Metabolomic and Lipidomic Approaches to Evaluate the Effects of *Eucommia ulmoides* Leaves on Milk Quality and Biochemical Properties

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Eucommia ulmoides leaves (EUL) contain a variety of natural bioactive compounds including chlorogenic acid, geniposide acid, and aucubin. These bioactive chemicals improve immune function and regulate lipid metabolism. The aim of this study was to investigate the effects of EUL on the biochemical properties of milk. Twenty Holstein dairy cows were randomly allocated to two groups fed a control (CTR, diet without EUL, n = 10) or EUL (diet containing 3% EUL, dry matter, n = 10) diet for 55 d. At the end of the experimental period (d 55), milk samples were collected and analyzed to determine their composition. Though levels of milk fat, protein, lactose, and total milk solids were similar between the groups, small molecules, metabolites, lipids, and cytokines differed. Compared with the CTR group, the EUL group had an improved cluster of differentiation (CD)4/CD8 ratio (P < 0.05) and lower interleukin (IL)-8 and IL-6 content (P < 0.05). Metabolomics analysis identified 14 metabolites including 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid (FC = 3.129), adrenic acid (FC = 2.830), and eicosapentaenoic acid (FC=1.685) as having significantly increased in the EUL group (P < 0.05) while 11 metabolites, including indole-2-carboxylic acid (FC = 0.636), cholic acid (FC = 0.430), and creatine (FC = 0.784) had significantly decreased (P < 0.05).

Based on a constructed metabolome map, linoleic acid metabolism had the highest impact value for EUL. A total of 21 lipid classes and 1,094 lipid species were detected in the milk by lipidomic analysis, among which 40 differed significantly between the CTR and EUL groups. The present findings showed that the EUL altered milk composition. Correlation analysis showed that 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid, adrenic acid, and eicosapentaenoic acid levels were negatively correlated with those of the inflammatory factors IL-6 and IL-8 (P < 0.05), indicating that EUL improved milk quality by reducing inflammatory factors and increasing the CD4/CD8 ratio. Overall, our data demonstrate that EUL had positive effects on milk antioxidant parameters, immune indices, and micro-composition metabolism, thereby improving milk quality.

**Keywords:** dairy cows, Eucommia ulmoides leaves, milk composition, milk metabolomics, milk lipidomics
INTRODUCTION

Eucommia ulmoides (Du-Zhong) is a well-known medical plant that has been used in Chinese medicine and food production since ancient times. In addition, Eucommia ulmoides has also been planted in Japan, Korea, and some other countries as a source for health drinks. Eucommia ulmoides leaves (EUL) are a facultative nutrient food source that contains natural bioactive compounds with multiple functions for healthcare (1). EUL contains more than 50 bioactive substances including chlorogenic acid, geniposide acid, and aucubin, which have antioxidant properties and improve immune function, as well as regulating lipid metabolism (2, 3). Supplementation with chlorogenic acid extract from EUL is reported to alleviate the negative effects of heat stress on meat quality and fatty acid profiles in broiler breast meat or mutton, by improving antioxidant status (4, 5). Additionally, fatty acid, amino acids, and polysaccharides were identified from EUL (6, 7). Zhu and Sun reported that EUL has rich polyunsaturated fatty acids (PUFA) such as linoleic acid and α-linolenic acid (7). Our previous research found that EUL can regulate lipid partitioning in sheep and improve mutton quality, as well as modulate glucose metabolism (8, 9). Therefore, raw EUL can be used as a high-quality animal foodstuff, or functional additive, for livestock.

Bovine milk is an important food source because milk contains all the essential nutrients for young people and other mammals (10); however, it is a mutable product that can be affected by many factors including diet, the environment, and physiological status. Recent studies have shown that forage quality has an important impact on the quality of milk. It was found that feeding low quality forage to cows not only exhibited low feed efficiency but also led to lower milk performance (11). The Chinese dairy industry suffers from limitations in forage quality. Until now, limited evidence has been available on the utilization of EUL to improve dairy cow milk composition and its biochemical properties. Therefore, understanding biochemical properties and metabolic mechanisms involved in milk production in response to EUL is helpful for developing strategies for effective, efficient utilization of the abundant EUL.

It is difficult to discern metabolic alterations of milk from cows fed specific diets using traditional measurement methods. Metabolomics and lipidomics analysis are effective approaches for studying metabolites in fluids and tissues, and other physiological and biochemical properties (12, 13). Therefore, in this study, metabolomics and lipidomics were used to assess the milk composition and metabolite profile of dairy cows in response to EUL, with the aim of evidencing its function on dairy cow metabolism.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

