

Lipo-nutritional profile analysis of bovine milk, goat milk, and giant panda milk through untargeted lipidomics

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Abstract: Breast milk plays a crucial role in the growth of captive giant pandas, particularly lipids crucial to brain, retinal, and central nervous system development. This study aimed to analyze the lipid differences among bovine milk, goat milk, and giant panda milk. Lipidomics was employed to conduct a comprehensive and systematic analysis of milk lipids from different animal sources. It enables rapid, high-throughput analysis and identification of the lipids present in the given material employing advanced instrumentation. Milk was analyzed through untargeted lipidomics. The results showed that giant panda milk was rich in phospholipids, including glycerophosphatidylcholines, glycerophosphatidylethanolamines, and glycerophosphatidylinositols, while bovine milk and goat milk were abundant in diacylglycerols and triacylglycerols (TAGs). The neutral and polar lipids in the three species showed that giant panda milk was richer in TAGs containing palmitic acid and sphingomyelins containing ultra-long-chain fatty acids. In the positive and negative ion modes, 223 and 128 significantly different lipids (SDLs) were found between bovine milk and giant panda milk, and 208 and 126 SDLs were found between goat milk and giant panda milk, which were highly correlated to five main pathways including sphingolipid metabolism, glycerophospholipid metabolism, metabolic pathways, glycerolipid metabolism, and inositol phosphate metabolism. Our work aimed to provide comprehensive lipid profiles of giant panda milk, bovine milk, and goat milk, and promote the development of giant panda milk alternatives.

Keywords: lipidomics; lipid analysis; diacylglycerols; triacylglycerols; phospholipids

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1 Introduction

Giant panda (*Ailuropoda melanoleuca*) is a kind of rare and vulnerable mammal that has become a flagship and umbrella species for global biodiversity conservation due to its extremely low reproductive rate and survival rate of the cubs^[1-2]. The neonates of the giant panda are extremely thin, weak, and rapidly growing, with a neonatal to maternal body mass ratio of approximately 1:1 000. Therefore, postnatal development is highly dependent on adequate breast milk, which provides nutrition, energy, and immune protection for cubs^[3-4]. Due to the improvement of giant panda captive breeding techniques, the probability of giving birth to twins has greatly increased. However, their postpartum lactation capacity is hard to meet the requirements of rapid growth and development for two cubs at the same time, and some pandas cannot even meet the requirements of one cub^[5-6]. In response to this situation, breeders have to artificially feed the cubs with frozen giant panda milk previously stored or milk powder. Meanwhile, bovine milk is an important source of breast milk substitute, and interest has been rising in recent years to explore the nutritive potential of bovine milk for feeding other animals^[7]. Also, goat milk is the third largest milk source in the world and is the dairy product closest to breast milk, which has received much attention due to its macro and micronutrient composition^[8-9]. Therefore, the research on giant panda milk, bovine milk, and goat milk is crucial to search for an

appropriate substitute for breast milk and promote the proliferation of captive giant pandas^[10].

Lipids, the main component of breast milk, account for 3%–5% of milk but provide more than 50% of energy requirements for neonates. In addition, they also contain essential fatty acids, fat-soluble vitamins, and hormones necessary for weight gain, organ development, and the development of the immune system^[11-12]. The lipids in milk mainly include neutral lipids such as diacylglycerols (DAGs), triacylglycerols (TAGs), free fatty acids (FFAs), cholesterol, and polar lipids such as phospholipids (PLs), sphingolipids (SPs), and glycolipids^[13]. Among these, TAG is quantitatively dominant and plays an important role in energy provision and storage^[14]. Meanwhile, glycerophospholipids (GPLs), SPs, and glycolipids are relatively low in content, but they are the major constituents of the milk fat globule membrane (MFGM) and are associated with neurodevelopment, cognitive development, and the protective effects of breast milk on neonates^[15]. In previous studies, pet or infant milk powder is often used as a source of milk substitute for giant pandas^[16]. These commercial formulas are usually templated on the breast milk of mother mammals, with vegetable oil as a supplement to make their lipid composition close to that of breast milk. However, these still differ significantly from the breast milk of mammal species in terms of lipid structure^[17-18]. Tu et al.^[19] have detected more than 60 TAGs in human milk compared to 50 TAGs in infant formulas. This deficiency resulted

in an insufficient supply of the essential trace lipids and bioactive compounds critical for cub development. Consequently, formulating milk substitutes replicating the lipid profile of giant panda milk could provide suitable nutrition for cubs. Comprehensive characterization of the lipid composition and concentration in giant panda milk, therefore, remains imperative to facilitate the development of effective nutritional solutions.

