

Article

Exploratory Lipidomic Comparison of Neutral and Polar Lipid Profiles Between Mare and Cow Milk Under a Shared Grazing Environment

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Abstract

This exploratory study used UHPLC-HRMS/MS-based non-targeted lipidomics to compare milk lipid profiles between eight Yili mares and eight Xinjiang Brown cows maintained under a shared grazing environment. A total of 2460 lipid molecules were annotated across all confidence levels, and 1013 high-confidence Grade A/B lipids were retained for differential analysis. Forty-five differential lipids were identified, including 21 enriched in mare milk and 24 enriched in cow milk. Cow-milk-enriched lipids were mainly triacylglycerols with low double-bond numbers, consistent with neutral lipid deposition and glycerolipid-related features. In contrast, mare-milk-enriched lipids were more widely distributed among polar-lipid and membrane-related classes, including phosphatidylethanolamine, lysophosphatidylethanolamine, lysophosphatidylcholine, phosphatidylinositol, and ceramide, and showed broader unsaturation patterns. KEGG analysis provided supplementary, positive-ion-mode pathway support for lipid digestion and absorption, glycerolipid metabolism, cholesterol metabolism, and lipolysis-related processes, whereas negative-ion-mode mappings were not statistically significant. Overall, Yili mare milk and Xinjiang Brown cow milk shared a similar major lipid framework but differed in selected neutral-lipid and polar-lipid molecular profiles under the sampled conditions. These candidate features require validation in larger, multi-breed, multi-season datasets before being generalized as robust markers of mare-versus-cow milk differences.

Keywords: mare milk; cow milk; non-targeted lipidomics; milk lipids; triacylglycerols; polar lipids



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1. Introduction

Cow milk is the most widely consumed commercial milk source worldwide, and its lipid composition has been systematically investigated. With the increasing development of specialty milk resources and the growing demand for diversified dairy products, mare milk, a representative traditional dairy product in pastoral areas of Central Asia and Xinjiang, China, has received increasing attention for its nutritional value and quality characteristics [1,2]. Previous studies have shown that mare milk differs from cow milk in protein composition, lactose content, vitamin composition, fatty acid profile, and digestion-related properties [2–4]. However, most existing comparisons remain focused on conventional composition or total fatty acids, leaving the intact molecular lipid differences between Yili mare milk and Xinjiang Brown cow milk insufficiently characterized.

Milk fat composition is influenced by animal species, lactation stage, feeding conditions, digestive physiology, and mammary lipid metabolism [5,6]. Cattle and horses are herbivorous livestock with distinct digestive systems: cattle are ruminants, in which dietary lipids are extensively modified by rumen microbial metabolism before absorption [7–9], whereas horses are hindgut-fermenting non-ruminants and lack ruminal biohydrogenation before intestinal absorption [10,11]. These physiological differences may influence the fatty-acyl substrates available for mammary lipid synthesis and contribute to differences in the molecular composition of milk lipids. In addition, comparing milk samples collected from animals maintained in the same grazing pasture can reduce variation associated with farm location, sampling environment, management practices, and concentrate supplementation; however, this design cannot completely eliminate dietary variation arising from species-specific grazing behavior, intake patterns, and digestive physiology [2,12,13].

Most previous comparisons of mare and cow milk have focused on conventional nutritional components or total fatty acid composition, which provides important but incomplete information on milk fat characteristics [1,3]. Milk fat is a complex lipid system composed of triacylglycerols, glycerophospholipids, sphingolipids, lysophospholipids, and other low-abundance lipid molecules [5,14]. These lipid molecules differ in their roles in energy storage, the structure of the milk fat globule membrane, lipid emulsification, digestion, and biological activity [15,16]. Therefore, reliance only on total fat content or fatty acid proportions is insufficient to reveal differences in neutral lipids, polar lipids, membrane-related lipids, and fatty-acyl chain structures. Non-targeted lipidomics provides an effective approach for characterizing intact lipid molecules, lipid subclass distributions, and milk-source-related lipid features [14,17].

In this study, UHPLC-HRMS/MS-based non-targeted lipidomics was used to compare the lipid molecular profiles of Yili mare milk and Xinjiang Brown cow milk collected from the same grazing pasture. The study focused on major lipid classes, high-confidence differential lipids, fatty-acyl chain characteristics, and lipid-related pathway mapping, with the aim of providing exploratory molecular evidence for milk-source characterization and specialty dairy resource evaluation.

2. Materials and Methods

2.1. Sample Collection

Milk samples were collected from Jiang’abulake Village, Yumin County, Tacheng Prefecture, Xinjiang, China (E 82.884197, N 46.170966). The sampling protocol was approved by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University (Approval No. 2023059). The mare milk group consisted of eight clinically healthy Yili mares, and the cow milk group consisted of eight healthy Xinjiang Brown cows. All animals were lactating females maintained under the same free-grazing system without supplementary concentrate or conserved forage feeding. Mineral blocks and drinking water were available to both groups during the sampling period. The selected animals were in mid-lactation, with days in milk ranging from approximately 90 to 140 days and parity ranging from 2 to 3. Animals with similar visually assessed body condition were randomly selected from the available lactating animals. Clinical health status was assessed before sampling based on normal appetite, absence of visible mastitis, and absence of clinical signs of disease. Individual age and numerical body condition score were not recorded at the time of sampling and were therefore not included as covariates in the statistical analysis.

All milk samples were collected on the same day during the morning milking period between 07:00 and 09:00 to minimize diurnal variation. Mare milk and cow milk samples were collected within the same time window to ensure temporal comparability. Milk was collected manually from each animal using routine farm milking procedures. For mare milk

collection, foals were not present during milking, and no pharmacological milk let-down treatment was used. Milk fraction, complete udder-emptying status, and individual milk yield were not recorded. After collection and thorough mixing, milk samples were filtered through sterile gauze, transferred into sterile 5 mL cryovials, immediately frozen in liquid nitrogen, transported to the laboratory on dry ice, and stored at -80°C until lipidomics analysis. Each animal was treated as an independent biological replicate, and no pooled experimental milk samples were used. The sampling procedure involved routine milk collection only and did not involve invasive manipulation, experimental treatment, or pain-inducing procedures.

2.2. Reagents and Instrumentation

HPLC-grade methanol, acetonitrile, isopropanol, methyl tert-butyl ether (MTBE), formic acid, ammonium acetate, and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The SPLASHTM LIPIDOMIXTM Mass Spec Standard was obtained from Avanti Polar Lipids (Birmingham, AL, USA) and used as the internal standard mixture for lipid extraction and relative quantification.

Chromatographic separation was performed using a VanquishTM UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectrometric detection was carried out using a Q ExactiveTM HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Lipid separation was achieved on an AccucoreTM C30 column (150 mm $\times$ 2.1 mm, 2.6 μm ; Thermo Fisher Scientific, Waltham, MA, USA). A Thermo ST16R refrigerated centrifuge (Thermo Fisher Scientific, Hennigsdorf, Brandenburg, Germany) and a Reacti-Therm nitrogen evaporator (Thermo Fisher Scientific, Hennigsdorf, Brandenburg, Germany) were used for sample preparation.

2.3. Lipid Extraction and Quality Control Sample Preparation

Lipid extraction was performed using an MTBE-based method described by Matyash et al. with modifications for milk samples [18]. Briefly, 100 μL of thawed milk sample was transferred into a glass centrifuge tube with a polytetrafluoroethylene-lined cap, followed by the addition of 0.75 mL of pre-cooled methanol. The mixture was vortexed for 30 s. Subsequently, 2.5 mL of pre-cooled MTBE and 10 μL of SPLASHTM LIPIDOMIXTM internal standard solution were added, and the mixture was shaken at room temperature for 1 h. Phase separation was induced by adding 0.625 mL of mass-spectrometry-grade water. After vortexing and standing for 10 min, the mixture was centrifuged at $1000 \times g$ for 10 min at 4°C .