All procedures were approved by the Animal Care and Use Committee of Henan Agricultural University (approval number: HENAU-2018-015). Twenty Holstein dairy cows of similar age (parity 2), lactation stage (150 ± 20 d), and milk production (25.2 ± 5.00 kg/d) were selected and randomly divided into two groups: CTR (control, diet without EUL, n = 10) and EUL (diet containing 3% EUL, dry matter, n = 10). The range of body condition scores of cows was 3.0–3.5. Before the formal experiment, we collected milk samples of experimental cows for milk composition analysis and found that there was no difference in milk composition before the EUL treatment (Supplementary Table 1). All the animals remained in good health throughout the experimental period with no visible deleterious health effects. This level of EUL inclusion (3%) in the diet was chosen based on the biochemical indicators, hormone concentrations, and immune parameters in blood results from preliminary studies (Supplementary Table 2). Eucommia trees were cultivated in Henan (34 39’ N, 112 24’ E). EUL were collected from Eucommia trees from September to October. After drying at 65°C for 72 h, EUL were crushed through a 40 mesh sieve to generate EUL powder, which was packaged and stored in waterproof bags until use. The composition and nutritional values of the EUL were as follows (dry matter basis, %): dry matter: 89.5%, crude protein: 8.3%, Ca: 2.06%, P: 0.15%, ether extract: 7.54% and its fatty acid composition is palmitic acid: 10.80%, hexadecatetraenoic acid: 0.85%, linoleic acid: 1.95%, and linolenic acid: 45.58%. Geniposide acid, chlorogenic acid, and aucubin in EUL were quantified by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) (ProNetsBio, Wuhan, China) using a Poroshell 120 SB-C18 reverse phase column (2.1 × 150, 2.7 um; AGLIENT, United States). The geniposide acid, aucubin, and chlorogenic acid content in EUL were 77.15 ± 8.94, 20.71 ± 0.82, and 2,264.16 ± 46.51 µg/g, respectively. EUL was supplemented to the diet according to weight and fed as a mixed ratio. The diet was fed to the cows twice daily (at 7:00 and 17:00 h), with ad libitum intake. During the experiment, feed intake was recorded daily allowing for approximately 5–10%orts. The detailed composition and nutrient content of the diets are presented in Table 1.

Cows were housed in a shaded open barn, with free access to water. The trial duration was 55 d. Cows were gradually adjusted to the experimental diet over a 14 d transition period. In the experiment, feed intake was recorded daily and nutrient content of the diets are presented in Table 1. The geniposide acid, aucubin, and chlorogenic acid content in EUL were 77.15 ± 8.94, 20.71 ± 0.82, and 2,264.16 ± 46.51 µg/g, respectively. EUL was supplemented to the diet according to weight and fed as a mixed ratio. The diet was fed to the cows twice daily (at 7:00 and 17:00 h), with ad libitum intake. During the experiment, feed intake was recorded daily allowing for approximately 5–10%orts. The detailed composition and nutrient content of the diets are presented in Table 1.

Sampling and Analysis

At the end of the trial, two 50 mL aliquot milk samples of individual cows were collected proportional to the yield (4:3:3, composite) in the morning, noon, and evening. Regular parameters, including fat, protein, lactose, total milk solids, and somatic cell count (SCC), were analyzed using a MilkoScan FT⁺ instrument (Foss Electric A/S, Foss, Hillerød, Denmark). Samples allocated for metabolomics and lipidomics were immediately stored in a liquid nitrogen container to minimize any possible metabolite degradation, then transferred to a cryogenic refrigerator and stored at −80°C until further analysis.

For biochemical analysis, 10 mL of the milk samples were skimmed by centrifugation at 3,000 g for 15 min at 4°C, and then centrifuged at 12,000 g for 30 min at 4°C using a high-speed freezing centrifuge (Avanti JXN-30, Beckman Coulter,
TABLE 1 | Experimental diet constituents.

| Item                          | CTR  | EUL  |
|-------------------------------|------|------|
| Ingredient (%, DM basis)      |      |      |
| Corn silage                   | 25   | 25   |
| Alfalfa hay                   | 20   | 20   |
| Oat hay                       | 7    | 4    |
| Eucommia ulmoides leaves      | 0    | 3    |
| Steam-flaked corn             | 20   | 20   |
| Cottonseed                    | 3    | 3    |
| Soybean meal                  | 12.7 | 12.7 |
| Beet pulp                     | 2.1  | 2.1  |
| Brewers grain                 | 3.9  | 3.9  |
| Molasses                      | 2    | 2    |
| Premix*                       | 4.3  | 4.3  |
| Nutrient composition          |      |      |
| DM, %                         | 66.28| 66.42|
| CP, % of DM                   | 15.06| 15.09|
| NDF, % of DM                  | 31.74| 31.04|
| ADF, % of DM                  | 20.68| 20.49|
| NFC, % of DM                  | 44.15| 44.61|
| Ca, % of DM                   | 0.59 | 0.63 |
| P, % of DM                    | 0.33 | 0.33 |
| NEL, Mcal/kg of DM            | 1.56 | 1.57 |

*Formula to provide (per kg of DM) 500,000–700,000 IU of vitamin A, 140,000–170,000 IU of vitamin D3, 2,000–4,000 IU of vitamin E, 7,000–9,000 mg of Zn, 40–80 mg of Se, 180 mg of I, 1,400–2,500 mg of Fe, 15–30 mg of Co, 1,400–2,500 mg of Mn, and 1,400–2,500 mg of Cu.