Lipidomics is a useful discipline that provides a comprehensive and systematic analysis of biological lipids^[20]. It enables rapid, high-throughput analysis and identification of the lipids present in the given material employing advanced instrumentation. At present, liquid chromatography-mass spectrometry (LC-MS) with high resolution and throughput has become the commonly used technique for lipidomic analysis, which provides the possibility for the detailed chemical structure characterization of lipid species in milk^[21–22]. In previous reports, the vast majority of milk lipidomic studies have focused on human milk and bovine milk^[11–13]. Yu et al.^[23] has reported that a total of 17 fatty acids were detected in giant panda milk, including 49.9% saturated fatty acids (SFAs), 33.7% monounsaturated fatty acids (MUFAs), and 20.7% polyunsaturated fatty acids (PUFAs). Due to the preciousness of giant panda milk, current studies have heretofore been confined to basic nutritional components^[24]. Our team previously investigated the differences in fatty acids between giant panda milk, bovine milk, and goat milk and found that giant panda milk contained more unsaturated fatty acids. However, fundamental differences in the lipid composition of giant panda milk, bovine milk, and goat milk have yet to be comprehensively analyzed.

In this work, ultra-high liquid performance-quadrupole-time of flight-mass spectrometry (UPLC-Q-TOF-MS) was used to characterize the lipid composition in giant panda milk, bovine milk, and goat milk, and to compare the main differential lipids, which could provide significant information for the future development of a giant panda milk substitute.

2 Materials and methods

2.1 Materials and reagents

Eight bovine milk samples from eight healthy Holstein bovines and eight goat milk samples from eight healthy Guanzhong dairy goats were collected on day 30 after delivery from local farms (Xi'an, China), and four giant panda milk samples from healthy Qinling giant pandas were collected on day 32 after delivery from the Qinling Giant Panda Research Center (Xi'an, China). Samples were collected utilizing the standardized manual milking technique, adhering to the previously established protocol, with no incidence of adverse symptoms, notably mastitis, observed throughout the process^[24]. Ensuring the preservation of sample integrity, all specimens were promptly transported to the laboratory within an insulated container maintained at 0 to 4 °C and subsequently stored at a temperature of –80 °C until the commencement of analytical procedures. Notably, each individual sample underwent testing in isolation, without any form of admixture, to maintain the highest level of accuracy and precision in the subsequent analyses.

LC-MS grade methanol and acetonitrile were purchased from Fluka of Honeywell (Seelze, Germany) and formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Lipid extraction

Lipid extraction was performed according to the method by Yu et al.^[25] with slight modifications. Briefly, the collected milk

samples were thawed on ice, and the lipid materials in milk were extracted with 50% (V/V) methanol. In brief, 120 µL of pre-cooled 50% (V/V) methanol was mixed with 20 µL of the sample, vortexed and shaken for 90 s, followed by incubation for 10 min at room temperature. Then the extraction mixture was stored overnight at –20 °C to precipitate the protein in the sample. On the next day, the mixture was centrifuged at $4\,000 \times g$ for 20 min, and the supernatant was taken and transferred to new 96-well plates. Then the supernatant was diluted with diluent (isopropyl alcohol:acetonitrile:water = 2:1:1, V/V). The samples were stored in a refrigerator at –80 °C prior to UPLC-MS analysis. In addition, pooled quality control (QC) samples, which were mixed with lipid extracts from eight bovine milk, eight goat milk, and four giant panda milk samples, were also prepared by combining 10 µL of each extraction mixture.

