-mL frozen storage tubes. Fecal samples and plasma samples were flash frozen in liquid nitrogen and then stored at −80°C for subsequent analyses. A total of 120 fecal and 120 blood samples from 30 peripartum dairy cows were collected.
at 4 time points. For the subsequent validation of the prediction models, additional 85 fecal and 85 blood samples were collected using the same method. Among these 85 samples, 83 samples were used for predicting NEFA levels, 18 samples were used for predicting OSI levels (16 samples were overlapped).

**Plasma Parameters Measurement**

An autoanalyzer 7020 instrument (Hitachi High-Technologies Corporation, Tokyo, Japan) was used to quantify glucose concentrations (GLU, #ZH2079T), nonesterified fatty acids (NEFA, #ZH2045Z), BHB (#ZH2029T), BUN (#ZH2017S), cholesterol (CHOL, #ZH2040Z), triglycerides (TG, #ZH2039Z), albumin (#ZH2013G), alanine aminotransferase (#ZH2001G), aspartate aminotransferase (#ZH2002G), total protein (#ZH2012G), superoxide dismutase (SOD, #ZH2058F), and creatinine (#ZH2020S2) in plasma with commercial kits (all from Ningbo Medical System Biotechnology Co. Ltd., Ningbo, China). Likewise, commercial assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were used to quantify concentrations of plasma catalase (#A007–1–1), total antioxidant capacity (T-AOC, #A015–2–1), glutathione peroxidase (#A005–1–2), malondialdehyde (MDA, #A003–1–2), haptoglobin (HPT, #H136), amyloid (SAA, #H134), and ceruloplasmin (CPL, #A029–1–1) following the manufacturer’s instructions. These methods have been validated in cattle and parameters’ values in these studies were subject to the normal range (Jiang et al., 2022; Gu et al., 2023a,b). Finally, plasma total oxidative status (TOS; #KC5100, Bensheim, Germany) was measured with commercial assay kits following manufacturer’s instructions (Shi et al., 2021). The oxidative status indicator (OSI) was defined as the ratio of TOS to T-AOC (Durgut et al., 2013; Sánchez-Rodríguez and Mendoza-Núñez, 2019).

**Microbial DNA Extraction, Sequencing, and Preprocessing Analysis**

Microbial DNA from fecal samples was extracted using the E.Z.N.A. Stool DNA Kit (#D4015, Omega Inc.). The universal 16S primers corresponding to the V3-V4 region (341F: 5’-CCTACGGGNGGCWGCAG-3’; 805R: 5’-GACTACHVGGGTATCTAATCC-3’) were used to amplify the bacterial 16S rRNA gene. Purification and quality testing were performed using AMPure XP beads (Beckman Coulter Genomics, Danvers, MA) and Qubit (Invitrogen). The PCR products were then pooled together and sequenced on an Illumina NovaSeq PE250, which was provided by LC-Bio Technology Co. Ltd., Hangzhou, China.

We used FLASH (V1.2.8) to demultiplex the raw sequence data into paired-end fastq files. Clean data were stored in the NCBI SRA database as described in the data available statement. De-noising and clustering algorithms that generate amplicon sequence variants (ASV) are included in DADA2 embedded in QIME2 (Callahan et al., 2016). SILVA (138 database, https://www.arbsilva.de; Quast et al., 2013) was referenced for taxonomic annotation. At each time point, microbial taxa with a relative abundance greater than 0.01% in more than 15 samples were used for further analysis. The filtered taxonomy matrix was stored in Supplemental Tables S3, S4, and S6 (https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). Alpha and diversities were calculated using QIME2 (Bolyen et al., 2019). To compare the Bray-Curtis dissimilarity of the microbial community we used the vegdist function in the R package vegan (V2.6-2, https://cran.r-project.org/web/packages/vegan/index.html; Dixon, 2003). The adonis test was used to determine significant differences caused by different categories of feed and host related metabolites. Finally, functional components of the microbiome were predicted by PICRUSt2 program.

**Co-occurrence Network Analysis**

To understand the microbial interaction patterns at all 4 time points, all the genera with an abundance greater than 0.01% in more than half of the samples from at least one of the 4 time points were retained for co-occurrence analysis. Spearman’s rank correlation coefficient between the genera were pair-calculated using rcoc function in the Hmics package (v4.6.0, https://cran.r-project.org/web/packages/Hmisc/index.html). In the rcoc function, the type arguments was set to Spearman. In the case of ties, the midranks method was used. The P-values were approximated using F-distribution. Robust correlations ($P < 0.05$, $|\rho| > 0.60$) were used to construct the networks.

**Fecal Metabolite Measurement and Data Analysis**

Frozen feces (100 mg) from each sample were thoroughly ground with liquid nitrogen, dissolved with 1 mL of 50% methanol buffer, and incubated for 10 min. After being stored at $-20^\circ$C overnight to precipitate out protein, the mixture was then centrifuged at 4,000 × g for 20 min at 4$^\circ$C. The supernatant was injected into the ultra-performance liquid chromatography system (SCIEX, UK) with an ACQUITY UPLC T3 column (100 mm × 2.1 mm, 1.8 μm, Waters, UK) for chromatographic reversed-phase separation. The TOF 5600 Plus high-resolution tandem mass spectrometer (SCIEX, Warrington, UK) with mass set at the 60 to
1,200 Da range was performed to obtain both positive and negative metabolic fragments eluted from the column. Peak detection and annotation were performed by CAMERA (Kuhl et al., 2012), MetaX toolbox in R and the CAMERA package. Each ion was identified by its retention time and mass-to-charge ratio. The raw metabolites matrix was stored in Supplemental Table S9 (https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a).

**Dynamic Clustering of Microbiota and Metabolites**

Kruskal-Wallis rank sum test was performed to select genera with significant changes among the 4 time points. Dynamics patterns in these 91 genera were identified by soft clustering function with Mfuzz in the Mfuzz package (V2.52.0; Kumar and Futschik, 2007). The number of clusters was determined by Dmin function in Mfuzz package with soft clustering for a range of cluster numbers c and reporting the minimum centroid distance. The elbow of the curve was used to determine the optimal cluster number for clustering. We optimized the setting of fuzzifier variables under elbow cluster classification at 3.60, 3.21, 3.86 for genera, host and microbiota co-metabolism, microbial metabolism, respectively.

**Metabolites Origin Tracing and Metabolic Function Analysis**

Identification of the origin of metabolites was accomplished in MetOrigin (http://metorigin.met-bioinformatics.cn/; Yu et al., 2022) with information from 7 well-known metabolite databases, which classified metabolites into 6 groups, including microbiota, host, co-metabolism, food, drug, and environment. We applied Deep MetOrigin Analysis, and chose Bos taurus (cow) as host parameter. Metabolomics pathway analysis (MetPA pathway topology analysis, https://www.metaboanalyst.ca/; Xia and Wishart, 2010) included in MetOrigin was applied to each metabolite from each cluster to identify metabolic pathways ($P < 0.05$).