California, USA). Supernatants were separated into several clean vials to measure biochemical indices, related hormones, and immune indicators. Biochemical parameters, including superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px); hormones, such as growth hormone (GH), triiodothyronine (T3), insulin (INS), glucagon (GC), cortisol (CORT), and adrenaline (ADR); and immune indicators, including immunoglobulin A (IgA), immunoglobulin G (IgG), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon-γ (IFN-γ), cluster of differentiation 4 (CD4), and cluster of differentiation 8 (CD8), among others, were tested using ELISA kits (Shanghai BlueGene Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's instructions. The inter- and intra-assay coefficient of variations of these kits were <12 and <10%, respectively. These kits had no obvious cross-reactivity.

Metabolomics
Sample Preparation and Extraction
Samples were thawed at 4°C and then shaken and mixed evenly. Milk samples (100 μL) were added to 400 μL of cold methanol/acetonitrile (1:1, v/v), and the mixture vortexed. Subsequently, all sample mixtures were stored at −20°C for 60 min, and then centrifuged at 14,000 g for 20 min at 4°C. Supernatants were collected, vacuum dried, and reconstituted with 100 μL water/acetonitrile (1:1, v/v). The reconstituted solution was vortexed and centrifuged at 14,000 g for 15 min at 4°C and the supernatant used for LC-MS/MS analysis.

Liquid Chromatography Positive Ion Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) Analysis
Prepared samples were separated using a UHPLC HILIC column (1.7 μm, 2.1 mm × 100 mm; Waters, Massachusetts, United States). Column temperature was 25°C and the injection volume was 2 μL. Mobile phase A comprised 25 mM ammonium acetate and 25 mM aqua ammonia, and mobile phase B was acetonitrile. To prevent the effects of fluctuations in instrument signal detection, a random sequence was used for continuous analysis of the samples. Processed sample stability was determined by repeated analysis of quality control milk samples. During the entire process of analysis, samples were placed in an automatic sampler at 4°C.

Samples separated by UHPLC were analyzed on a Triple TOF 5600 mass spectrometer (AB SCIEX, Boston, United States). ESI positive and negative modes were used and reaction monitoring parameters were as follows: ion source gas 1, 60 psi; ion source gas 2, 60 psi; curtain gas (CUR), 30 psi; source temperature, 600°C; ion spray voltage floating, ± 5,500 V (positive and negative modes); time-of-flight mass spectrometry (TOF MS) scan m/z range, 60–1,000 Da; product ion scan m/z range, 25–1,000 Da; TOF MS scan accumulation time, 0.20 s/spectra; and product ion scan accumulation time, 0.05 s/spectra. Information-dependent acquisition (IDA) was used for secondary mass spectrometry, and a high-sensitivity model was adopted. The de-clustering potential was ± 60 V (positive and negative modes), with collision energy 35 ± 15 eV. The IDA parameters were as follows: exclude isotopes within 4 Da; candidate ions to monitor per cycle, 6.

Lipidomics
Sample Preparation and Extraction
The samples were thawed slowly at 4°C and thoroughly vortex mixed. Milk samples (100 μL) were added to 200 μL water and vortexed. Cold methanol (200 μL) was then added to the composite milk samples and re-vortexed. The composite milk samples were then added to 800 μL methyl tert butyl ether and thoroughly vortex mixed. All sample mixtures were placed in an ultrasound water bath at 4°C for 20 min, and stored at 25°C for 30 min, before being centrifuged at 14,000 g for 15 min at 10°C. The supernatant was then decanted and freeze-dried in nitrogen and reconstituted with 300 μL 90% isoopropanol/acetonitrile (1:1, v/v). The reconstituted solution was vortexed and centrifuged (14,000 g, for 15 min at 10°C). The resulting supernatant was used for LC-MS/MS analysis.
The temperature of the column was set at 45°C and the injection volume was 3 µL. The mobile phase consisted of phases A and B. Mobile phase A was constituted using ammonium acetonitrile and isopropanol (acetonitrile: isopropanol = 3:7, v/v) and mobile phase B was ammonium formate acetonitrile isopropanol solution (acetonitrile: isopropanol = 1:9, v/v).

The samples were separated by UHPLC and analyzed on the Q Exactive Plus mass spectrometer (Thermo Scientific, Massachusetts, United States). The electrospray ionization (ESI) source conditions were as follows in the positive mode: heater temperature = 300°C; sheath gas flow rate = 45 arb; auxiliary gas flow rate = 15 arb; sweep gas flow rate = 1 arb; spray voltage = 3.0 kV; capillary temperature = 350°C; S-lens radio frequency (RF) level = 50%; and MS1 scan ranges = 200–1,800. In the negative mode: heater temperature = 300°C; sheath gas flow rate = 45 arb; auxiliary gas flow rate = 15 arb; sweep gas flow rate = 1 arb; spray voltage = 2.5 kV; capillary temperature = 350°C; S-Lens RF level = 60%; and MS1 scan ranges = 250–1,800. The mass charge ratio of lipid molecules and lipid fragments was determined according to the following protocol: 10 fragments (MS2 scan, high-energy collisional dissociation) were collected after each full scan. The resolution of MS was 70,000, and that of MS2 was 17,500.