The upper organic phase was collected, and the lower aqueous phase was re-extracted with 1 mL of MTBE/methanol/water (10:3:2.5, $v/v/v$). The organic phases from the two extractions were combined and dried under a gentle stream of nitrogen. The dried lipid residues were reconstituted in 100 μL of isopropanol, vortexed, and transferred into autosampler vials for UHPLC-HRMS/MS analysis.

Procedural blanks were prepared using the same extraction procedure without milk samples to monitor background contamination. A pooled quality control (QC) sample was prepared by mixing equal aliquots of all processed sample extracts. All experimental samples were analyzed in a randomized injection sequence to reduce injection-order bias. Two pooled QC injections were performed at the beginning of the analytical sequence to equilibrate the LC-MS system and evaluate instrument performance. During routine analysis, one pooled QC sample was injected after every three experimental samples to monitor analytical stability throughout data acquisition. All samples were analyzed within a single randomized LC-MS sequence; therefore, no additional batch correction was applied, and potential signal drift was monitored using repeated pooled QC injections.

2.4. UHPLC-HRMS/MS Analysis

UHPLC-HRMS/MS analysis was performed using a C30 reversed-phase lipidomics method with minor modifications based on previously reported untargeted lipidomics methods [19]. Chromatographic separation was performed on an Accucore™ C30 column. The column temperature was maintained at 40 °C, the flow rate was set at 0.35 mL/min, and the injection volume was 5 µL. Mobile phase A consisted of acetonitrile/water (60:40, *v/v*) containing 0.1% formic acid and 10 mM ammonium acetate. Mobile phase B consisted of isopropanol/acetonitrile (90:10, *v/v*) containing 0.1% formic acid and 10 mM ammonium acetate.

The gradient elution program was as follows: 30% B at 0–2 min, 43% B at 5 min, 55% B at 5.1 min, 70% B at 11 min, 99% B at 16–18 min, returned to 30% B at 18.1 min, and maintained at 30% B until 20 min for column re-equilibration.

Mass spectrometric analysis was performed in both positive and negative ion modes. The main MS parameters were as follows: sheath gas, 40; sweep gas, 0; auxiliary gas rate, 10 in positive ion mode and 7 in negative ion mode; spray voltage, +3.5 kV in positive ion mode and −3.5 kV in negative ion mode; capillary temperature, 320 °C; heater temperature, 350 °C; S-lens RF level, 50; scan range, *m/z* 114–1700; automatic gain control target, 3×10^6 ; normalized collision energies, 22, 24, and 28 eV; maximum injection time, 100 ms; isolation window, 1 *m/z*; MS/MS automatic gain control target, 2×10^5 ; and dynamic exclusion time, 6 s.

2.5. Lipid Identification and Semi-Quantitative Analysis

Raw MS data files were imported into LipidSearch 5.1 software for lipid identification, peak extraction, peak alignment, and semi-quantitative analysis. Lipid annotation was performed by matching accurate precursor mass, adduct ion information, retention time, and MS/MS fragment information against the LipidSearch database. The precursor ion mass tolerance and product ion mass tolerance were both set at 5 ppm. Peak alignment among samples was performed using a retention time tolerance of 0.05 min and a signal-to-noise threshold of 3. The adduct ions observed in the final annotation tables included $M + H$, $M + Na$, $M + NH_4$, and $M + H - H_2O$ in positive ion mode, and $M - H$, $M + HCOO$, $M - CH_3$, $M - 2H$, and $M + CH_3COO$ in negative ion mode. The detected lipid classes included glycerolipids, glycerophospholipids, sphingolipids, fatty acids, and related low-abundance lipid classes. For lipids with available MS/MS spectra, experimental fragment ions were matched with database fragment ions and collision-energy information to support MS/MS-level annotation. Background ions detected in procedural blank samples were removed before downstream analysis. Isotopic peaks, in-source fragments, and duplicate annotations were processed according to the default annotation-filtering workflow in LipidSearch 5.1. Missing values generated during peak alignment were handled using the default peak-filling procedure in LipidSearch based on the expected *m/z* and retention-time window. The filled quantitative matrices were then used for downstream statistical analysis. Data quality was further evaluated using pooled QC samples. QC relative standard deviation (RSD) was calculated for peak-aligned lipid features to describe the overall analytical reproducibility. The QC RSD values of annotated lipids are provided in Table S1 and were used as quality-control information for data interpretation.

The overall lipid profile was characterized using all annotated lipid molecules. To improve the reliability of differential lipid analysis, only high-confidence lipids annotated as LipidSearch Grade A or Grade B were retained for subsequent statistical analysis. In this study, Grade A annotations were considered to have stronger MS/MS fragment support for lipid class and fatty-acyl-chain assignment, whereas Grade B annotations provided

lipid-class assignment with partially supported fatty-acyl information [20]. These Grade A/B lipids were therefore treated as the high-confidence subset for differential screening.

Quantitative values were obtained by integrating chromatographic peak areas and normalized using the SPLASH™ LIPIDOMIX™ internal standard mixture. Because this internal standard mixture does not provide an exact isotope-labeled internal standard for every lipid subclass detected in the non-targeted dataset, normalization was performed using the available internal standards as class representatives or, where applicable, as structurally related references. The normalized peak areas were interpreted as internal-standard-normalized semi-quantitative abundances rather than absolute lipid concentrations. Because ionization efficiency, adduct formation, and internal-standard coverage differ among lipid classes and ionization modes, the normalized values were mainly used to compare the same lipid species or closely related lipid species across samples, rather than to make direct absolute comparisons among chemically distinct lipid subclasses or between ionization modes.

2.6. Statistical Analysis

Positive and negative-ion mode datasets were analyzed separately because lipid coverage and signal distribution differ between ionization modes. Before multivariate analysis, normalized lipid abundance data were log-transformed and scaled. Principal component analysis (PCA) was performed in R version 4.5.1 to assess sample distribution and QC stability. Orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed using SIMCA 14.1 to evaluate lipidomic separation between mare milk and cow milk. Model quality was evaluated using R^2X (cum), R^2Y (cum), and Q^2 (cum). The OPLS-DA models were assessed using seven-fold cross-validation in SIMCA 14.1, and model significance was evaluated by cross-validated analysis of variance (CV-ANOVA). Model overfitting was further evaluated using 200-permutation testing. Variable importance in projection (VIP) values were obtained from the corresponding OPLS-DA models.

For univariate analysis, a two-sided two-sample *t*-test was used to compare normalized lipid abundances between mare milk and cow milk. The resulting *p*-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method, separately for the positive- and negative-ion mode datasets. Because of the small sample size, normality and variance assumptions could not be robustly assessed for every lipid feature. Therefore, the *t*-test results were not interpreted alone but were combined with VIP, FDR-adjusted *p*-values, and fold-change thresholds to prioritize exploratory differential lipid candidates. Fold change (FC) was calculated as Ma/Co , where *Ma* represents Yili mare milk, and *Co* represents Xinjiang Brown cow milk. Differential lipids were screened using $VIP > 1.0$, FDR-adjusted $p < 0.05$, and $FC \geq 1.5$ or $FC \leq 0.666$. Lipids with $FC \geq 1.5$ were considered enriched in mare milk, whereas lipids with $FC \leq 0.666$ were considered enriched in cow milk.

Volcano plots were generated using the R package ggplot2 (version 4.0.3). Hierarchical clustering heat maps were generated in R using the pheatmap package after Z-score normalization. Pearson correlation analysis was performed to assess correlations among differentially abundant lipids, and correlation plots were generated in R.

KEGG pathway mapping and exploratory enrichment analysis were performed separately for positive-ion and negative-ion mode datasets using the KEGG database accessed on 2 January 2026. KEGG-mappable differential lipid entries were used as the foreground set, and all KEGG-mappable annotated lipid entries in the corresponding ion mode were used as the background set. Pathway enrichment was evaluated using a hypergeometric test. Because adjusted pathway *p*-values were not available, pathways with unadjusted

$p < 0.05$ were reported as nominally enriched, and KEGG results were interpreted as exploratory pathway-level evidence.