**Construction of Bayesian Networks**

Bayesian networks were constructed to infer important interactions between fecal microenvironment and host physiological parameters. From each cluster, the relative importance of the first principal component was calculated to represent the cluster. The network topology was interpreted as successional dependency of these variables throughout the peripartum period (Scutari, 2010). The Bayesian network’s structure was constructed using a tabu search algorithm in the bnlearn package (v4.8.3 https://cran.r-project.org/web/packages/bnlearn/index.html; Scutari, 2010) after 2,000 bootstrap replicates. We performed model averaging to save significant arcs. Finally, all parameters of the Bayesian network were calculated using bn.fit function.

**Microbiability Calculations**

Microbiability is used to describe the capacity or ability of the microbiota to influence or contribute to a certain outcome or parameter. The linear mixed-effects model (LMM) was used to correct for fixed effects such as parity and milk yield, and to calculate the contribution of microbial random effects to the variance of blood indicators:

$$y = Kc + ms + u + e,$$

where $y$ is the phenotype NEFA, BHB, OSI, HPT, SAA; $e$ is the vectors of the fixed covariates, consisting of parity and milk yield; $K$ is the fixed effects matrix, including coefficients related to $c$; $ms$ is the random effects vector, representing the random effects from microbiota; $u$ is the random intercept vector, representing the random intercepts for each observation; and $e$ is the error vector, representing the unexplained random errors. Gut microbes in each animal are considered random effects, which follow the distribution $ms \sim N(0, \text{Var}_m)$ (Xue et al., 2020).

**Comparison of Prediction Performance Between Different Machine-Learning Models**

Ahead model construction, we divided dairy cows into high- and low-level cows based on the level of OSI and NEFA at the +7 d time point respectively. A level of 0.8 mmol NEFA was chosen as the demarcation level for high and low NEFA (Roberts et al., 2012; Kerwin et al., 2022a). As for OSI, which is rarely used in dairy cows, there is no empirical data to be used for dividing into high and low oxidative status. Therefore, the third quartile of OSI (0.745) in the entire peripartum period was used as cutoff value for the high and low OSI levels. To find a better classification strategy to identify high and low OSI levels, multiple statistical and machine-learning models were applied: random forests (RF), radial support vector machines (SVM radial), K nearest neighbors, linear support vector machines (SVM linear), linear discriminant analysis, eXtreme gradient boosting (XGboost), naive Bayesian, generalized additive model, and logistic regression. The synthetic minority oversampling technique (SMOTE) algorithm was used to balance positive and negative
samples using the SMOTE function in the DMwR package (v0.4.1, https://github.com/haghibinh/ DMwR). Then, we applied 10-fold cross-validation for predicting high or low NEFA levels at real time, and 5-fold cross-validation for predicting high or low OSI levels 1 wk after the birth. Cross-validation was applied using the function caret() in the caret (v6.0–94, https://cran. r-project.org/web/packages/caret/). All models were run using default parameters and by including all variables without filtering to allow comparisons between the models. The machine-learning models were built using the following packages: caret (v6.0–91), e1071 (v1.7–9, https://CRAN.R-project.org/package = e1071), kknn (v1.3.1, https://cran.r-project.org/web/packages/ kknn/), randomForest (v4.7–1 https://cran.r-project. org/web/packages/randomForest/), xgboost (v1.6.0.1 https://cran.r-project.org/web/packages/xgboost/index.html), glmnet (v4.1–4, https://cran.r-project. org/web/packages/glmnet/index.html); Friedman et al., 2010), MASS (v7.3–56, https://cran.r-project.org/ web/packages/MASS/index.html) and mboost (v2.9–6, https://github.com/boost-R/mboost/).

Model Predictions for NEFA and OSI Levels

After model comparison, RF and SVM radial models were selected for predicting NEFA and OSI. We created a SVM radial classification to identify high or low NEFA and OSI levels in host peripheral blood using the tune.svm function in the e1071 package. 10-fold cross-validation was applied and feature filtering was done for each cross-validation using the rfe function in the caret package. The features within the top 15 highest accurate coefficients from each cross-validation were selected for final model construction. For OSI levels at +7 d classification, SVM radial classification was used, and common top variables from 5-fold validation were ultimately included in the models. To validate the effectiveness of the model, we collected 85 additional blood and corresponding fecal samples from the peripartum cows (Supplemental Table S2; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a) in the same dairy farm. After measuring their NEFA, OSI and microbial amplicon data using the same methods, we used our trained model for prediction.

Statistical Analysis

Kruskal-Wallis rank sum test was applied to perform comparisons among 4 time points, which was done using the kruskal.test function within the package stats in R (V4.1.0). Significant differences from the multiple comparisons were further analyzed by pairwise comparison using Dunn’s test, which was conducted with dunntest function in the FSA (V0.9.3, https://cran.r-project.org/web/packages/FSA/index.html) package. Following pairwise comparison, the absolute highest values were identified and the number of highest values at −21, −7, +7, +21 d were determined. Correlation analysis was performed using Spearman rank correlation, and the asymptotic P-value was calculated using corPvalueStudent function in Weighted Gene Co-expression Network Analysis (WGCNA; Langfelder and Horvath, 2008). In all models, P-values < 0.05 after Bonferroni correction were considered as significance, and 0.05 ≤ P ≤ 0.10 was considered a significant trend.

RESULTS

Dynamics of Fecal Microbial Composition and Microbiota Abundance During Peripartum Period

A total of 10,095,354 raw reads were obtained from the sequencing data of 120 fecal samples, collected from 30 dairy cows at 4 peripartum time points. After retaining microbial taxa with a relative abundance greater than 0.01%, a total of 8,418,878 reads were retained after quality control, and 32,580 ASV were assigned from these reads (Supplemental Table S3). Our results show that Chaol indices at −21 and −7 d were significantly higher than at +7 d (P = 0.033 and P < 0.001) and +21 d (P = 0.018 and P < 0.001), −21 d did not differ significantly from −7 d (P > 0.05; Figure 1A). The Shannon indices trend resembled Chaol. The results of microbiota β-diversity among the 4 time points showed significant differences (Anoism P < 0.001, R = 0.49; PERMANOVA P < 0.001, R² = 0.18). Bray-Curtis distance from other time points to −21 d increased significantly over time (distance to −21 d: −7 d lower than +7 d, P = 0.0012; +7 d lower than +21 d, P = 0.0012; Figure 1B). In addition, Bray-Curtis distance differed most between −7 to +7 d (PERMANOVA P < 0.001, R² = 0.15).