**Statistical Analysis**

The raw MS data for the metabolomics were converted to MzXML files using ProteoWizard MS Convert and processed using XCMS for feature detection, retention time correction, and alignment. The metabolites were identified by accuracy mass (<25 ppm) and MS/MS data that were matched with our standards database. LipidSearch software version 4.1 (Thermo Scientific™) was used for feature detection, retention time correction, and alignment.

In the extracted ion features, only variables having more than 50% of the non-zero measurement values in at least one group were retained. The data were normalized by the total peak area. SIMCA-P14.1 (Umetrics, Umea, Sweden) was used for pattern recognition multivariate statistical analysis. After Pareto scaling, principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed. PCA was used to visualize the dataset, including the similarities and differences. The PLS-DA model was validated by 7-fold permutation tests. OPLS-DA was performed to maximize the covariance between the measured data and the response variable. To avoid over-fitting, model validity was evaluated by a permutation test (14). Significantly different metabolites were metabolites with variable influence on projection (VIP) values from the OPLS-DA model >1.0 and p-values <0.05 for two-tailed Student’s t-tests of the raw data.

Differential metabolites were further identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) online database (http://www.kegg.jp/). The KEGG pathway enrichment of the differentially expressed metabolites was assessed using Fisher’s Exact Test; P < 0.05 was the significance threshold. The volcano map was created using R software (Version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria).

Differences in milk composition, biochemical indices, hormones, and immune parameters were analyzed by t-test (SPSS version 18, IBM, New York, NY, United States). Milk composition data, biochemical parameters, hormone levels, and immune indices were the test variables, and the EUL and

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**TABLE 2** | Effects of EUL on dry matter intake and milk yield and general composition (n = 10).

| Item                  | CTR | EUL   | SEM   | P   |
|-----------------------|-----|-------|-------|-----|
| Dry matter intake (kg/d) | 19.81 | 19.62  | 0.91  | 0.562 |
| Milk yield (kg/d)      | 23.95 | 24.64  | 1.42  | 0.636 |
| Milk fat (%)           | 6.07  | 5.59   | 0.284 | 0.116 |
| Milk protein (%)       | 3.34  | 3.44   | 0.068 | 0.176 |
| Milk lactose (%)       | 4.83  | 4.79   | 0.076 | 0.612 |
| Total milk solids (%)  | 14.60 | 13.93  | 0.357 | 0.093 |
| Somatic cell count, SCC (10^5/mL) | 21.67 | 14.62  | 3.92  | 0.102 |

**TABLE 3** | Biochemical indicators, hormone concentrations, and immune parameters in milk (n = 10).

| Items                  | CTR    | EUL    | SEM    | P    |
|------------------------|--------|--------|--------|------|
| Antioxidant parameters  |        |        |        |      |
| SOD (µg/mL)            | 1.14   | 1.37   | 0.105  | 0.059 |
| GSH-Fx (ng/mL)         | 15.38  | 16.51  | 0.964  | 0.271 |
| GR (ng/mL)             | 1.97   | 1.48   | 0.284  | 0.112 |
| MDA (ng/mL)            | 5.56   | 5.24   | 0.312  | 0.327 |
| Hormone concentrations  |        |        |        |      |
| GH (ng/mL)             | 4.76   | 5.15   | 0.858  | 0.662 |
| T3 (ng/mL)             | 168.05 | 185.60 | 15.74  | 0.291 |
| INS (ng/mL)            | 0.63   | 0.80   | 0.067  | 0.028 |
| GC (ng/mL)             | 2.56   | 2.61   | 0.285  | 0.86  |
| CORT (ng/mL)           | 102.69 | 95.34  | 7.61   | 0.357 |
| ADR (ng/mL)            | 8.25   | 8.37   | 0.886  | 0.896 |
| Immune indicators      |        |        |        |      |
| IgA (mg/mL)            | 0.98   | 0.94   | 0.068  | 0.619 |
| IgG (mg/mL)            | 3.72   | 4.20   | 0.448  | 0.309 |
| TNF-α (pg/mL)          | 88.61  | 86.14  | 6.69   | 0.72  |
| IL-1β (pg/mL)          | 73.93  | 58.57  | 12.13  | 0.234 |
| IL-6 (pg/mL)           | 191.31 | 105.43 | 20.76  | 0.008 |
| IFN-γ (pg/mL)          | 55.54  | 63.78  | 5.98   | 0.198 |
| CD4 (ng/mL)            | 3.97   | 4.24   | 0.461  | 0.573 |
| CD8 (ng/mL)            | 1.99   | 1.63   | 0.197  | 0.102 |
| CD4/CD8                | 2.00   | 2.62   | 0.174  | 0.005 |

CTR, basal diet; EUL, basal diet + 3% Eucommia ulmoides leaves; SEM, Standard error of mean.
CTR were used as grouping variables. Differences were reported as significant at $P < 0.05$; $P < 0.01$ was considered highly significant and $0.05 \leq P < 0.10$ was designated as a tendency toward significance.