3. Results

3.1. Validation of Analytical Methods

To evaluate the stability and reproducibility of the lipidomics platform, pooled quality-control samples were analyzed throughout the analytical workflow. The total ion chromatograms of the QC samples showed stable signal responses and consistent chromatographic retention patterns in both positive and negative ion modes, indicating good instrumental stability during data acquisition (Figure 1).

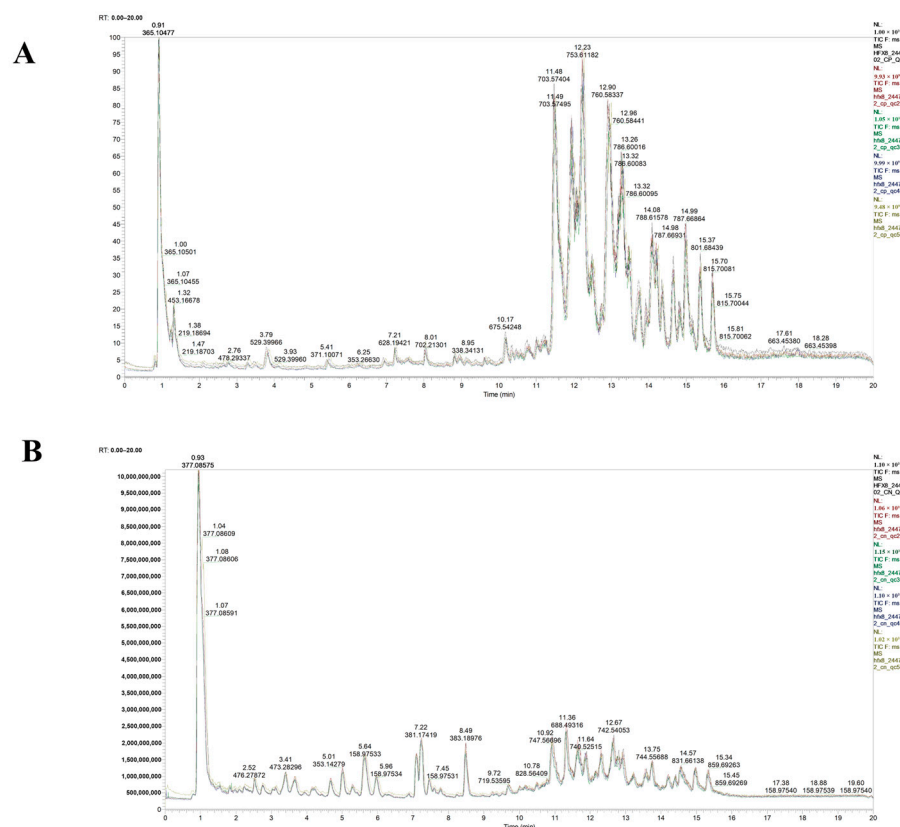


Figure 1. Total ion chromatograms of quality control samples. Total ion chromatograms of quality control samples acquired in positive ion mode (A) and negative ion mode (B).

PCA further showed that the QC samples clustered closely in both ion modes, whereas mare milk and cow milk samples showed distinct distribution tendencies, suggesting that the observed group separation was unlikely to be driven primarily by instrumental fluctuations (Figure 2A,B). The correlation heat maps of QC samples showed high consistency among repeated QC injections, with correlation coefficients close to 1.00 in both positive and negative ion modes (Figure 2C,D). The RSD distribution of peak intensities showed that 85.99% of lipid features in positive ion mode and 85.78% in negative ion mode had RSD values below 30% (Figure 3). The QC RSD values of individual annotated lipids were further included in Table S1 to provide feature-level information on analytical reproducibility.

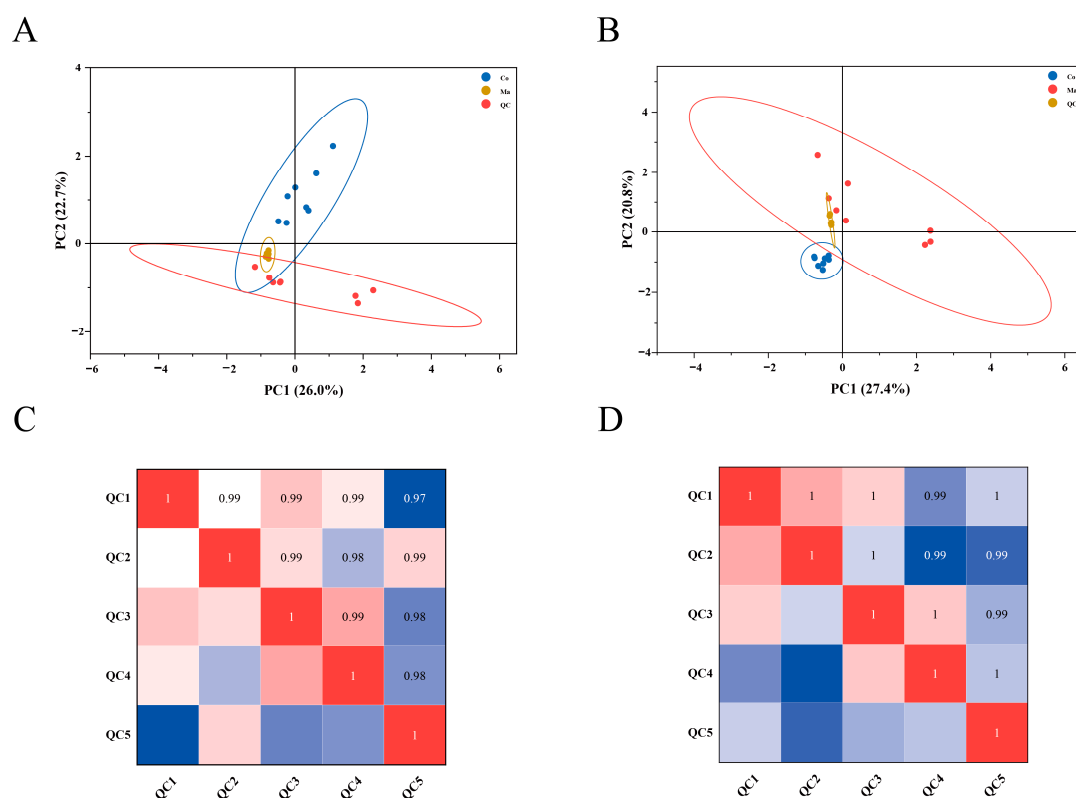


Figure 2. Reliability of the analytical method. PCA score plots and correlation heat maps of mare milk and cow milk samples under positive and negative ion modes. PCA score plots under positive ion mode (A) and negative ion mode (B); correlation heat maps of QC samples under positive ion mode (C) and negative ion mode (D). QC: quality control; RSD: relative standard deviation; Ma: Yili mare milk; Co: Xinjiang Brown cow milk.