In terms of microbial composition, 24 phyla were identified within the individuals (Supplemental Figure S1A, https://doi.org/10.6084/m9.figshare.24460333.v1, Zhu, 2023b); Supplemental Table S4). The top 10 phyla included Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria, Actinobacteria, Verrucomicrobia, Candidatus saccharibacteria, Tenericutes, Kiritimatielaeota and Fibrobacteres which accounted for a total of 98.5% of phyla on average abundance. Firmicutes and Bacteroidetes were the 2 dominant phyla (Figure 1C). Multiple comparisons showed that the abundance of different phyla was elevated at each time point. Spirochaetes was higher on +21 d than on −21 d (P = 0.0016), and −7 d (P < 0.001). Bacteroidetes
was higher on +7 d higher than on −21 d (P < 0.001) and −7 d (P < 0.001). Fibrobacteres was higher on −7 d than on +21 d with (P = 0.012). None of a single phylum was found higher on −21 d than on +7 d. Some phyla were significantly higher on both −7 and −21 d,

such as Firmicutes on −21 d higher than +7 d (P < 0.001) and on −7 d higher than on +7 d (P < 0.001). Verrucomicrobia were higher on −21 d than on +7 d (P < 0.001, +21 d P < 0.001) and on −7 d higher than on +7 d (P < 0.001) and on +21 d (P < 0.001).
Similarly, some phyla were higher both on +7 and on +21 d: Actinobacteria (on +21 d higher than −7 d; \( P < 0.001 \) and −21 d; \( P < 0.001 \)). Likewise, they were higher on +7 d than −7 d (\( P < 0.001 \) and −21 d; \( P < 0.001 \)). Similarly, Tenericutes were higher on +21 d than on −7 d (\( P < 0.001 \) and on −21 d; \( P < 0.001 \)) and higher on +7 d than −7 d (\( P = 0.018 \) and −21 d; \( P < 0.001 \); Supplemental Table S5; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a).

In addition, 106 genera were identified after filtering from each time point (Supplemental Figure S1A, Supplemental Figure S2, https://doi.org/10.6084/m9.figshare.24460333.v1, Zhu, 2023b; Supplemental Table S6). For the 106 genera, 91 of which differed significantly between multiple points as from Kruskal-Wallis test (\( P < 0.05 \)), (Supplemental Table S6). These 91 genera were clustered into 4 dynamic modules. Microbiota cluster (MicroC) 1 includes 24 genera that were most abundant on −21 d, which belonged to Firmicutes and Proteobacteria phyla. Ruminococcaceae unclassified, the most abundant genus, also belonged to MicroC1 (Figure 1D, Supplemental Table S7; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). Genera in MicroC2 were enriched on +21 d, and belonged to Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Candidatus Saccharibacteria, and Tenericutes.

 Genera in MicroC3 reached their peak abundance on −7 d, with the most complex distribution of 9 phyla (Figure 1E, Supplemental Table S7). Genera in MicroC4 were Firmicutes, Actinobacteria, Bacteroidetes, Fibrobacteres, Spirochaetes, and Tenericutes, which elevated to the highest abundances on +7 d. Microbiota cluster 4 was the only cluster with more Bacteroidetes than Firmicutes (Figure 1E, Supplemental Table S7).

Interaction Between Fecal Microbiota and Metabolic Microenvironment

We constructed a co-occurrence network from 106 genera at the 4 time points separately. Each time point had particular connective patterns. The total number of edges at each point were 48, 74, 19, 71, corresponding to time points between −21 and +21 d (Supplemental Figure S3, https://doi.org/10.6084/m9.figshare.24460333.v1; Zhu, 2023b). The network of genera had the lowest number of edges on +7 d and a lower degree of centrality compared with other time points (+7 d was lower than −21 d; \( P = 0.14 \); −7 d; \( P = 0.0011 \); and +21 d; \( P = 0.020 \); Supplemental Table S8; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). Also, it had the lowest number of genera involved in the network. The proportion of positive edges (89.5%) was the highest on +7 d. On −7 d the degree of centrality was higher than on −21, +7, +21 d and average levels were higher on −7 d, \( P = 0.077 \), than on −21 d, \( P = 0.0055 \), and +7 d, \( P = 0.0011 \), and +21 d, \( P = 0.085 \), while the proportion of positive edges was lower (74.3%; Figure 2A). A total of 1,182 metabolites belonged to 32 categories of MS2class in the Human Metabolome Database (https://hmdb.ca/ ) resulted from the metabolome (Supplemental Figure S1B, Supplemental Table S9). The results of metabolic sources analysis showed that 23 metabolites derived from host, 88 from microbiota, 200 were shared by both the host and microbiota co-metabolism, 138 from drugs, 356 from feed and 9 from environment (Figure 2B; Supplemental Table S9). PERMANOVA analysis showed that feed (\( P < 0.001 \)), host (\( P < 0.001 \)), microbiota (\( P < 0.001 \)) and both microbiota and host (\( P < 0.001 \)) related metabolites differed significantly among the 4 time points (Supplemental Figure S4, https://doi.org/10.6084/m9.figshare.24460333.v1, Zhu, 2023b; Supplemental Table S10; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). Three categories of feed-related metabolites, including sterol lipids (\( R^2 = 0.094, P = 0.016 \)), organooxygen compounds (\( R^2 = 0.056, P = 0.018 \)) and phenols (\( R^2 = 0.026, P = 0.043 \)) significantly contributed to microbial variations at genera level (Figure 2C; Supplemental Table S10). We focused on the microbial related metabolite variation. In total, 216 microbiota-related metabolites significantly differed among the 4 time points (Kruskal-Wallis \( P < 0.05 \)). These metabolites were combined into 6 major categories according to the Human Metabolome Database material classification, including 20 amino acids, 32 aromatic compounds, 50 carbohydrates and derivatives, 75 lipids, and 18 other kinds of metabolites. Among them, aromatic compounds, carbohydrates and derivatives and lipid categories were mainly composed of benzene and substituted derivatives (18 in 32), carboxylic acids and derivatives (20 in 50) and glycerophospholipids (30 in 75; Figure 2D, Supplemental Table S9, Supplemental Table S11; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). Different time point differences were found in these 6 metabolic categories. The aromatic compounds peaked on −7 d (−21, −7, +7, +21 d: 12, 17, 11, 14 Dunn’s test \( P < 0.05 \) after Bonferroni correction). Lipid metabolites, carbohydrate metabolites and derivatives were more abundant on +7 and +21 d (lipids −21, −7, +7, +21 d: 20, 21, 44, 32; carbohydrate and derivatives −21, −7, +7, +21 d: 13, 11, 27, 27 \( P < 0.05 \)). Amino acids were more prevalent on +21 d (−21, −7, +7, +21 d: 4, 4, 15; Figure 2E). These 216 microbiota-related metabolites showed different temporal patterns. 143 significantly different metabolites shared between hosts and microbiota were divided into 5 clusters. Co-metabolism clusters (MetaC) 1, 2, 3, 4, 5 (Co-MetaC1,
 Alteration of Functionality of Fecal Microbiota and Metabolites During Peripartum Period