Simple correlations between differential metabolites and inflammatory factors (IL-6 and IL-8) were analyzed with Pearson correlation analysis, and multiple linear regressions among differential metabolites and inflammatory factors (IL-6 and IL-8) were analyzed by stepwise correlation analysis.

**RESULTS**

**Effects of EUL on General Milk Composition**

General milk composition in the CTR and EUL groups are presented in Table 2. These data demonstrate that total milk solids exhibited a trend toward increasing ($P = 0.093$) in the EUL group. There were no significant differences in general milk fat, protein, or lactose between groups ($P > 0.10$).

**Effects of EUL on Biochemical Indicators, Hormone Concentrations, and Immune Parameters in Milk**

Though the general composition remained stable, the specific composition and properties, such as antioxidant parameters, immune indicators, and relevant hormones, showed a large difference between groups (Table 3). These data demonstrate that the EUL group exhibited a trend toward increased SOD levels ($P = 0.059$). Further, INS was significantly increased in the EUL group compared with the CTR group ($P < 0.05$), whereas there were no significant differences in other hormone indices. Levels of the immune indicators, IL-6 and IL-8, were substantially reduced ($P < 0.05$), whereas the CD4/CD8 ratio was markedly increased ($P < 0.05$) in the EUL group relative to the CTR group; other immune indicators did not exhibit significant differences.

**Metabolomics Analysis of Differential Metabolites in the CTR and EUL Groups**

OPLS-DA results for the two groups showed clear separation and discrimination between the CTR and EUL groups, indicating that there was a notable difference between them (Supplementary Figure 1). Figure 1 shows different metabolites according to FC $> 1.5$ and $P < 0.05$ in positive and negative mode between two groups. Metabolites meeting the criteria, a variable importance in projection (VIP) value $> 1$, and $P < 0.05$ indicated a significant difference between the groups (Table 4). Twenty-five differential metabolites were identified between the CTR and EUL groups; 14, including 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid (FC $= 3.129$), adrenic acid (FC $= 2.830$), and eicosapentaenoic acid (FC $= 1.685$), were significantly increased in the EUL group whereas 11 metabolites, including indole-2-carboxylic acid (FC $= 0.636$), cholic acid (FC $= 0.430$), and creatine (FC $= 0.784$), were significantly decreased ($P < 0.05$).

**Pathways Analysis of Differential Metabolites**

Attributive analysis using KEGG identified 25 differential metabolites as enriched in 13 metabolic pathways (Figure 2) involved in amino acid metabolism, carbohydrates metabolism, fatty acid metabolism, and energy metabolism, indicating that EUL influences many biochemical metabolic pathways to affect milk quality. Visually interpreting a metabolome map, linoleic acid metabolism, and retrograde endocannabinoid signaling were the pathways most impacted by EUL (Figure 2). The ABC transporters are one of the most impacted pathways by EUL.

**Lipidomics**

Lipid composition of the milk, determined by lipidomic analysis, is presented in Supplementary Figure 2. A total of 21 lipid classes, including 1,094 lipid species, were detected in the
TABLE 4 | Differential metabolites in milk between the EUL and CTR groups (n = 10, CTR and n = 8, EUL).