2.3 UPLC condition

The chromatographic separations were performed using a Thermo Scientific UltiMate 3000 HPLC, and all samples for the UPLC-MS system were collected according to the equipment instructions. For the purpose of reversed-phase separation, an ACQUITY UPLC BEH C₁₈ column (100 mm × 2.1 mm, 1.8 µm, Waters, USA) was employed. Each sample was injected with a precise volume of 4 µL, ensuring optimal column utilization. The column temperature was meticulously maintained at 35 °C to stabilize the chromatographic performance, while a flow rate of 0.4 mL/min was utilized to ensure efficient and reproducible elution. The mobile phase consisted of solvent A (0.1% formic acid in distilled water) and solvent B (0.1% formic acid in acetonitrile). Gradient elution conditions were set as follows: 0–0.5 min, 5% B; 0.5–7 min, 5% to 100% B; 7–8 min, 100% B; 8–8.1 min, 100% to 5% B; 8.1–10 min, 5% B.

2.4 High-resolution MS condition

The metabolites eluted from the column were detected by a triple TOF 6600 Plus high-resolution mass spectrometer (SCIEX, Framingham, MA, USA). Q-TOF was operated in positive (POS) and negative (NEG) ion modes, respectively. The specific parameters were as follows: curtain gas was set to 30 psi, ion source gas 1 was set to 60 psi, ion source gas 2 was set to 60 psi, and the interface heater temperature was 650 °C. The ion spray voltage float value was set to 5 000 V in POS ion mode or –4 500 V in NEG ion mode. The MS data were collected in the information-dependent acquisition mode. The TOF mass scan range was m/z 60–1 200. The survey scans were acquired in 150 ms and as many as 12 product ion scans were collected with a threshold of 100 counts/s and 1+ charge-state. The total cycle time was fixed at 0.56 s. Four times bins were summed for each scan at a pulse frequency value of 11 kHz through monitoring of the 40 GHz multichannel time-to-digital converter detector with four-anode/channel detection. Dynamic exclusion was set for 4 s. During the acquisition process, the mass accuracy was calibrated at every 20 sample intervals. In addition, to assess the stability of the UPLC-MS throughout the acquisition process, a QC sample (a collection of all samples) was taken at every 10 sample intervals.

2.5 Lipid data processing

The acquired MS data were preprocessed using XCMS software, which encompassed peak detection, peak grouping, retention time (RT) correction, secondary peak grouping, and annotation of isotopes and adducts. Raw UPLC-MS data files were converted to

mzXML format and subsequently processed with the XCMS, CAMERA, and metaX toolkits, implemented via R software. Ions were identified based on the combination of RT and m/z values. The intensity of each peak was recorded, generating a three-dimensional matrix that included arbitrarily assigned peak indices (RT- m/z pairs), sample names (observations), and ion intensity information (variables).

The intensity data for each peak were further refined using the metaX toolkit. Features detected in fewer than 50% of the QC samples or 80% of the biological samples were excluded. Missing values in the remaining peaks were interpolated using the K -nearest neighbor algorithm to enhance data quality. The preprocessed data were subjected to principal component analysis (PCA) to identify outliers and assess batch effects, and PCA scores were calculated in RStudio software with the `prcomp` and `variance` functions, while the hierarchical clustering analysis method using the Ward linkage criterion clusters the objects according to similarities of the distance criterion using the smallest error of the sum of squares. QC data were fitted using a quality-controlled LOESS signal correction method, aligned with the injection sequence to minimize temporal drift in signal intensity. Additionally, relative standard deviations of metabolic profiles were computed for all QC samples, and those exceeding 30% were excluded. Differences in metabolite concentrations between the two species were assessed using the Student's t -test, with P -values adjusted for multiple comparisons using the false discovery rate approach (Benjamini-Hochberg). Compounds were filtered from the raw data by analysis of variance (ANOVA) and fold change of variance to obtain significantly differential lipids (SDLs) in bovine milk, goat milk, and giant panda milk samples, defining compounds with a ratio ≥ 2 or $\leq 1/2$ and $P < 0.05$ as SDLs^[26].

2.6 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). The data were analyzed by ANOVA, and $P < 0.05$ was considered significantly different. In addition, searching for the pathways was performed via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps. The graphs were constructed using GraphPad Prism 8 software.