The microbial community functional capacity was predicted using PICRUSt2 at each time point (Supplemental Table S14; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). No significantly different MetaCyc pathways were observed between −21 and −7 d. Moreover, the pathways related to glycolysis, pentose phosphate pathway, carbohydrate degradation and parts of fermentation increased significantly between −7 to +7 d, including PWY-5484 (glycolysis II from fructose 6-phosphate, \( P < 0.001 \)), GLYCOLYSIS (glycolysis I from fructose 6-phosphate, \( P = 0.0072 \)), PWY-621 (sucrose degradation III sucrose invertase, \( P < 0.001 \)), PWY-5384 (sucrose degradation IV sucrose invertase, \( P = 0.0017 \)), PENTOSE PHOSPHATE PATHWAY (pentose phosphatase pathway, \( P = 0.0011 \)), ANAEROFRUCAT-PWY (homolactic fermentation, \( P = 0.0031 \); Figure 3; Supplemental Table S15; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). However, some of the pathways belong to the tricarboxylic acid cycle (TCA) and fermentation was significantly downregulated between −7 to +7 d, and included PWY-7254 (TCA cycle VII acetate-producers, \( P = 0.042 \)), P105-PWY (TCA cycle IV 2-oxoglutarate decarboxylase, \( P = 0.010 \)), PWY-5676 (acetyl-CoA fermentation to butanoate II, \( P = 0.0043 \)), PWY-5677 (sucinate fermentation to butanoate, \( P = 0.017 \)), P163-PWY (L-lysine fermentation to acetate and butanoate, \( P = 0.015 \)) and PWY-7094 (fatty acid salvage, \( P = 0.0020 \); Supplemental Table S15). The microbial functional pathways on +21 d were similar with those on +7 d, but some differences in AA biosynthesis and TCA cycle were observed, including upregulated PWY-6628 (super-pathway of L-phenylalanine biosynthesis, \( P = 0.030 \)), PWY-6630 (super-pathway of L-tyrosine biosynthesis, \( P = 0.030 \)), P105-PWY (TCA cycle IV 2-oxoglutarate decarboxylase, \( P < 0.001 \)) and PWY-7003 (glycerol degradation to butanol, \( P = 0.0031 \); Figure 3, Supplemental Table S15).

In addition, metabolic pathways derived from metabolites clusters demonstrated that lipid metabolism, AA metabolism, nucleotide metabolism were functionally different as well as energy metabolism (MEPA enrichment analysis \( P < 0.05 \)). Microbial and metabolites pathways shared with the host (co-metabolism metabolites) were functionally enriched. Different clusters were characteristic for different categories of function. The main functional component of co-metabolism Co-MetaC1 was nucleotide metabolism (highest counts of matched metabolites 5 in 18) including purine metabolism (\( P < 0.001 \)), Co-MetaC2 dominant functional components were carbohydrate metabolism and AA metabolism, derived from counts of matched metabolites, carbohydrate metabolism 14 in 54, AA metabolism 13 in 54 and mainly included alanine, aspartate and glutamate metabolism (\( P < 0.001 \)) and citrate cycle (\( P = 0.0013 \)). Co-MetaC3, 4 and 5 were dominated by AA metabolism (33 in 71), lipid metabolism (13 in 17) and carbohydrate metabolism (5 in 18), which mainly consisted of aminoacyl-tRNA biosynthesis (\( P < 0.001 \)), glycerocephospholipid metabolism (\( P < 0.001 \)), amino sugar and nucleotide sugar metabolism (\( P < 0.001 \)). The primary function of the MetaC1, 2, 3, 4, 5, 6, 7 was biodegradation of xenobiotics and metabolism (from counts of matched metabolites in all clusters 21 in 25) which mainly included aminobenzoate degradation (\( P < 0.001 \); Figure 4; Supplemental Table S16; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a).

Core Intestinal Energy Metabolic Pathways and Related Pathways

Based on the PICRUSt2 result, we identified the major pathways from each metabolic cluster including energy, lipid, AA, and aromatic compounds metabolism. Interestingly, several highly enriched pathways shared intermediate metabolites with TCA cycle and glycolysis, including alanine, aspartate and glutamate metabolism (Co-MetaC2 \( P < 0.001 \), Co-MetaC3 \( P = 0.0093 \)), phenylalanine metabolism (Co-MetaC3 \( P = 0.0016 \), MetaC7 \( P = 0.010 \)), glycerocephospholipid metabolism (Co-MetaC1 \( P < 0.001 \), Co-MetaC2 \( P = 0.0020 \), Co-MetaC3 \( P = 0.027 \), Co-MetaC4 \( P < 0.001 \), Co-MetaC5 \( P = 0.033 \)), aminobenzoate metabolism (MetaC2 \( P < 0.001 \), MetaC3 \( P < 0.001 \), MetaC7 \( P = 0.019 \)), TCA cycle (Co-MetaC2, \( P = 0.0013 \)) and glycolysis (Co-MetaC3 \( P = 0.011 \); Figure 5A). The general temporal trend of the pathways could be derived from the temporal clusters of metabolites. As for alanine, aspartate and glutamate metabolism, glutamate, N-acetylglutamate, citrulline, arginine came from Co-MetaC2, 4, 3, 3, respectively, which reached the highest values between +7 and +21 d. Phenylalanine metabolism, phenylalanine, trans-3-hydroxy-cinnamate, all belonging to Co-MetaC3, peaked on +7 and +21 d. Glycero-
Figure 2. The variation of microbial interactions and the fecal metabolome of 30 cows at −21 d, −7 d, +7 d, and +21 d relative to calving. A: The 106 genera’s co-occurrence network edge statistics comparison among 4 time points. B: Number of metabolites from different sources analyzed by MetOrigin. C: Distribution and contribution (Adonis R^2) of feed-related metabolites significantly affected microbiota community. D: The 6 categories derived from 216 differential microbial sourced metabolites. E: Circle map displayed the summaries of metabolites reaching the highest values at 4 time points. F: Temporal clustering of differential microbial metabolites. The network at each time point was constructed from 106 genera, using (|ρ| > 0.6 and P < 0.05) as edge screening criterion. Moreover, the contribution of metabolites to genera level microbiota was analyzed by PERMANOVA (P < 0.05), using the first principal component value of the metabolic abundance matrix as the factor representing the category of metabolites. Multiple comparisons were done by Kruskal-Wallis rank sum test and pairwise comparison using Dunn’s test. P-value (*P < 0.05) after Bonferroni correction was set as significant cutoff. Adonis R^2: in adonis analysis, R-square indicates to what extent the independent variable explaining the variations between samples.
pholipid metabolism pathway consists of metabolites such as lecithin (lysophosphatidylcholine [LysoPC] and lysophosphatidylethanolamine [LysoPE]), 1-acyl-sn-glycero-3-phosphoethanolamine, which belonged to Co-MetaC4 and 2, and reached the highest expression on +7 and +21 d. Aminobenzoate metabolism includes benzoly-formate, benzamide, terephthalate, benzoate, 4-Hydroxy-benzoate, phenol, pyruvate, which are metabolites from MetaC2, 3, 1, 7. They were highest on −21, −7, +7, and +21 d. Metabolites from the TCA cycle and glycolysis were observed postpartum on +7 and +21 d, including succinate, 2-Oxo-glutarate, citrate, pyruvate, Acetyl-CoA, acetaldehyde, which belonged to Co-MetaC2, 2, 3, 2, 4. Pyruvate, Acetyl-CoA and acetaldehyde, which served as the key metabolic link in the TCA cycle, were shared in all the 5 pathways. The critical metabolites of each pathway also significantly correlated with host biochemical parameters, including phenol (NEFA R = −0.500, P < 0.001, OSI R = 0.26, P < 0.0038), phenylalanine (NEFA R = 0.36, P < 0.001, OSI R = −0.42, P < 0.0038), Lyso PC 15:0 (lecithin; NEFA R = −0.51, P < 0.001, HPT R = 0.19, P < 0.043, OSI R = −0.214, P < 0.019), Lyso PE 9:0 (lecithin; NEFA R = −0.51, P < 0.001, OSI R = −0.214, P = 0.0026), citric acid (NEFA R = 0.60, P < 0.001) and succinic acid (NEFA R = 0.47, P < 0.001; Figure 5B; Supplemental Table S17; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a).