| Description | VIP   | FC    | FDR  | m/z    | Rt (s) |
|-------------|-------|-------|------|--------|--------|
| Beta-citronellol | 2.325 | 0.732 | 0.021 | 198.18 | 53.21  |
| L-carnitine    | 1.967 | 1.774 | 0.022 | 144.10 | 659.24 |
| L-palmitylcarnitine | 2.082 | 2.086 | 0.022 | 400.34 | 362.44 |
| 5-aminopentanoic acid | 1.224 | 1.327 | 0.039 | 118.09 | 674.23 |
| Indole-2-carboxylic acid | 2.310 | 0.636 | 0.039 | 162.05 | 756.55 |
| PC (16:0/16:0) | 1.887 | 1.558 | 0.039 | 309.97 | 309.97 |
| Levonordefrin  | 2.240 | 0.559 | 0.039 | 206.08 | 360.44 |
| 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine | 1.808 | 1.701 | 0.039 | 301.65 | 301.65 |
| 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine | 1.909 | 1.561 | 0.039 | 306.81 | 306.81 |
| 5-aminopentanoic acid | 2.214 | 1.327 | 0.039 | 118.09 | 674.23 |
| Indole-2-carboxylic acid | 2.310 | 0.636 | 0.039 | 162.05 | 756.55 |
| PC (16:0/16:0) | 1.887 | 1.558 | 0.039 | 309.97 | 309.97 |
| Levonordefrin  | 2.240 | 0.559 | 0.039 | 206.08 | 360.44 |
| 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine | 1.808 | 1.701 | 0.039 | 301.65 | 301.65 |
| 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine | 1.909 | 1.561 | 0.039 | 306.81 | 306.81 |
| D-lactose      | 1.526 | 1.353 | 0.047 | 325.11 | 798.38 |
| Guanosine      | 1.656 | 0.555 | 0.048 | 266.09 | 269.68 |
| 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid | 6.411 | 3.129 | 0.000 | 756.55 | 309.97 |
| Eicosapentaenoic acid | 2.708 | 1.685 | 0.005 | 301.22 | 98.31  |
| Arachidonic acid (peroxide free) | 8.231 | 2.281 | 0.000 | 303.23 | 97.21  |
| Norethindrone acetate | 7.347 | 3.272 | 0.005 | 331.26 | 97.21  |
| 16-hydroxypalmitic acid | 4.191 | 0.694 | 0.021 | 329.25 | 96.52  |
| Adrenic acid   | 4.352 | 2.830 | 0.053 | 339.20 | 60.62  |
| Arachidonic acid (peroxide free) | 4.352 | 2.830 | 0.053 | 339.20 | 60.62  |
| Norethindrone acetate | 7.347 | 3.272 | 0.005 | 331.26 | 97.21  |
| Eicosapentaenoic acid | 2.708 | 1.685 | 0.005 | 301.22 | 98.31  |
| 16-hydroxypalmitic acid | 4.191 | 0.694 | 0.021 | 329.25 | 96.52  |
| Alpha-D-glucose | 4.498 | 0.430 | 0.039 | 452.28 | 399.55 |
| Cholic acid    | 4.498 | 0.430 | 0.039 | 452.28 | 399.55 |
| Benzoic acid   | 4.498 | 0.430 | 0.039 | 452.28 | 399.55 |
| 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine | 1.392 | 1.516 | 0.039 | 111.01 | 538.84 |
| Dihydroxyacetone | 2.713 | 0.742 | 0.039 | 111.01 | 840.83 |
| Creatine      | 1.235 | 0.784 | 0.039 | 130.06 | 629.63 |
| Cytidine monophosphate | 2.039 | 0.439 | 0.047 | 613.14 | 796.84 |
| N-acetylneuraminic acid | 2.039 | 0.439 | 0.047 | 613.14 | 796.84 |
| N-acetylmannosamine | 4.745 | 0.603 | 0.047 | 220.08 | 486.82 |

CTR, basal diet; EUL, basal diet + 3% Eucommia ulmoides leaves; VIP, variable importance in projection; FC, fold-change, mean value of peak area obtained from the EUL group/mean value of peak area obtained from the CTR group; FC values > 1 indicate that metabolites are present at higher levels in the EUL group than the CTR group and FC values < 1 indicate that metabolites are present in the EUL group less than the CTR group. m/z, mass-to-charge ratio; Rt, retention time.

The Correlation Analysis Between Differential Metabolites and Inflammatory Factors

Pearson correlation analysis was used to assess the relationships between differential metabolites and inflammatory factors (IL-6 and IL-8) (Table 6). Metabolites and lipid species, such as 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid, adrenic acid, and eicosapentaenoic acid up-regulated in EUL, were negatively correlated with the inflammatory factors IL-6 and IL-8 (P < 0.05). Conversely, the metabolites 16-hydroxypalmitic acid and alpha-D-glucose, which were significantly down-regulated in EUL, were positively correlated with IL-6 and IL-8 (P < 0.05). Further, multiple linear regression analysis was used to conduct stepwise correlations between differential metabolites selected based on Pearson correlation analysis and inflammatory factors (Table 7).
There was a negative correlation between eicosapentaenoic acid and IL-6 \((P < 0.05)\) whereas 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid, PC (16:0/16:0), and adrenic acid were negatively correlated with IL-8 \((P < 0.05)\), while 16-hydroxypalmitic acid and IL-8 were positively correlated \((P < 0.05)\). These findings indicate that EUL may improve milk quality by reducing the levels of inflammatory factors.

DISCUSSION

Milk composition can be affected by several factors such as genetics, the physiological and nutritional status of the animal, animal management, and environmental conditions \((15, 16)\). EUL is a dual functional feed, which provides both nutrition and bioactive components such as geniposide acid, aucubin, and chlorogenic acid, which have specific functions in enhancing immunity. Chlorogenic acid can increase antioxidation capacity; for example, by improving SOD and GSH-Px activities and reducing oxidation products, such as MDA, in pigs, chickens, and sheep, thereby improving product quality \((4, 5, 17)\). In China, EUL is routinely served as food, tea, medicine, and animal feed additives in rural communities \((7, 18)\). Therefore, it may have an influence on the metabolism and chemical and physical properties of dairy cows.