3 Results and discussion

3.1 Methodology verification

The reproducibility of the sample preparation procedure and the reliability of the method were assessed by chromatograms of QC samples and PCA^[27]. Five identical QC samples were equally mixed with lipid extracts from eight bovine milk, eight goat milk, and four giant panda milk samples. The base peak chromatograms in POS and NEG ion mode of five QC samples were compared respectively (Figure 1). The RT and peak area of each chromatographic peak largely overlapped, indicating that the experiment had excellent stability. The peaks obtained from the experimental samples and QC samples were fetched, and PCA was performed. The variance contributions of PC1 and PC2 were 40.52% and 15.82%, respectively (Figure 2). The five QC samples were clustered together and highly overlapped, indicating the high reliability of the analytical methods used in this study.

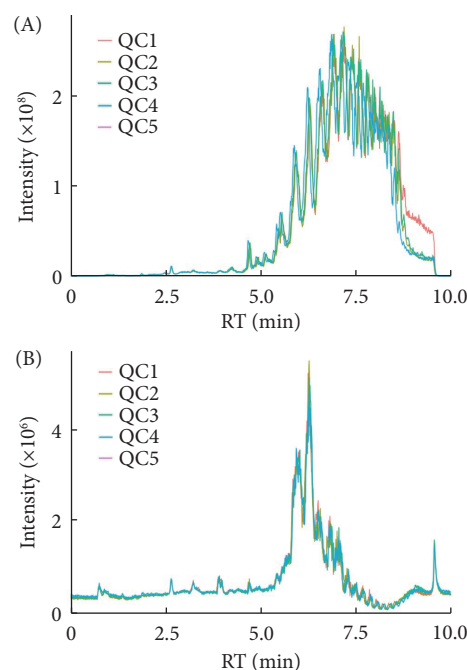


Figure 1 Total ion chromatograms of QC samples in (A) POS and (B) NEG ion modes.

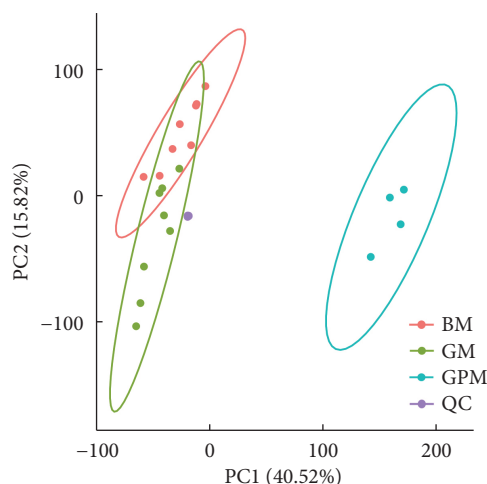


Figure 2 PCA score plot of the bovine milk, goat milk, giant panda milk, and QC samples. BM, GM, and GPM represent bovine milk, goat milk, and giant panda milk, respectively.

3.2 Lipid identification in the milk samples

Untargeted lipidomic analysis based on UPLC-Q-TOF-MS was performed on bovine milk, goat milk, and giant panda milk. Typical chromatograms of these three species samples in POS and NEG ion modes are shown in Figure S1. The lipid species and relative contents detected in POS and NEG ion modes are shown in Figure 3. In total, 860 lipids in bovine milk, goat milk, and giant panda milk were discovered and classified into 18 subclasses, including 51 DAGs, 193 TAGs, 89 glycerophosphatidylcholines (PCs), 75 glycerophosphatidylethanolamines (PEs), 24 glycerophosphatidylinositols (PIs), 21 glycerophosphatidylserines (PSs), 17 glycerophosphatidylglycerols (PGs), 26 lysophosphatidylcholines (LPCs), 15 lysophosphatidylethanolamines (LPEs), 84 sphingomyelins (SMs), 22 ceramides (Cers), 44 hexosylceramides (HexCers), 7

dihexosylceramides (Hex2Cers), 45 monogalactosyldiacylglycerols (MGDGs), 22 seminolipids (SMGDGs), 41 FFAs, 55 sulfatides (SHexCers), and 29 sterols (STs). The PC, PE, PI, PS, LPC, LPE, SM, Cer, and Hex2Cer were identified in both POS and NEG ion modes.