Figure 3. Variation of microbial function predicted by PICRUSt2. Microbial function displayed by Metacyc pathway abundance at −21 d, −7 d, 7 d, 21 d relative to calving. A total of 120 fecal samples from 30 cows at 4 time points were used for function prediction. Time 1, 2, 3, 4 refer to −21 d, −7 d, +7 d, +21 d, respectively. The size of red dot represents abundance of Metacyc pathways.
Correlation Analysis of Microbiota, Microbial Metabolites, and Host OSI, NEFA, and HPT

Next, we wanted to know how changes in microbiota and metabolites in the fecal microenvironment interacted with the host phenotypes in Table 1. From +7 to +21 d, body weight decreased ($P = 0.046$), and milk yield increased ($P = 0.0018$). Body condition score declined significantly from −21 to +21 d ($P < 0.001$).

Indicating energy metabolism, NEFA and BHB showed the highest concentration on +7 d (NEFA +7 d higher than −21, −7, and +21 d, $P < 0.05$; BHB +7 d higher than −21 d $P < 0.001$, −7 d $P < 0.001$, +21 d $P = 0.047$). Correspondingly, glucose, cholesterol, triglyceride showed lower concentration on +7 d (GLU, +7 to −21 d $P < 0.001$, +7 to −7 d $P < 0.001$, +7 to +21 d $P = 0.11$; CHOL, +7 to −21 d $P < 0.001$, +7 to +21 d $P < 0.001$; TG, +7 to −21 d $P < 0.001$, +7 to −7 d $P < 0.001$). As for oxidative status, OSI showed the lowest concentration on +21 d (+21 d lower than +7 d $P < 0.001$, −7 d $P < 0.001$, +21 d $P < 0.001$). Superoxide dismutase stayed higher on +7 d, +21 d (+7 d higher than −21 d $P = 0.0095$, −7 d $P = 0.11$, +21 d higher than −21 d $P < 0.001$, −7 d $P = 0.0061$). For indicators of inflammation, HPT reached its peak on +7 d (+7 d higher than −21 d, $P = 0.0088$; −7 d, $P = 0.12$; +21 d, $P = 0.0010$). Amyloid and CPL stayed higher status on +7 d (SAA +7 to −21 d, $P < 0.001$; SAA +7 to −21 d $P = 0.24$, +7 to −7 d $P = 0.0061$, +7 to +21 d $P = 0.015$). To assess the contribution of fecal microbiota to the host blood parameters after correcting the factors variation of parity milk yield and weight (Table 2), LMM was applied to calculate microbiability. Microbiability reached 17.0% contribution variance to NEFA, 49.0% to OSI, 21.4% to BHB, 23.3% to HPT, 9.6% to SAA after calving. Potential correla-
tion among OSI, NEFA, MicroC, and MetaC from previous sequences analyses was examined using a Bayesian network. Interestingly, OSI, NEFA were mainly regulated by the fecal microenvironment and served as endpoints of the network (Figure 6A). Bayesian network structure results (bootstrapping number = 2,000, Supplemental Figure S5A, https://doi.org/10.6084/m9.figshare.24460333.v1; Zhu, 2023b) showed that NEFA could potentially be moderated by microbiota from MicroC4 (arc strength = 0.665) and metabolites from

Figure 5. Core metabolic pathways (lipid, AA, aromatic compounds, and energy metabolism) from metabolite clusters with increasing trend at +7 and +21 d relative to calving. A: Kyoto Encyclopedia of Genes and Genomes (KEGG) map of core microbial metabolism pathways of lipid, AA, aromatic compounds and energy metabolism. The metabolites within the differential microbial metabolites are given in blue, and the metabolites shared in energy metabolism are colored in red. Unaffected metabolites from the 4 time points are in gray. B: Correlation between the key metabolites in these pathways and NEFA, HPT, OSI. All of the 120 fecal samples and blood samples were applied for correlation analysis, and the displayed correlation was tested by Spearman’s rank correlation with Bonferroni correction ($P < 0.05$). OSI: Oxidative status indicator. NEFA: Nonesterified fatty acids. HPT: haptoglobin.
MetaC2 (arc strength = 0.676). OSI potential regulators included microbiota from MicroC2 (arc strength = 0.789) and metabolites from MetaC7 (arc strength = 0.531). Haptoglobin was regulated by MicroC1 (arc strength = 0.641; Supplemental Table S18; Zhu, 2023a). Further, Kyoto Encyclopedia of Genes and Genomes enrichment analysis suggested that MetaC2 was mainly involved in degradation of aromatic compounds such as aminobenoate degradation ($P < 0.001$) and benzoate degradation ($P = 0.0082$). Co-metabolism cluster 7 was likewise involved in phenylalanine metabolism ($P = 0.001$), aminobenoate degradation ($P = 0.019$) and furfuraldehyde degradation ($P = 0.028$; Figure 6B). Because NEFA and OSI are endpoints in the network, we addressed if NEFA and OSI could be predicted by fecal microbiota and metabolites in the pre-natal period. Indeed, prenatal NEFA did not differ significantly ($P = 0.55$) between the groups according to the warning value on +7 d (0.8 mmol NEFA, 0.745 OSI), as described in the methods sections on model prediction, but prenatal OSI tended to differ significantly ($P = 0.071$; Figure 6C).