In our study, IL-6 and IL-8 were lower in the EUL group than in the CTR group, indicating that the bioactive components in EUL, such as chlorogenic acid and aucubin, act as inhibitors of inflammatory factors as well as improving antioxidation, eliminating free radicals, and upregulating SOD activity \((19, 20)\). Chlorogenic acid can significantly suppress mRNA expression of the proinflammatory molecules IL-6 and IL-8, thus alleviating inflammatory responses in bovine mammary epithelial cells \((21, 22)\). The CD4/CD8 ratio is another indicator reflecting physiological immune status \((23)\). In the present study, CD4/CD8 was significantly increased in the EUL group, indicating that EUL improves mammary gland immunity and milk quality.

Metabolites reflect alterations in the metabolism of dairy cows. Based on the current study, 25 differential metabolites were identified between the CTR and EUL groups including amino acids, carbohydrates, and lipids. Our data demonstrate that INS was significantly increased in the EUL group compared...
with the CTR group, whereas Alpha-D-glucose was significantly reduced. As the nutrients in milk are transported from the blood, variations in the blood also affect the composition of the milk. According to previous reports, EUL significantly reduced blood glucose levels and significantly increased INS (Taurine, L-Lysine) and carbohydrates (D-Lactose) between the CTR and EUL groups. This may partly explain the variations in milk transfer of compounds is mainly mediated by two transporter superfamilies: ATP-binding cassette and Solute Carrier (25). In our study, one of the most impacted pathways of EUL was the ABC transporters, which was mainly associated with changes in amino acids (Taurine, L-Lysine) and carbohydrates (D-Lactose) between the CTR and EUL groups. This may partly explain the variations in the levels of Alpha-D-glucose and INS between CTR and EUL groups.
Milk fat content and milk fatty acid composition are mainly affected by animal diet (26). In the current study, polyunsaturated fatty acids (PUFA), including 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid, arachidonic acid, eicosapentaenoic acid, and adrenic acid, were increased in the EUL group. Short-chain fatty acids and UFA are beneficial to human health (27). Eicosapentaenoic acid and 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid are n-3 PUFA molecules, which have benefits in preventing cardiovascular disease and in being anti-inflammatory (28), and were found to have significantly increased in the present study, indicating that the nutritional value of milk was improved in the EUL group. In previous studies, it was found that the amount of fatty acid in feed determines the fatty acid profile in dairy products (29). EUL groups are rich in linolenic acid, which is the precursor of 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid and eicosapentaenoic acid, which explains the increase of 7Z, 10Z, 13Z, 16Z, 19Z-docosapentaenoic acid and eicosapentaenoic acid in milk. In line with this, Zhou et al. also observed that chlorogenic acid increased C18:3n-3 content in the liver of rats (30). Adrenic acid is an essential n-6 unsaturated fatty acid associated with boosting immunity, brain and nervous system development, and prevention of cardiovascular disease and diabetes (31), and was enriched in the EUL group. This is related to linolenic acid and linoleic acid, the precursors of adrenic acid, which are abundant in the EUL group. The increase of PUFA in the EUL group is favorable because a high content of PUFA has become a desirable nutritional characteristic in livestock products for consumers (32). The most enriched pathway in our study was the linoleic acid metabolism signaling pathway, which was mainly associated with changes in PUFA between the EUL and CTR groups. The increasing content of PUFA in the EUL group was likely related to the following factors: (1) EUL are rich in PUFA, such as linoleic acid, α-linoleic acid, and adrenic acid, which can increase the levels of precursors (7); (2) active substances in EUL, such as lignin, iridoid terpenes, and flavonoids, can affect fat metabolism and SFA synthesis (2, 33).

Lipidomics can be used to quantify total lipid components (34). Milk fat is an important source of essential fatty acids and a carrier of the lipid soluble vitamins, A, D, and E (35). The main component of milk fat is triglyceride (TG), which accounts for ~95% of total milk fat. Compared with the CTR group, 40 differential metabolites were identified in the EUL group by lipidomics analysis. EUL had a significant

FIGURE 4 | Correlation analysis of lipid molecules in the CTR and EUL groups. Map illustrating the correlations among lipid species differing significantly between EUL and CTR groups. The color of the points represents the degree of correlation, varying from −1 to 1, from jujube red to blue, respectively.
effect on TG composition in milk. Twenty-three types of TG with polyunsaturated bonds were increased and TG (18:0/16:0/16:0)+\(\text{NH}_4\) decreased; however, total TG content was not significantly altered. This is because chlorogenic acid supplementation reduced the SFA content, which is consistent with previous studies on broiler chicken meat (5). Furthermore, TG (18:0/16:0/16:0)+\(\text{NH}_4\) was negatively correlated with the 39 other differential lipids. It was reported that consumption of TG types with more carbon atoms and unsaturated bonds are associated with a lower incidence rate of type 2 diabetes (36). Hence, EUL substantially influenced milk lipid composition and may benefit the health of milk consumers.