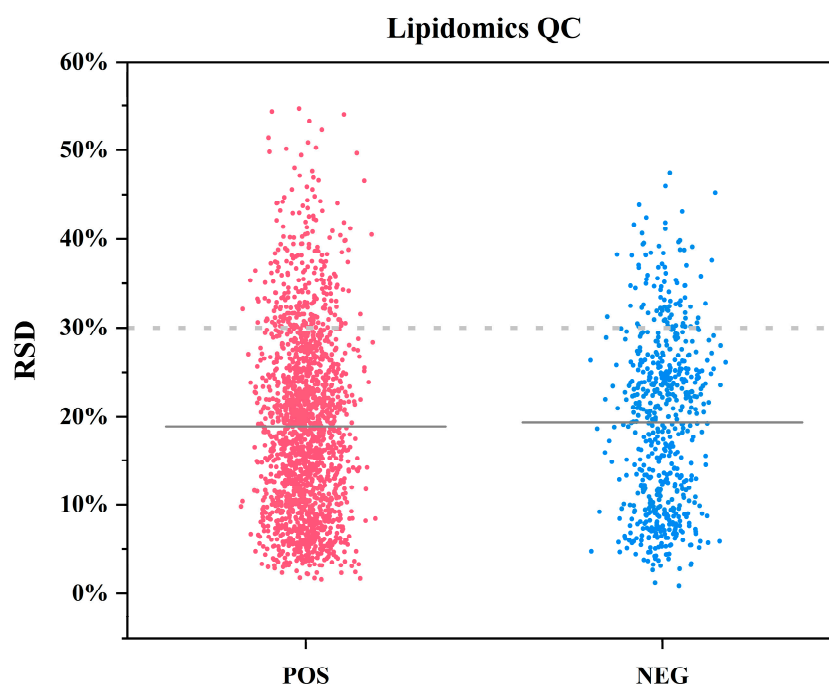


Figure 3. Relative standard deviation of peak intensities in QC samples. Distribution of RSD values for peak-aligned lipid features in QC samples acquired in positive and negative ion modes. The dashed line indicates the commonly used 30% RSD reference threshold. RSD: relative standard deviation.

3.2. Lipid Analysis of Mare and Cow Milk Samples

A total of 2460 lipid molecules were annotated across all annotation confidence levels in mare milk and cow milk samples, including 1792 lipids in positive ion mode and 668 lipids in negative ion mode. These 2460 lipids represented the complete lipid annotation results rather than the high-confidence Grade A/B subset used for subsequent differential lipid analysis. The complete lipid annotation and internal-standard-normalized semi-quantitative abundance matrix are provided in Table S1.

The annotated lipids were assigned to multiple lipid classes. The top 16 lipid classes were selected for visualization because they contained relatively large numbers of annotated lipid molecules. These classes included triacylglycerol (TG), phosphatidylcholine (PC), diacylglycerol (DG), phosphatidylethanolamine (PE), ceramide (Cer), phosphatidylserine (PS), sphingomyelin (SM), fatty acid (FA), lysophosphatidylcholine (LPC), phosphatidic acid (PA), phosphatidylinositol (PI), bis (methyl) phosphatidic acid (BisMePA), lysophosphatidylethanolamine (LPE), methyl phosphatidylcholine (MePC), cardiolipin (CL), and monohexosylceramide (Hex1Cer). Among these classes, TG contained the largest number of annotated lipid molecules, with 873 molecules, followed by PC, DG, and PE, with 449, 281, and 235 molecules, respectively (Figure 4A).

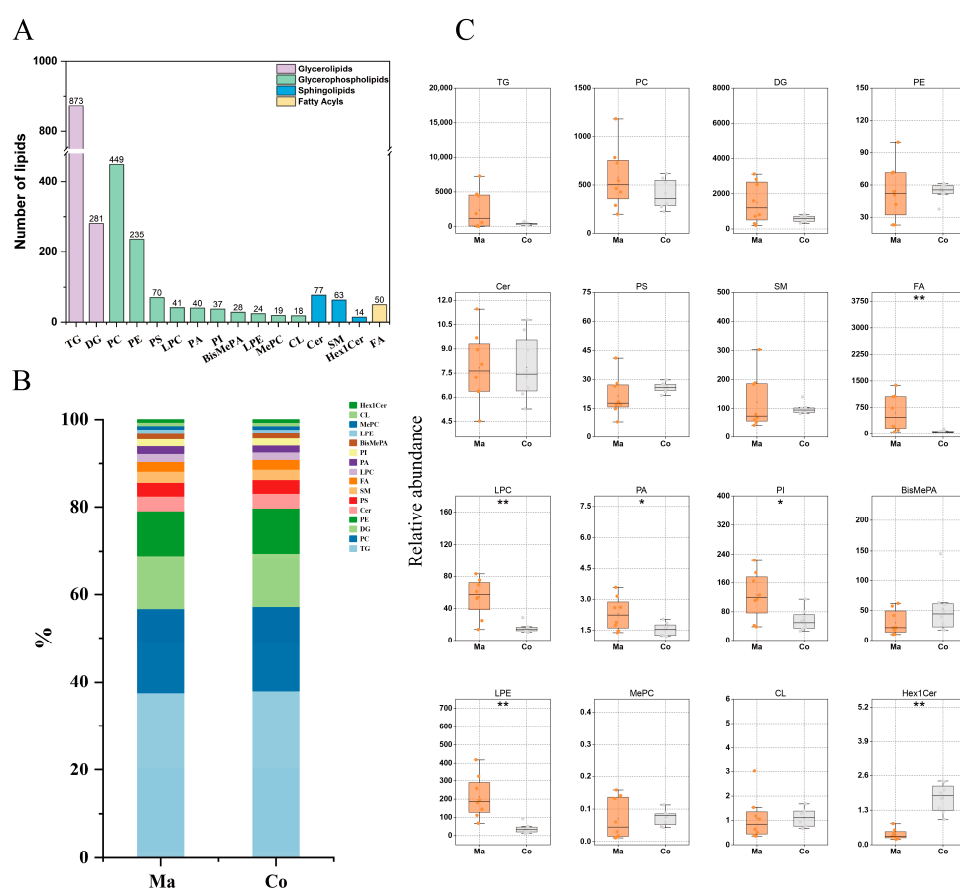


Figure 4. Overall lipid annotation and lipid-class composition in mare and cow milk. (A) Number of lipid molecules in the top 16 lipid classes among all 2460 annotated lipids. (B) Internal-standard-normalized semi-quantitative abundance composition of the top 16 lipid classes in mare and cow milk. (C) Comparison of internal-standard-normalized semi-quantitative abundances of the top 16 lipid classes between mare and cow milk. The top 16 lipid classes were selected according to the number of annotated lipid molecules. The abundance of each lipid class in each sample was calculated as the sum of internal-standard-normalized semi-quantitative values of all lipid molecules within that class. Data are presented as box plots. Group comparisons were performed using the Wilcoxon rank-sum test. * $p < 0.05$; ** $p < 0.01$. Ma: Yili mare milk; Co: Xinjiang Brown cow milk.

The normalized semi-quantitative abundance composition showed that mare milk and cow milk shared a broadly similar lipid-class framework, with TG, PC, DG, and PE being the dominant lipid classes in both milk types (Figure 4B). However, lipid-class-level comparisons revealed clear differences between the two groups. Mare milk showed relatively higher abundances of several polar lipid-related classes, including LPC, LPE, PA, and PI, whereas cow milk showed a higher relative abundance of Hex1Cer (Figure 4C). These results indicate that Yili mare milk and Xinjiang Brown cow milk shared a similar major lipid-class framework but differed in selected lipid subclasses within this semi-quantitative analytical framework.

3.3. Multilevel Statistical Analysis of Mare and Cow Milk Lipidomes

To improve the reliability of the differential lipid analysis, only lipids annotated as LipidSearch Grade A or Grade B were retained for subsequent statistical analysis. After confidence-level filtering, 632 lipids were retained in positive ion mode, including 437 Grade A and 195 Grade B lipids, and 381 lipids were retained in negative ion mode, including 252 Grade A and 129 Grade B lipids. In total, 1013 high-confidence Grade A/B lipids were used for subsequent statistical analysis. The high-confidence Grade A/B lipid list is provided in Table S2.

PCA score plots showed group-distribution differences between mare milk and cow milk samples in both positive and negative-ion modes. In positive ion mode, PC1 and PC2 explained 28.4% and 23.6% of the total variation, respectively (Figure 5A). In negative ion mode, PC1 and PC2 explained 27.4% and 22.8% of the total variation, respectively (Figure 5B). Compared with PCA, OPLS-DA showed clearer group separation between mare milk and cow milk samples. The positive-ion-mode OPLS-DA model had $R^2Y = 0.977$ and $Q^2 = 0.858$ (Figure 5C), and the negative-ion-mode model had $R^2Y = 0.986$ and $Q^2 = 0.940$ (Figure 5D). The predictive significance of the OPLS-DA models was supported by CV-ANOVA in positive-ion mode ($F = 5.2715$, $p = 0.0206$) and negative-ion mode ($F = 17.9362$, $p = 0.000267$).