### Table 1. Comparison of plasma physiological parameters, inflammation, oxidative status, and phenotypic characteristics

<table>
<thead>
<tr>
<th>Item</th>
<th>−21 d</th>
<th>−7 d</th>
<th>+7 d</th>
<th>+21 d</th>
<th>$P$-value</th>
<th>SEM</th>
</tr>
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<tr>
<td>Physiological parameters</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>21.6$^a$</td>
<td>18.8$^b$</td>
<td>18.8$^b$</td>
<td>17.8$^b$</td>
<td>&lt;0.001</td>
<td>4.81</td>
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<tr>
<td>AST, U/L</td>
<td>63.8$^a$</td>
<td>66.0$^b$</td>
<td>115$^a$</td>
<td>93.3$^a$</td>
<td>&lt;0.001</td>
<td>33.6</td>
</tr>
<tr>
<td>ALB, g/L</td>
<td>33.4</td>
<td>33.7</td>
<td>33.2</td>
<td>33.8</td>
<td>0.703</td>
<td>6.7</td>
</tr>
<tr>
<td>TP, g/L</td>
<td>82.4$^a$</td>
<td>73.6$^b$</td>
<td>69.8$^b$</td>
<td>77.7$^b$</td>
<td>&lt;0.001</td>
<td>6.7</td>
</tr>
<tr>
<td>BUN, mM</td>
<td>4.88</td>
<td>4.5</td>
<td>4.46</td>
<td>4.76</td>
<td>0.327</td>
<td>1.02</td>
</tr>
<tr>
<td>CREA, μM</td>
<td>90.0$^a$</td>
<td>103$^a$</td>
<td>90.9$^b$</td>
<td>69.8$^a$</td>
<td>&lt;0.001</td>
<td>17.7</td>
</tr>
<tr>
<td>GLU, mM</td>
<td>4.24$^a$</td>
<td>4.11$^a$</td>
<td>3.54$^b$</td>
<td>3.89$^b$</td>
<td>&lt;0.001</td>
<td>0.416</td>
</tr>
<tr>
<td>CHO, mM</td>
<td>3.40$^a$</td>
<td>3.05$^b$</td>
<td>2.64$^b$</td>
<td>4.36$^a$</td>
<td>&lt;0.001</td>
<td>0.938</td>
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<tr>
<td>TG, mM</td>
<td>0.303$^a$</td>
<td>0.281$^b$</td>
<td>0.105$^b$</td>
<td>0.126$^b$</td>
<td>&lt;0.001</td>
<td>0.104</td>
</tr>
<tr>
<td>BHB, mM</td>
<td>0.429$^b$</td>
<td>0.434$^a$</td>
<td>0.852$^a$</td>
<td>0.590$^b$</td>
<td>&lt;0.001</td>
<td>0.256</td>
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<tr>
<td>NEFA, mM</td>
<td>0.137$^b$</td>
<td>0.285$^b$</td>
<td>0.719$^a$</td>
<td>0.497$^a$</td>
<td>&lt;0.001</td>
<td>0.342</td>
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<tr>
<td>Inflammation biomarkers</td>
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<td></td>
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</tr>
<tr>
<td>SAA, μg/mL</td>
<td>30.9$^b$</td>
<td>35.2$^a$</td>
<td>37.6$^a$</td>
<td>34.6$^a$</td>
<td>&lt;0.05</td>
<td>6.63</td>
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<tr>
<td>CPL, U/L</td>
<td>21.9$^a$</td>
<td>20.3$^b$</td>
<td>25.2$^b$</td>
<td>21.1$^b$</td>
<td>&lt;0.05</td>
<td>6.2</td>
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<tr>
<td>HPT, U/L</td>
<td>300$^a$</td>
<td>318$^{ab}$</td>
<td>364$^a$</td>
<td>293$^b$</td>
<td>&lt;0.001</td>
<td>76.5</td>
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<td>Oxidative status biomarkers</td>
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<td>CAT, U/mL</td>
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<td>1.09</td>
<td>1.09</td>
<td>1.08</td>
<td>0.489</td>
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<td>GSH.PX, U/mL</td>
<td>24.1</td>
<td>26.3</td>
<td>29.1</td>
<td>24.4</td>
<td>0.482</td>
<td>9.63</td>
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<tr>
<td>MDA, nmol/mL</td>
<td>1.50$^b$</td>
<td>3.28$^a$</td>
<td>3.57$^a$</td>
<td>3.32$^a$</td>
<td>&lt;0.001</td>
<td>1.91</td>
</tr>
<tr>
<td>TOS, μM</td>
<td>0.326$^a$</td>
<td>0.223$^a$</td>
<td>0.331$^a$</td>
<td>0.072$^b$</td>
<td>&lt;0.001</td>
<td>0.238</td>
</tr>
<tr>
<td>OSI$^a$</td>
<td>0.691$^a$</td>
<td>0.453$^b$</td>
<td>0.650$^b$</td>
<td>0.141$^a$</td>
<td>&lt;0.001</td>
<td>0.353</td>
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<tr>
<td>SOD, U/mL</td>
<td>174$^a$</td>
<td>177$^b$</td>
<td>188$^a$</td>
<td>194$^a$</td>
<td>&lt;0.001</td>
<td>18.7</td>
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<td>T-AOC, mM</td>
<td>0.481$^b$</td>
<td>0.496$^b$</td>
<td>0.517$^{ab}$</td>
<td>0.534$^a$</td>
<td>&lt;0.001</td>
<td>0.0404</td>
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<td>Performance parameters</td>
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<tr>
<td>BW, kg</td>
<td>690 (calving day)</td>
<td>630</td>
<td>&lt;0.001</td>
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<tr>
<td>Milk yield, kg/d</td>
<td>39</td>
<td>46</td>
<td>&lt;0.001</td>
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<tr>
<td>BSC$^3$</td>
<td>3.21</td>
<td>2.76</td>
<td>&lt;0.001</td>
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</table>

$^a$Superscript letters in the table represent significant pairwise differences (Dunn’s test). Groups sharing the same letter are not significantly different from each other, whereas groups with different letters are significantly different at a predetermined level of significance ($P < 0.05$). If a group is labeled with 2 letters, it indicates that this group does not significantly differ from 2 distinct groups labeled with each of the individual letters. The superscript letters “ab” indicate that the group labeled with “ab” does not differ significantly from both the groups labeled with “a” and “b.”

$^b$ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALB = albumin; TP = total protein; CREA = creatinine; GLU = glucose concentrations; CHOL = cholesterol; NEFA = non-esterified fatty acids; SAA = amyloid; CPL = ceruloplasmin; HPT = haptoglobin; CAT = plasma catalase; GSH.PX = glutathione peroxidase; MDA = malondialdehyde; TOS = plasma total oxidative status; OSI = oxidative status indicator; SOD = superoxide dismutase; T-AOC = total antioxidant capacity.

$^a$Oxidative status indicator = total oxidative status per total antioxidant capacity.

$^b$Body condition was scored following the method described by Edmonson et al. (1989), using a 5-point scale (1 = thin, 5 = fat) at 3 time points (0600, 1400, and 2000 h).