Simple correlation and multiple linear regression analyses of differential metabolites with IL-6 and IL-8 revealed significant negative correlations of eicosapentaenoic acid, PC (16:0/16:0), and adrenic acid with IL-6 and IL-8, whereas 16-hydroxypalmitic acid was significantly positively correlated with IL-6 and IL-8. Some studies have reported that PUFA plays a key role in modulation of the immune system and inflammatory responses (37). Rezamand et al. reported that α-linolenic acid-enriched diets down-regulated expression of IL-6 and IL-8 pro-inflammatory factors (38). In our study, we found that eicosapentaenoic acid and adrenic acid significantly negatively correlated with inflammatory factors, but 16-hydroxypalmitic acid significantly positively correlated with inflammatory factors. Therefore, eicosapentaenoic acid, PC (16:0/16:0), and adrenic acid may serve as potential biomarkers for better milk microcomposition and quality. Overall, our data suggest that the ingredients of EUL in the diet of dairy cows could endow milk with some desirable traits and benefit consumer health. However, the weak point of this work is that before the EUL treatment, the only baseline data researched was on general milk composition, which did not include data from metabolomics and lipidomics analyses. In the future, we will carry out a time-course study to further clarify the associated underlying mechanisms of EUL in regulating milk quality.

### CONCLUSIONS

In summary, these results suggest that EUL reduced levels of proinflammatory factors IL-6 and IL-8 and increased the ratio of inflammatory inhibitory factors CD4/CD8. EUL had positive effects on unsaturated fatty acid and micro-component contents, which can improve the nutritional value of milk. The underlying

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**TABLE 6** | Simple correlation analysis between differential metabolites and inflammatory factors.

| Inflammatory factors | Differential metabolites | Correlation coefficient | \(P\) |
|----------------------|--------------------------|-------------------------|-------|
| IL-6                 | 7Z, 10Z, 13Z, 16Z, 19Z-Docosapentaenoic acid | -0.693 | 0.013 |
|                      | PC (16:0/16:0)            | -0.688 | 0.012 |
|                      | Adrenic Acid             | -0.631 | 0.028 |
|                      | Arachidonic Acid (peroxide free) | -0.734 | 0.007 |
|                      | Eicosapentaenoic acid    | -0.705 | 0.01  |
|                      | 16-Hydroxypalmitic acid  | 0.642 | 0.024 |
|                      | Alpha-D-Glucose          | 0.733 | 0.007 |
|                      | Cytidine monophosphate N-acetylneuraminic acid | 0.687 | 0.014 |
|                      | TG (4:0/16:0/19:1)+\(\text{NH}_4\) | -0.669 | 0.017 |
|                      | TG (10:0/18:1/18:1)+\(\text{NH}_4\) | -0.58  | 0.048 |
|                      | TG (15:0/16:0/18:1)+\(\text{NH}_4\) | -0.577 | 0.05  |
|                      | TG (18:0/16:0/16:0)+\(\text{NH}_4\) | 0.587 | 0.045 |
|                      | SM (d18:1/14:0)+\(\text{HCOO}\) | -0.617 | 0.032 |
| IL-8                 | 7Z, 10Z, 13Z, 16Z, 19Z-Docosapentaenoic acid | -0.772 | 0.003 |
|                      | PC (16:0/16:0)            | -0.773 | 0.003 |
|                      | Adrenic Acid             | -0.745 | 0.005 |
|                      | Norethindrone Acetate     | -0.658 | 0.002 |
|                      | Eicosapentaenoic acid    | -0.627 | 0.03  |
|                      | 16-Hydroxypalmitic acid  | 0.807 | 0.002 |
|                      | Alpha-D-Glucose          | 0.749 | 0.005 |

**TABLE 7** | Multiple linear regression analysis of the relationships among differential metabolites and inflammatory factors.

| Inflammatory factors | Differential metabolites | Correlation coefficient | \(P\) |
|----------------------|--------------------------|-------------------------|-------|
| IL-6                 | Eicosapentaenoic acid    | -0.705 | 0.01  |
| IL-8                 | 7Z, 10Z, 13Z, 16Z, 19Z-Docosapentaenoic acid | -0.772 | 0.003 |
|                      | PC (16:0/16:0)            | -0.773 | 0.003 |
|                      | Adrenic Acid             | -0.745 | 0.005 |
|                      | 16-Hydroxypalmitic acid  | 0.807 | 0.002 |
mechanisms are related to the rich, natural, bioactive ingredients that serve multiple functions in EUL.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All procedures were approved by the Animal Care and Use Committee of Henan Agricultural University, Henan Agricultural University, Zheng Zhou, China (approval number: HENAU-2018-015).

AUTHOR CONTRIBUTIONS

ZT: conceptualization, data curation, formal analysis, methodology, and writing-original draft. LW: project administration and validation. HD: funding acquisition and supervision. GY, TF, HL, and YS: formal analysis and visualization. SL and LZ: formal analysis, visualization, and validation. TG: project administration, funding acquisition, and supervision. ZT: conceptualization, data curation, formal analysis, supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.644967/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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