The 200-permutation tests were used to assess potential model overfitting. The R^2 and Q^2 intercepts were 0.798 and -0.661 in positive ion mode, respectively (Figure 5E), and 0.795 and -0.729 in negative ion mode, respectively (Figure 5F). The negative Q^2 intercepts suggested that no obvious overfitting was observed in the permutation tests.

3.4. Identification of Significantly Different Lipids Between Mare and Cow Milk

Differential lipids between mare milk and cow milk were screened separately in positive and negative ion modes using the criteria of $FC \geq 1.5$ or $FC \leq 0.666$, $VIP > 1.0$, and FDR-adjusted $p < 0.05$. In positive ion mode, 24 differential lipids were identified, including 4 lipids enriched in mare milk and 20 lipids enriched in cow milk (Figure 6A). In negative ion mode, 21 differential lipids were identified, including 17 lipids enriched in mare milk and 4 lipids enriched in cow milk (Figure 6B). Overall, 45 differential lipids were screened from the two ion modes, including 21 mare-milk-enriched lipids and 24 cow-milk-enriched lipids. Detailed information on these differential lipids, including lipid species, lipid class, ion mode, annotation confidence level, adduct ion, molecular formula, retention time, m/z value, QC RSD, sample-level normalized abundances, total carbon number, total double bonds, VIP, FC, enrichment direction, and FDR-adjusted p -value, is shown in Table S3.

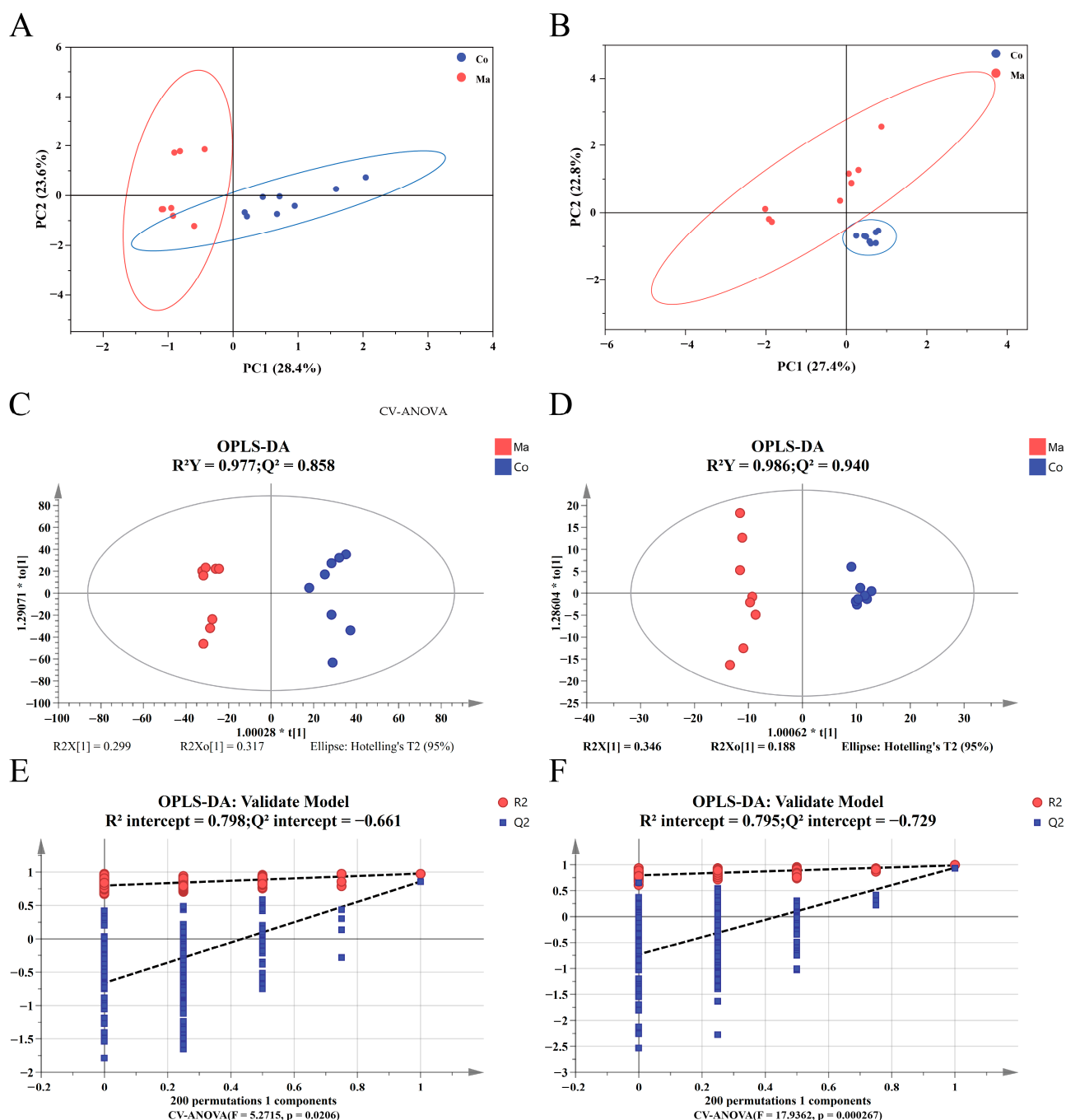


Figure 5. Multivariate statistical analysis of mare milk and cow milk samples. PCA score plots of mare milk and cow milk samples acquired in positive ion mode (A) and negative ion mode (B). OPLS-DA score plots obtained in positive ion mode (C) and negative ion mode (D). Two-hundred-permutation test plots for the OPLS-DA models in positive ion mode (E) and negative ion mode (F). The OPLS-DA models were evaluated using seven-fold cross-validation, CV-ANOVA, and permutation testing. Ma: Yili mare milk; Co: Xinjiang Brown cow milk.

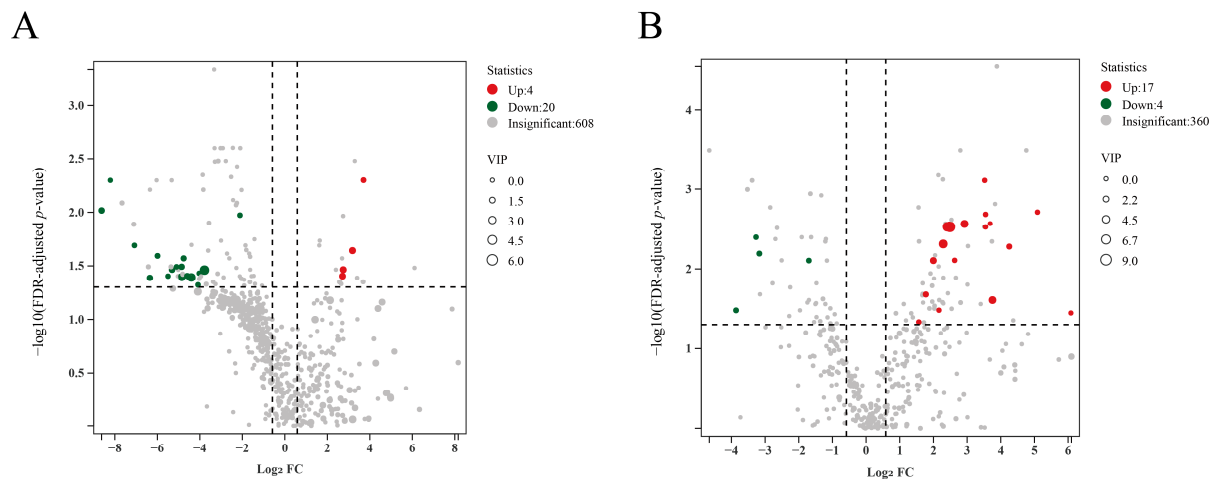


Figure 6. Screening of differential lipids between mare milk and cow milk samples under positive and negative ion modes. Volcano plots of differential lipids between mare milk and cow milk samples acquired in positive ion mode (A) and negative ion mode (B). Differential lipids were screened using VIP > 1.0, FDR-adjusted $p < 0.05$, and $FC \geq 1.5$ or $FC \leq 0.666$. Ma: Yili mare milk; Co: Xinjiang Brown cow milk.