### Prediction of Postpartum Phenotypes Based on Fecal Microbiota and Metabolites Using Machine-Learning Models

Nine machine-learning and statistical models were applied to predict blood NEFA and OSI separately. The predictive power of each model with default parameters for the NEFA prediction was determined by an average
value under the 10-fold cross-validation without feature selection. The highest accuracy of 0.91 was observed in the SVM radial model approach (Figure 7A). After 10 times, feature selection using the training set of 10-fold cross-validation, 4 microbiota and 2 metabolites with the top 10 highest Gini coefficient (Figure 7B). The final SVM radial model revealed that 2 metabolites including 3-hydroxy-2-methylpyridine-4,5-dicarboxylate and pyrocatechol, and 3 microbiota including *Mycoplasma*, *Porphyromonas*, and *Paludibacter* contributed to the discrimination of high and low NEFA status (AUC = 1; accuracy = 1.00 in training set; AUC = 1; accuracy = 1.00 in testing set; Figures 7C–E). Since the accuracy in the test set was equal to that in the training set, to eliminate the problem of over-fitting, 85 additional blood and fecal samples from the peripartum cows were applied for external validation (Supplemental Table S2). A total of 83 additional dairy cows were applied in NEFA validation. Our model had an accuracy of 0.963 for predicting NEFA among the external peripartum data (Supplemental Table S19; https://doi.org/10.6084/m9.figshare.21606732.v3; Zhu, 2023a). The SVM model with radial basis function (kernel) was used for predicting OSI prenatally (Figure 7F). *Akkermansia*, *Desulfovibrio*, *Oxobacter* and m-hydroxycinnamic acid were the common top microbiota from 5-fold cross-validation and a model built with these features had an accurate postpartum discrimination ability (AUC = 0.969; accuracy = 0.976 in training set; AUC = 1; accuracy = 1.00 in testing set; Figures 7G, 7H, 7I, and 7J). The model test using external samples also showed an accuracy of 0.889 and AUC 0.929.

### DISCUSSION

Various factors influencing oxidative status, inflammation and NEB, in the peripartum period of dairy cows are interrelated (Sordillo and Raphael, 2013; Putman et al., 2018). In our study, we observed the significant rise in milk yield and decline in body weight after calving, which have been shown to be an important contributor to the rise of NEFA and BHB after calving. While the body fat mobilized is also a major sources of oxidative status (Sordillo and Raphael, 2013). The upward trend of the concentration of MDA from −7 to +7 d corroborated the previous studies. However, OSI on −21, −7 d shows no significant difference with +7 d. This could due to weaker natural antioxidant defense system, reflected in lower SOD and T-AOC before calving. oxidative status is often accompanied by inflammation, and positive acute phase proteins like SAA and HPT showed similar trend with MDA. Fecal microbiota, important markers of host oxidative status, inflammation, NEB and metabolism (Singh et al., 2017; Vallianou et al., 2021), provide a new tool for understanding and mitigating peripartum oxidative status, NEB and inflammation in dairy cows.

Previous studies have shown that fecal microbiota are better indicators of host health than rumen microbiota in ruminants (Shen et al., 2017; Wallace et al., 2019). In the present study, we describe the variation between sequential stages in peripartum period in microbiota and metabolites in dairy cows during transition. We found that oligotrophic phyla declined from −21 and −7 to +7 and +21 d, and carbohydrate dependent genera rose at +7 and +21 d, especially on +7 d. Fecal metabolites from each time point mainly consisted of aromatic compounds, lipids and carbohydrates and amino acids. We identified the relationships between relevant pathways within aromatic compounds, lipids and AA metabolism for supplementing and promoting energy metabolism, which may potentially relate to host peripartum oxidative status, inflammation, NEB. The relationship between fecal microenvironment and host OSI and NEFA was confirmed by Bayesian network. Finally, an identification model of host OSI and NEFA with high accuracy based on fecal microbiota and metabolites was constructed.

The temporal variation of fecal microbiota in dairy cows during the peripartum period was carefully described in our study. In contrast to rumen microbiota remodeling between −21 and −7 d as shown in the study of Bach et al. (2019), hindgut microbiota are slower in adapting to the new lumen contents and they show an imbalance on +7 and +21 d (Huang et al., 2020). The decrease in the abundance of oligotrophic phyla such as *Verrucomicrobia* and increase of copiotrophic *Bacteroidetes* after parturition is corresponding to feed changes from forage to concentrate (Serena et al., 2018; Bach et al., 2019). At genus level, *MicroC4* was the only cluster dominated by *Bacteroidetes*, with high relative abundances of *Bacteroidales unclassified, Lach-
The microbiota have been known for the utilization of carbohydrates and production of organic acids (Blanco-Morales et al., 2020). A co-occurrence network comparison revealed vulnerable and uniform interactions between microbiota on +7 d, which further illustrate the remodeling of hindgut microbiota under the effect of a rapid feed dietary shift. Investigating functional dissimilarity of intestinal microbiota by PICRUSt2, we found that the microbial function is kept stable prenatally. Remarkably, energy metabolism including glycolysis and TCA cycle pathway increased between −7 and +7 d, and reached higher values between +7 and +21 d, reflecting the adaptability of microbiota in response to high energy requirements between +7 and +21 d.

Microorganisms generally infiltrate the hindgut metabolic microenvironment. Three different feed-related categories of metabolites, sterol lipids, organooxygen compounds and phenols, all derived from soybean, grain, hay and their metabolic products, were identified as significant factors shaping the microbiota community during the peripartum period and indicating a major role for feed for microbiota. Sterols lipids were the major factors for microbiota variation in this study. Plant sterols were reported to have antioxidant and anti-inflammatory activities (Vilahur et al., 2019), and can be absorbed in the hindgut (Samtiya et al., 2021). However, plant sterols also caused the reduc-