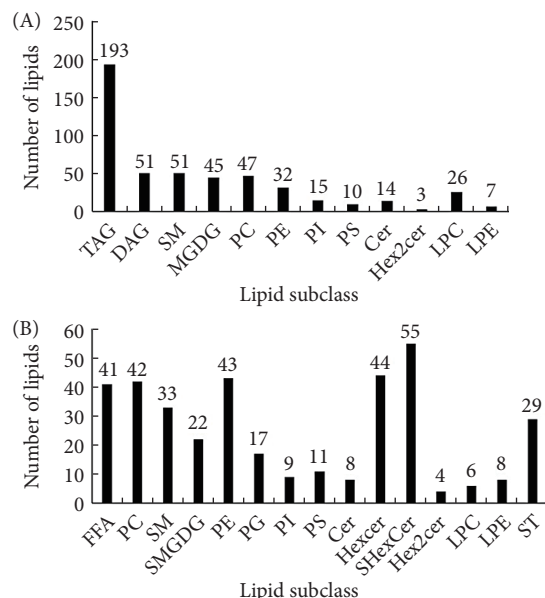


Figure 3 Number of lipids in each lipid subclasses of milk samples identified in (A) POS and (B) NEG ion modes.

It is well known that milk is a complex mixture of lipids containing thousands of species. Although many researchers have focused on these lipids in milk, there are still many low-content lipids that remain unknown^[13,28]. Compared with conventional approaches, UPLC-Q-TOF-MS enables the simultaneous identification of hundreds of lipids, including both high-content and low-content lipids^[11]. Previously, the lipid profile of bovine colostrum and mature milk was comprehensively characterized by Li et al.^[28] and a total of 335 lipid molecules in 13 subclasses were detected. Zhang et al.^[16] characterized the lipid composition of colostrum and mature milk in giant pandas, and detected 403 lipid molecules, including PLs, SPs, glycerolipids, FFAs, and STs. By contrast, the lipids detected in this study are similar in subclasses but with a greater quantity. For example, more lipid molecules were found in the subclasses of FFA, PC, PI, and so on than the results by Zhang et al.^[16]. The vast number of lipids identified herein could substantially improve our understanding of the composition of bovine milk, goat milk, and giant panda milk, providing a basis for better utilization of lipids in them.

3.3 Lipid analysis in bovine milk, goat milk, and giant panda milk

The sum of all lipids in the same subclass was calculated to estimate the relative content of each subclass^[28]. The composition and relative content of lipid components in milk are shown in Figure 4. In the POS ion mode (Figure 4A), the total TAG relative content of bovine milk, goat milk, and giant panda milk were 95.43%, 96.64%, and 79.17%, respectively; and the total DAG relative content were 2.57%, 1.35%, and 2.04%, respectively, indicating that glycerides (GLs) are predominant components in milk lipids. Despite containing lower levels of GLs, GLs were still the most abundant lipid in giant panda milk, which was consistent with the fact of lipid

composition in milk^[13]. Unusually, giant panda milk contains more SPs and PLs, with significantly higher levels of PC, PE, and SM than bovine milk and goat milk ($P < 0.05$). These lipids are the main constituents of goat milk, which ensures the stability of the oil-in-water emulsion of milk^[13,29]. A higher level of SPs means that SP pathways were the most significantly relevant metabolic pathways, which was strongly associated with energy balance in dairy cows. Therefore, we suppose this significant change in SP metabolism may be associated with alterations in the size of milk fat globules during lactation. In the NEG ion mode (Figure 4B), the relative content of four lipids, including PC, PE, PI, and FFA, in giant panda milk was significantly greater than that in bovine milk or goat milk ($P < 0.05$). The most prevalent PL class in giant panda milk was PC (36.91%), followed by PE (19.44%). The relative contents of these lipids in giant panda milk were significantly higher than those in bovine milk or goat milk ($P < 0.05$). There were higher levels of PLs in greater amounts in goat milk (55.63%) compared to bovine milk (42.25%). The reason for this is that goat milk had a relatively small size and higher total amount of fat globules, resulting in higher PL content than that in bovine milk^[30–31]. It could be assumed that giant panda milk comprised more small fat globules due to its higher amount of PLs (72.58%). Therefore, it is recommended that feeding pandas based on bovine milk or goat milk should be supplemented with PL-rich substances.