3.5. Identification of Differential Lipid Species

To further analyze the relative abundance distribution of differential lipids in mare and cow milk samples, hierarchical clustering analysis was performed separately for the differential lipids screened in positive and negative ion modes. The results showed group-related clustering patterns in both ion modes (Figure 7A,B). In positive ion mode, Co-enriched differential lipids were mainly represented by TG molecules, whereas several Ma-enriched DG and LPE molecules showed higher relative abundances in mare milk (Figure 7A). In negative ion mode, Ma-enriched differential lipids were mainly distributed among polar-lipid-related classes, including LPE, LPC, PI, PE, and Cer, whereas several PC molecules were relatively enriched in cow milk (Figure 7B). This abundance pattern was consistent with the enrichment direction indicated by FC values in Table S3.

Correlation analysis showed that differential lipids exhibited correlations in abundance (Figure 7C,D). In positive ion mode, most TG molecules showed positive correlations with one another (Figure 7C). In negative ion mode, several lipids with similar abundance patterns also showed relatively clustered correlation patterns (Figure 7D). These results indicated that part of the differential lipid set displayed coordinated abundance variation across samples, especially within the same lipid class or among closely related lipid classes.

To further characterize the fatty-acyl chain structural features of differential lipids, the total number of double bonds was used as the x -axis and the total number of fatty-acyl carbon atoms as the y -axis to display the structural distribution of differential lipids by lipid class (Figure 7E). Co-enriched differential lipids were mainly concentrated in the TG class, with total carbon numbers ranging mainly from 24 to 45 and total double-bond numbers mostly concentrated at 0–1. In contrast, Ma-enriched differential lipids showed a broader structural distribution across lipid classes. LPE and LPC molecules generally had relatively low total carbon numbers, mainly ranging from 14 to 20, whereas PC, DG, PI, Cer, and PE molecules mostly had total carbon numbers above 30. In addition, Ma-enriched differential lipids exhibited a broader distribution of total double-bond counts, with several molecules containing two or more double bonds. These results suggested that the differences between mare and cow milk were reflected not only in lipid abundance and class composition, but also in fatty-acyl chain length and unsaturation characteristics.

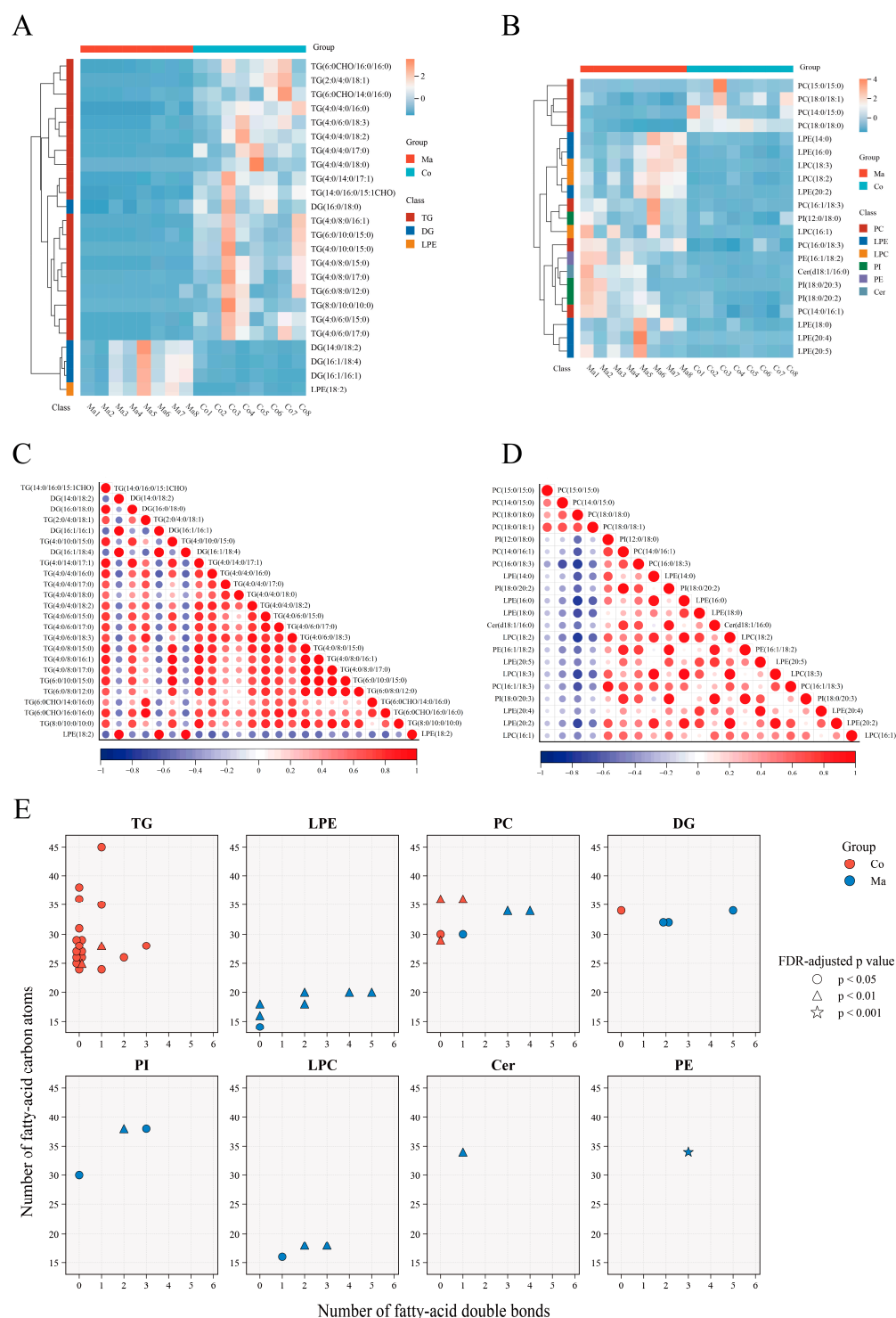


Figure 7. Clustering patterns, correlation structure, and fatty-acyl chain characteristics of differential lipids between mare and cow milk. Hierarchical clustering heat maps of differential lipids in positive ion mode (A) and negative ion mode (B). Correlation matrices of differential lipids in positive ion mode (C) and negative ion mode (D). Distribution of total carbon numbers and total double-bond numbers of differential lipids by lipid class (E). Ma: Yili mare milk; Co: Xinjiang Brown cow milk.

3.6. KEGG Pathway Mapping and Enrichment Analysis

KEGG pathway analysis was performed for KEGG-mappable differential lipid entries in the positive- and negative-ion-mode datasets. In positive ion mode, seven pathways were nominally enriched based on unadjusted $p < 0.05$, including insulin resistance, regulation of lipolysis in adipocytes, cholesterol metabolism, vitamin digestion and absorption,

glycerolipid metabolism, fat digestion and absorption, and metabolic pathways (Figure 8; Supplementary Table S4). Among these pathways, fat digestion and absorption, glycerolipid metabolism, cholesterol metabolism, and regulation of lipolysis in adipocytes were most directly related to lipid digestion, transport, storage, or mobilization. Insulin resistance and vitamin digestion and absorption were retained as pathway-mapping results, but they were not interpreted as direct evidence of disease- or endocrine-related effects in milk.