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**Figure 6.** Correlation assessment of dynamic microbiota, microbial metabolites clusters (Co-Meta clusters, Meta clusters in Figure 2) and OSI, NEFA, HPT. A: Bayesian networks generated from HPT, OSI, NEFA, and dynamic microbial and metabolic clusters. B: Functional enrichment of metabolic clusters (MetaC7, MetaC2). C: Variation of NEFA and OSI of the 30 dairy cows between high and low groups based on NEFA and OSI at +7 d (Time 3) relative to calving. The structure of Bayesian networks was analyzed using tabu algorithm with arc strength >0.5 under 2,000 times Botstrapping based on the relative abundance of genera microbiota and blood index. OSI: Oxidative status indicator. NEFA: nonesterified fatty acids. HPT: haptoglobin.
Figure 7. The performance of different prediction models of host OSI, NEFA levels in blood based on microbes and microbial metabolites within related clusters (MicroC2, MetaC7 for OSI prediction, MicroC4, MetaC2 for NEFA prediction). A and F: Prediction accuracy using different models and variables for NEFA and OSI respectively. B and G: The selected variables for the support vector machine (SVM) radial model and their importance biomarkers for predicting NEFA and OSI, respectively. C and H: Receiver operating characteristic (ROC) curve of the performance of the SVM radial model for NEFA and OSI in the training data set, respectively. D and I: ROC curve of the performance of the SVM radial model for NEFA and OSI in the testing data set, respectively. E and J: ROC curve and confusion matrix of the performance of the SVM radial model for NEFA and OSI in external data set, respectively. OSI: Oxidative status indicator. NEFA: nonesterified fatty acids. HPT: haptoglobin. Sensitivity: true positive rate. Specificity: true negative rate.
lipid and AA metabolism. As recently reported, upshifted from aromatic compounds metabolism toward postnatal periods, the energy metabolism pathways metabolism. Moreover, between the prenatal and the but also by intermediate metabolites of energy metabolism between +7 and +21 d were not only glycolysis. It is interesting that the upregulated energy metabolic pathways are involved in the TCA cycle and Interestingly, these major downstream metabolites of +7 d (Co-MetaC4). Lysophosphatidylethanolamine and LysoPC were the 2 largest components within the glycerophospholipids, which were upregulated on +7 and +21 d (belonging to Co-MetaC2 and 4), and have been reported to readily cross the enterocyte membrane by passive diffusion (Stahl et al., 1999). Lysophosphatidylcholine can efficiently drive intestinal lipid absorption by promoting lipid uptake and enforcing the lipoprotein effect. Lysophosphatidylethanolamine is known for inducing nonalcoholic fatty liver disease by suppressing liver lipolysis and fatty acid biosynthesis (Nakano et al., 2009; Yamamoto et al., 2022), which can potentially accelerate the mobilization of body fat and the deposition of liver fat between +7 d, +21 d. For aromatic metabolism, aminobenzoate degradation was the major pathway enriched on −21 d, −7 d. As the newest vitamin B, aminobenzoate is widely used as supplement in dairy cows since it is involved in gluconeogenesis and it increases milk production (Preynat et al., 2009). Interestingly, these major downstream metabolites of metabolic pathways are involved in the TCA cycle and glycolysis. It is interesting that the upregulated energy metabolism between +7 and +21 d were not only dominated by a higher proportion of carbohydrates, but also by intermediate metabolites of energy metabolism from lipids, amino acids and aromatic compounds metabolism. Moreover, between the prenatal and the postnatal periods, the energy metabolism pathways shifted from aromatic compounds metabolism toward lipid and AA metabolism. As recently reported, upregulated glycolysis of fecal microbiota may interfere with many important biosynthetic pathways that are critical for intestinal cell proliferation as observed in human studies (Wang et al., 2019). Some microbial respiration products, such as peroxisome proliferator activated receptors were reported to be activated by nicotinamide adenine dinucleotide and ATP to induce peroxidase proliferation, thus affecting the intracellular energy balance of the host (Scatena et al., 2008; Tian et al., 2022), as shown in the correlation analysis (Figure 5B, Supplemental Table S17).

Previous studies have detailed the relationships between host blood parameters and fecal microbiota in humans and monogastric animals (Schulfer et al., 2019; Ni et al., 2021). Here, we explored correlation between the dynamics of fecal microbiome and 3 predominant blood OSI, HPT and NEFA levels during the peripartum period of dairy cows. Interestingly, we noticed that fecal microbiota is one of the potential factors regulating blood indicators. Thus, hindgut microbiota and their metabolites might be one of the potential factors responsible for oxidative status, inflammation and the NEB in peripartum dairy cows. The high accuracy of our machine-learning model relates to reliable fecal parameters for high level of OSI and NEFA prediction and diagnosis. Moreover, the drivers for NEFA prediction including carbohydrate metabolism related genera and phenylalanine metabolism dependent metabolites, provide further evidence for the effects of these pathways on the host. Although our model demonstrates a potential association between prenatal microbiota and postnatal OSI, our data are far from sufficiently explaining the regulatory mechanism. The difficulties with obtaining the relative abundances beyond “-omics” data and the insufficient data volume relating to high costs challenge the sequencing approach to predict OSI and NEFA. We set out to build predictive models using abundance ratios of microbiota and their metabolites for the predominant and stable genera in the fecal.

Taken together, by characterizing the composition and function of microbiota and metabolites in the intestinal microenvironment of dairy cows during the peripartum period, we explored potential correlation and assessed the predictive power of dynamic fecal characteristics with host oxidative status, NEB and inflammation. These insights of ruminant fecal microenvironments between sequential stages in peripartum period not only suggest a relationship between the intestinal microenvironment and the OSI, NEFA level in ruminants, but also complement the importance of further research on hindgut microbiota for ruminant health. Thereby extended knowledge and improved modeling will provide tools to lower and ultimately prevent peripartum oxidative status, NEB in dairy cows.
CONCLUSIONS

Our study reveals variations in hindgut microbiota composition, interactions, and metabolism during the peripartum period in dairy cows. These findings highlight the crucial role of microbiota and their metabolites in peripartum metabolic disturbances during the transition phase. We identified key metabolites associated with co-metabolism pathways such as amino acids, lipids, aromatic compounds, and energy metabolism. These metabolites, including phenol, phenylalanine, LysoPC 15:0, LysoPE 9:0, citric acid, and succinic acid, showed strong correlations with the host’s oxidative status and NEB. Specific microbiota clusters (2, 4, and 7) and MetaC2, 4, and 7 were also associated with changes in oxidative status and energy metabolism. Furthermore, 3-hydroxy-2-methylpyridine-4,5-dicarboxylate, pyrocatechol, Mycoplasma, Porphyromonas, and Paludibacter within NEFA and OSI-related clusters demonstrated high accuracy in predicting NEFA (82.4%) and OSI (88.9%) levels. Overall, our study provides a theoretical foundation for implementing preventive protocols to regulate peripartum oxidative status and NEB in dairy cows in the future.

ACKNOWLEDGMENTS

Our research was supported by grants from the China Agriculture (Dairy) Research System (CARS-36, Beijing, China), and the “Hundred Talents Program” Research Professor Start-up Fund of Zhejiang University (Hangzhou, China). We thank the staff of the Hangjiang Dairy Farm (Hangzhou, China) for milking and taking care of the dairy cows. We also be grateful to the members of the Institute of Dairy Science, Zhejiang University, for their assistance with sampling and analysis of the samples. Data availability statement: A total of 120 samples’ raw pair-end 16S rRNA gene sequencing data were used in this study. Eight of the samples at +7 d were downloaded from our previously published data in China National Microbiology Data Center (NMDC; https://nmdc.cn/; BioProject NMDC10018108; Sample number NMDC20047188; Accession number NMDC40021951, NMDC40021953, NMDC40021956, NMDC40021960, NMDC40021963, NMDC40021966, NMDC40021967, NMDC40021969). The remaining 112 samples’ data were deposited in NMDC Data Center (BioProject number NMDC10018108; Sample number NMDC20047188) with accession number NMDC40027296-NMDC40027407. The authors have not stated any conflicts of interest.

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