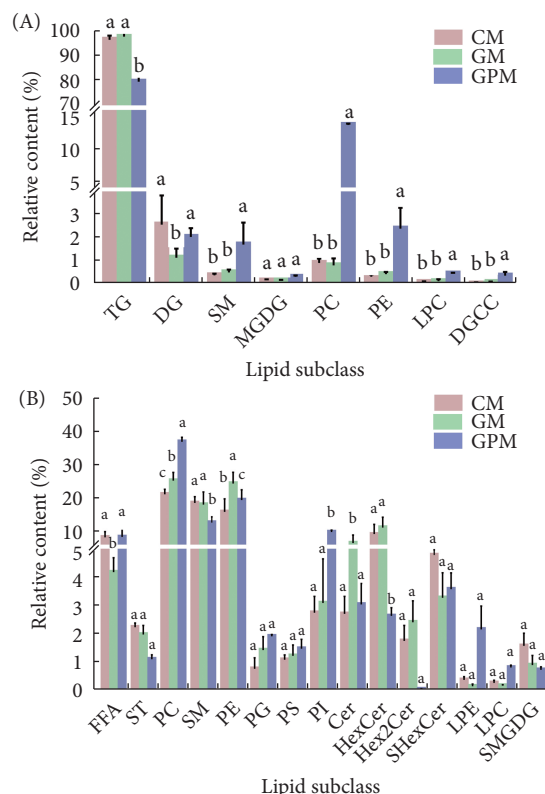


Figure 4 Relative contents of lipids in each lipid subclasses of bovine milk, goat milk, giant panda milk identified in (A) POS and (B) NEG ion modes. BM, GM, and GPM represent bovine milk, goat milk, and giant panda milk, respectively. Bars with different lowercase letters indicate significant differences between groups ($P < 0.05$).

Heatmap analysis was performed on the top 10% lipids of each subclass in different samples to highlight variations in lipid profiles of bovine milk, goat milk, and giant panda milk samples (Figure 5). The results combined with PCA (Figure 2) showed that giant panda

milk was significantly different from bovine milk and goat milk in terms of lipid composition and content, and bovine milk had a similar lipid distribution to goat milk. This could be explained by the physiological differences that bovines and goats are ruminants, whereas giant pandas are non-ruminants; hence, they have different processes of lipid synthesis^[32]. In summary, there remain differences in the lipid composition of the three types of milk, and a closer relation between bovine milk and goat milk lipid content was obviously observed than with giant panda milk.

3.4 Neutral lipid species analysis in bovine milk, goat milk, and giant panda milk

In-depth analysis of individual lipids highlighted differences in neutral lipids between bovine milk, goat milk, and giant panda milk, with individual lipids in the top 10% of neutral lipids shown in Table S1. The DAG, which has two fatty acyl substituents on the glycerol backbone, plays important roles in both the biosynthesis of neutral lipids such as TAG and products of metabolism of TAG, as well as being precursors of certain GPLs. TAG represents the dominant constituents of milk fat and is important for the texture,

flavor, and nutritional value of milk and related products. The most prevalent TAG types in giant panda milk samples were as follows: TAG 8:0/12:0/18:1 (9.703%), TAG 8:0/10:0/18:1 (6.054%), TAG 16:0/18:1/18:1 (5.618%), TAG 10:0/10:0/18:2 (5.025%), and TAG 16:0/16:1/18:1 (4.825%). The TAG 8:0/12:0/18:1 and TAG 8:0/10:0/18:1 in bovine milk and goat milk were 5.411% and 5.060%, 4.269% and 3.992% respectively. Although there is a lack of evidence of TAG 16:0/18:1/18:1 structure in giant panda milk, it is possible that it is 1,3-dioleoyl-2-palmitoylglycerol (OPO), which is rich in *sn*-2 palmitic acid (PA) and reduces stool hardness in neonates and promotes the absorption of fat and calcium^[31–32]. Currently, in some formulas, OPO is added to better mimic the TAG composition of natural breast milk^[18,33], and the same technology can be used in giant panda milk substitutes. In addition, TAG 8:0/10:0/14:0 and TAG 10:0/12:0/14:0 were the most abundant TAG species in goat milk (6.996%) and bovine milk (6.776%), respectively, but were very low in giant panda milk samples (0.450% and 4.573%). DAG and TAG species, which both contain PA, were significantly higher in giant panda milk compared to bovine milk and goat milk.