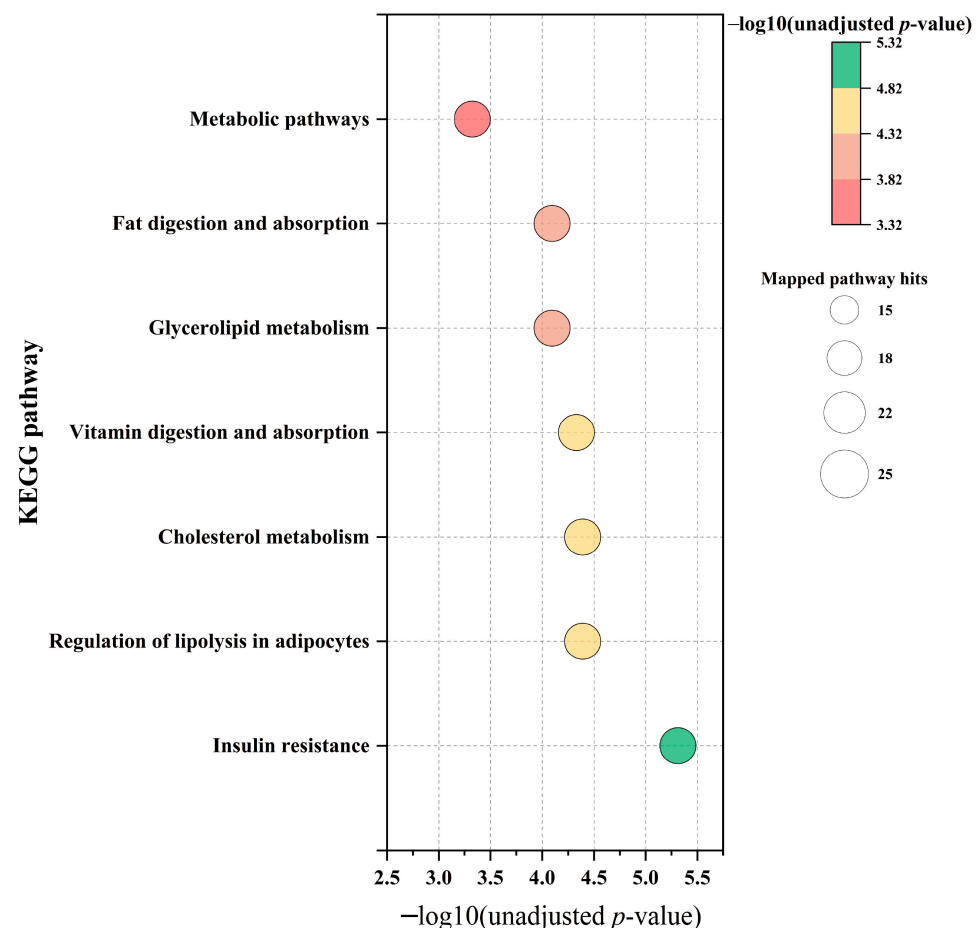


Figure 8. KEGG pathway enrichment analysis of KEGG-mappable differential lipid entries between mare milk and cow milk in positive ion mode. Only nominally enriched pathways with unadjusted $p < 0.05$ are shown. Bubble size represents the number of mapped pathway hits, and bubble color represents $-\log_{10}(\text{unadjusted } p\text{-value})$.

In negative ion mode, no pathway reached an unadjusted $p < 0.05$. Therefore, the negative-ion-mode KEGG results were retained only as exploratory pathway-mapping information in Supplementary Table S4 and were not emphasized in the biological interpretation. Overall, KEGG analysis provided exploratory and supplementary pathway-level support for the lipidomics results, but these findings should be interpreted cautiously because only unadjusted p -values were available.

4. Discussion

This study used UHPLC-HRMS/MS-based non-targeted lipidomics to compare the lipid molecular composition of Yili mare and Xinjiang Brown cow milk under shared grazing conditions. The results showed that although the two milk types shared a similar major lipid molecular framework, they differed in selected lipid classes, fatty-acyl chain structures, and exploratory pathway mapping. Compared with conventional analyses of

total fat content or total fatty acid composition, analyses at the intact lipid molecular level can further reveal differences among milk sources in neutral lipids, polar lipids, membrane-related lipids, and fatty acyl chain structures [14,21]. Therefore, the main significance of this study is not to demonstrate an absolute nutritional advantage of one milk source over another, but to characterize candidate lipid molecular allocation differences between Yili mare milk and Xinjiang Brown cow milk under the sampled shared-grazing conditions.

At the lipid-class level, TG, PC, DG, and PE were the main lipid classes in mare and cow milk, indicating that the two milk types shared a similar basic milk fat framework. TG is the major neutral lipid in milk fat and is mainly involved in milk fat energy storage and the formation of the milk fat globule core [22]. PC and PE are important phospholipid components of the milk fat globule membrane and are closely associated with interfacial stability, emulsification properties, and lipid digestion processes [23]. DG is an important intermediate in glycerolipid metabolism and is also related to TG synthesis and lipid remodeling [24]. Therefore, the consistency of the major lipid classes in mare and cow milk conforms to the general compositional characteristics of mammalian milk fat. However, class-level comparisons of all annotated lipids further revealed higher normalized relative abundances of several polar lipid-related subclasses, including LPC, LPE, PA, and PI, in mare milk, whereas Hex1Cer showed a higher normalized relative abundance in cow milk. Although the overall relative abundances of these subclasses were lower than those of TG, PC, DG, and PE, their differences indicate that the two milk types differed to some extent in low-abundance polar lipids and membrane-related lipid composition. LPC and LPE may be associated with phospholipid hydrolysis, membrane lipid turnover, and phospholipid remodeling, whereas PA and PI are related to membrane lipid metabolism and phospholipid signaling regulation [25,26]. Hex1Cer is a glycosphingolipid and an important component of milk fat globule membrane- and cell membrane-related lipids [27]. Its relative enrichment in cow milk may reflect differences between cow and mare milk in sphingolipid metabolism, milk fat globule membrane composition, or membrane lipid components derived from mammary epithelial cells. Thus, mare and cow milk are not completely different in their main milk lipid framework; rather, they exhibit different lipid subclass allocation patterns within a shared framework, and these differences may be related to digestive strategy and mammary lipid secretion characteristics. These subclass-level differences should be interpreted within the internal-standard-normalized semi-quantitative framework, rather than as absolute concentration differences among chemically distinct lipid classes.

The distribution of differential lipid classes further showed that cow-milk-enriched differential lipids were mainly concentrated in the TG class and were characterized by low double-bond counts, consistent with the characteristics of milk fat synthesis in ruminants. At the level of specific lipid molecules, TGs relatively enriched in cow milk mainly included TG (2:0/4:0/18:1), TG (4:0/4:0/17:0), TG (4:0/8:0/16:1), TG (6:0/8:0/12:0), and TG (8:0/10:0/10:0). Most of these molecules contained short- or medium-chain fatty-acyl groups, and their total double-bond numbers were mostly concentrated at 0–1. Therefore, the enrichment of these low-unsaturation TG molecules indicates that the differences in cow milk were more oriented toward neutral lipid deposition, milk fat globule core composition, and energy-storage-related lipid allocation. In addition, some low-double-bond PC molecules in cow milk, such as PC (15:0/15:0), PC (14:0/15:0), PC (18:0/18:0), and PC (18:0/18:1), also showed relative enrichment. PC is an important phospholipid component of the milk fat globule membrane and is associated with interfacial stability and emulsification properties [15,23]. Therefore, changes in these PC molecules indicate that cow milk differed not only in TG-type neutral lipids but also potentially in the fatty-acyl chain composition of some membrane-related phospholipids. Fatty acids in ruminant milk fat are

mainly derived from two pathways: one involves de novo synthesis in the mammary gland using substrates such as acetate and β -hydroxybutyrate, mainly producing short- and medium-chain fatty acids; the other involves mammary uptake of long-chain fatty acids from the blood, derived from the diet or body fat mobilization, followed by participation in TG synthesis [6,28]. In cattle, forage fiber is fermented by rumen microorganisms, producing volatile fatty acids, particularly acetate and butyrate. Acetate and β -hydroxybutyrate provide important carbon sources for de novo synthesis of short- and medium-chain fatty acids in the mammary gland [29]. Meanwhile, dietary unsaturated fatty acids undergo microbial lipolysis and biohydrogenation in the rumen before intestinal absorption, thereby altering the fatty-acyl substrates available for mammary lipid synthesis [7,9]. Therefore, the enrichment of cow-milk-related TG species with low double-bond numbers may reflect the combined influence of rumen fermentation, mammary de novo fatty acid synthesis, ruminal biohydrogenation, and TG assembly.