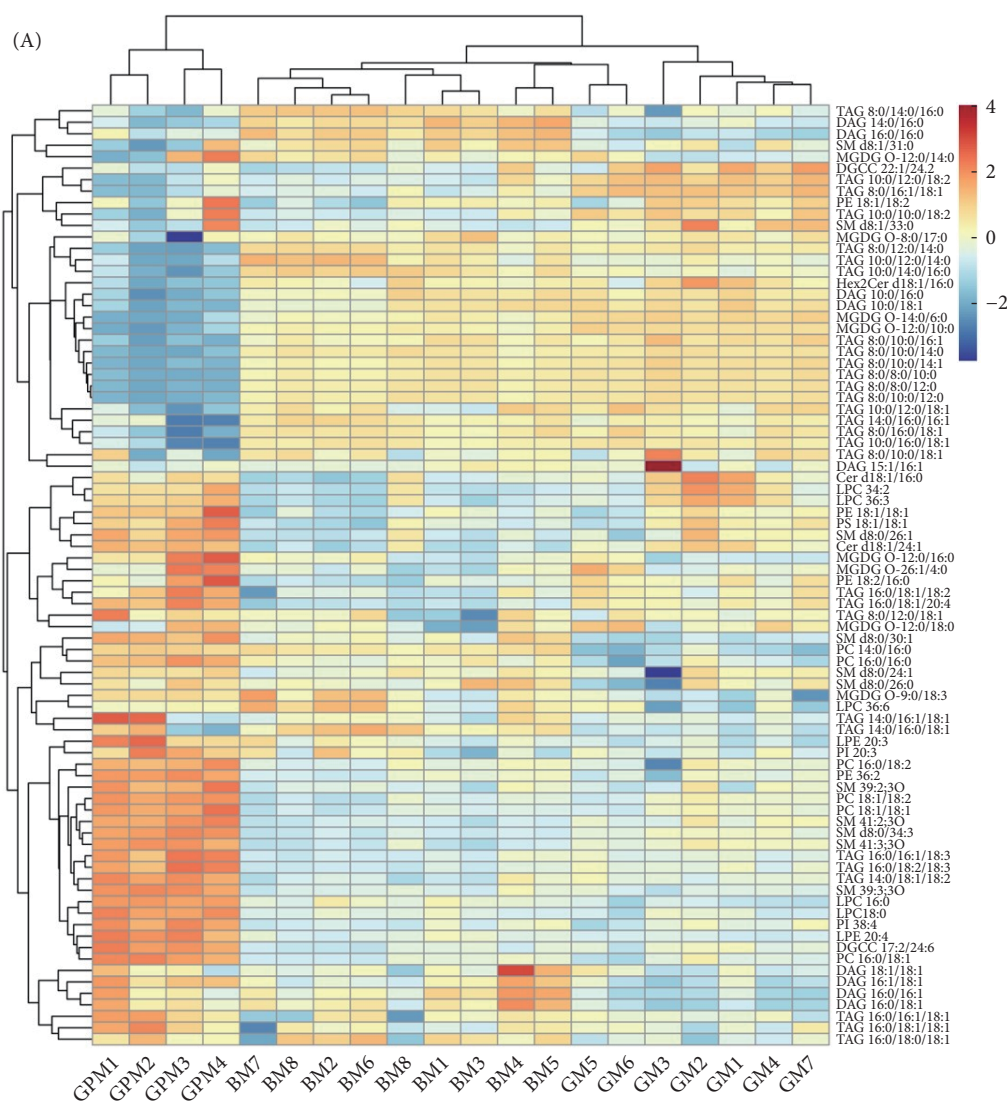


Figure 5 Heatmap analysis of the top 10% of individual lipids of each type in bovine milk, goat milk, giant panda milk in (A) POS and (B) NEG ion modes. BM, GM, and GPM represent bovine milk, goat milk, and giant panda milk, respectively.