Unlike cow milk, mare-milk-enriched differential lipids were more widely distributed in polar-lipid or membrane-lipid-related classes, including PE, LPE, LPC, PI, and Cer, and showed higher double-bond numbers and a broader unsaturation distribution. At the level of specific lipid molecules, differential lipids relatively enriched in mare milk included LPE (20:2), LPE (20:4), LPE (20:5), LPE (18:2), LPC (18:2), LPC (18:3), PE (16:1/18:2), PI (18:0/20:2), PI (18:0/20:3), PC (16:1/18:3), and Cer (d18:1/16:0). Among them, LPE (20:4), LPE (20:5), LPC (18:2), LPC (18:3), PE (16:1/18:2), and PC (16:1/18:3) contained relatively more unsaturated bonds, indicating that differential lipids enriched in mare milk were not only concentrated in polar-lipid and membrane-related lipid classes, but also showed more pronounced unsaturation at the fatty-acyl chain level. Glycerophospholipids such as PE, PC, and PI are important structural lipids of the milk fat globule membrane [30]; LPE and LPC are related to membrane lipid turnover, phospholipid remodeling, and lipid interfacial properties [31,32]; and Cer belongs to the sphingolipid class and participates in membrane structural stability and lipid signaling regulation [33]. Therefore, the relative enrichment of unsaturated fatty-acyl-chain-containing lysophospholipids such as LPE (20:4), LPE (20:5), LPC (18:2), and LPC (18:3) in mare milk may indicate more pronounced membrane-related lipid turnover, milk fat globule membrane interfacial lipid remodeling, and retention of unsaturated fatty-acyl chains. Horses are monogastric hindgut-fermenting herbivores; lipids and digestible nutrients are mainly absorbed in the small intestine, whereas the cecum and colon are primarily responsible for hindgut fermentation of some fibrous substrates [10,34]. Compared with ruminants, horses lack rumen microbial hydrogenation of dietary unsaturated fatty acids before absorption; therefore, the fatty acid composition entering absorption and subsequent mammary lipid metabolism may differ from that in cattle. This digestive physiological difference may contribute to the higher degree of lipid unsaturation in mare milk [11]. Overall, these mare-milk-enriched polar and unsaturated lipid molecules are more directly interpreted in relation to digestive physiology, mammary lipid handling, phospholipid remodeling, and milk fat globule membrane composition, rather than as evidence of broad functional or nutritional superiority.

KEGG pathway analysis was used as a supportive mapping approach to place the differential lipids into a broader metabolic context. In positive ion mode, differential lipids were nominally enriched in pathways such as fat digestion and absorption, glycerolipid metabolism, vitamin digestion and absorption, cholesterol metabolism, and regulation of lipolysis in adipocytes, and these pathways were mainly contributed to by TG and DG differential lipids. This result is consistent with the enrichment of TG-class differential lipids in cow milk, the low-double-bond neutral lipid characteristics, and glycerolipid-metabolism-related changes, suggesting that cow-milk differential lipids were more closely associated with a TG/DG-dominated metabolic background involving neutral lipid deposition, lipid

digestion and absorption, and lipid mobilization [24]. In contrast, negative-ion-mode differential lipids were mapped to several pathways, but these mappings did not reach statistical significance. Therefore, the negative-ion-mode KEGG results were considered exploratory pathway-mapping information and were not used as primary biological evidence. Overall, KEGG interpretation in this study should be treated as supplementary pathway-level support and should be integrated with lipid-class distribution, differential lipid species, and fatty-acyl chain structural characteristics.

This study has several limitations. First, the sample size was relatively limited, and all milk samples were collected from a single grazing region and a specific lactation stage; therefore, the differential lipid species identified here should be regarded as candidate features that require validation in larger, multi-breed, multi-season populations. Second, because this study compared Yili mares with Xinjiang Brown cows, species- and breed-associated effects could not be fully separated, and the shared grazing environment reduced but did not eliminate possible dietary variation related to selective grazing behavior and individual intake differences. Third, the non-targeted lipidomics data were based on internal-standard-normalized semi-quantitative abundances rather than absolute quantification of all lipid subclasses; therefore, unusual short-chain TG annotations and lipid species with “CHO” notation should be interpreted with caution until further confirmed by targeted lipidomics or representative MS/MS evidence. Despite these limitations, this study provides exploratory lipidomics evidence for understanding lipid subclass distribution, differential lipid composition, and fatty-acyl chain characteristics in Yili mare and Xinjiang Brown cow milk under shared grazing conditions. These findings may support future studies on mare milk quality evaluation, milk-source discrimination, targeted lipid validation, and specialty dairy resource development. From an applied perspective, these candidate lipid features may contribute to lipid-based quality assessment and authenticity evaluation of specialty dairy products, although their practical use requires further targeted quantification and external validation.

5. Conclusions

This exploratory study used UHPLC-HRMS/MS-based non-targeted lipidomics to compare the milk lipid profiles of Yili mares and Xinjiang Brown cows maintained under shared grazing conditions. The two milk types shared a broadly similar major lipid framework, but differed in selected neutral-lipid, polar-lipid, and fatty-acyl-chain features. Cow milk showed a greater association with TG-dominated, low-unsaturation neutral lipid features, whereas mare milk showed a greater contribution of polar-lipid and membrane-related lipid features. KEGG analysis provided supplementary positive-ion-mode pathway support but should be interpreted with caution, as the pathway evidence was exploratory and based on unadjusted *p*-values. Overall, this study provides candidate lipidomic features that may support future studies on mare milk quality evaluation, milk-source discrimination, and specialty dairy resource development. Larger studies across different breeds, farms, seasons, lactation stages, and feeding systems are still needed before these features can be generalized as robust markers of mare-versus-cow milk differences.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16141540/s1>, Table S1: Complete lipid annotation and internal-standard-normalized semi-quantitative abundance matrix of all annotated lipids in mare and cow milk. Table S2: High-confidence LipidSearch Grade A/B lipid list used for differential lipid analysis. Table S3: Differential lipid species between mare milk and cow milk. Table S4: KEGG pathway mapping and exploratory enrichment results of differential lipids in positive and negative ion modes.

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Abbreviations

The following abbreviations are used in this manuscript:

UHPLC-HRMS/MS	Ultra-high-performance liquid chromatography-high-resolution tandem mass spectrometry
QC	Quality control
CV	Coefficient of variation
RSD	Relative standard deviation
PCA	Principal component analysis
OPLS-DA	Orthogonal partial least-squares discriminant analysis
CV-ANOVA	Cross-validated analysis of variance
VIP	Variable importance in projection
FC	Fold change
FDR	False discovery rate
KEGG	Kyoto Encyclopedia of Genes and Genomes
MTBE	Methyl tert-butyl ether
MS/MS	Tandem mass spectrometry
Ma	Yili mare milk
Co	Xinjiang Brown cow milk
BisMePA	Bis(methyl) phosphatidic acid
Cer	Ceramide
CL	Cardiolipin
DG	Diacylglycerol
FA	Fatty acid
GL	Glycerolipids

GP	Glycerophospholipids
Hex1Cer	Monohexosylceramide
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MePC	Methyl phosphatidylcholine
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
SM	Sphingomyelin
SP	Sphingolipids
ST	Sterol lipids
TG	Triacylglycerol

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