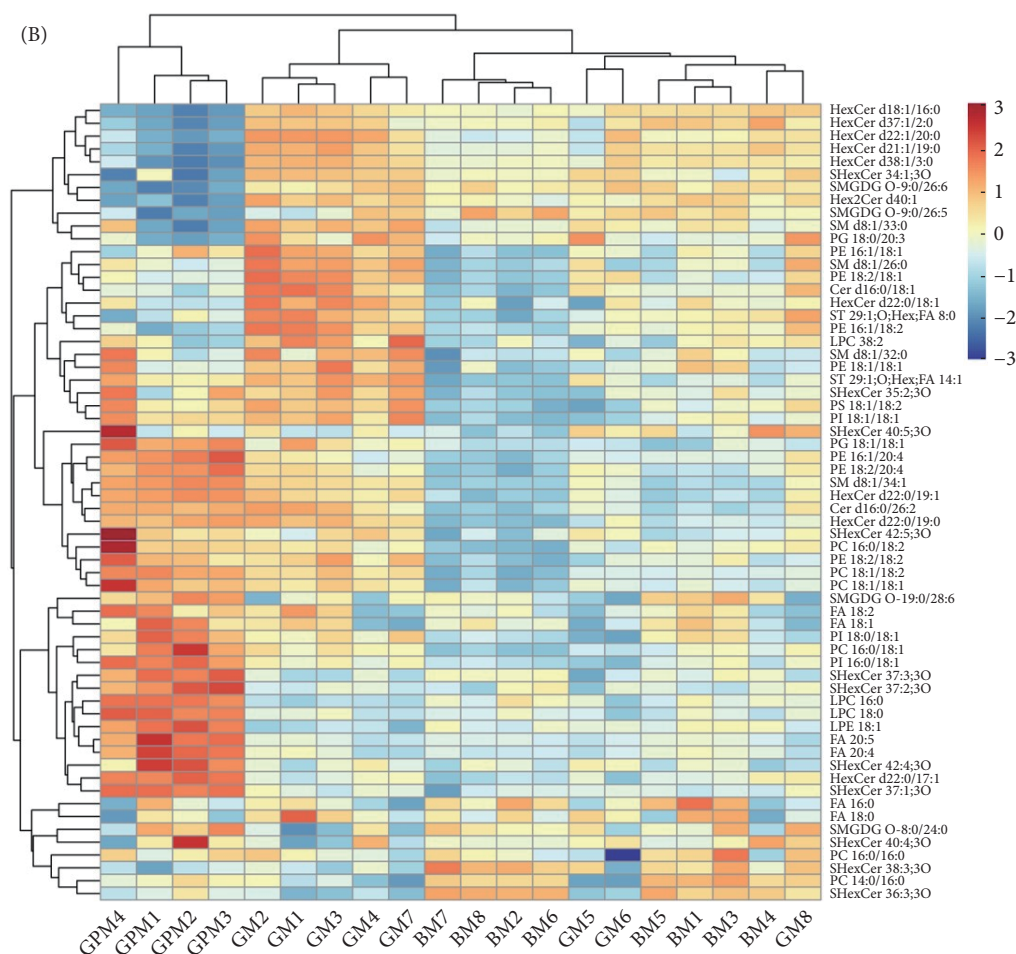


Figure 5 (Continued)

3.5 Polar lipid species analysis in the bovine milk, goat milk, and giant panda milk

GPLs and SPs are quantitatively the most important PLs in milk^[29]. GPLs are one of the essential components of milk lipids. PC, PE, PI, and PS are typical GPLs^[12,14]. PC is mainly associated with SFAs^[32]. Both POS and NEG ion mode results (Tables S1 and S2) showed that PC 16:0/18:1 was the most abundant PC type in giant panda milk (16.436% and 19.150%), bovine milk (23.956% and 25.568%), and goat milk (26.926% and 25.558%), and its relative content in giant panda milk was significantly lower than that of bovine milk and goat milk. Secondly, PC 16:0/18:2 and PC 18:1/18:1 were the second most abundant PC lipids (13.664% and 11.332%, 14.822% and 14.389%) in the giant panda milk samples, and their proportion in giant panda milk was close to that of goat milk but higher than that of bovine milk. Generally, PE lipids (< 300 ng/mL) are esterified to eicosapentaenoic acid and docosahexaenoic acid in mature human milk, both of which are important for brain development and the reduction of inflammatory events in infants^[18]. In the POS ion mode, PE 18:1/18:1 was the most abundant PE type in giant panda milk (16.433%), which was close to goat milk (19.786%) and lower than bovine milk (21.273%). In the NEG ion mode, PE 18:1/18:1, the most prevalent type of PE contained in the milk of these three species, was less abundant in giant panda milk (45.962%) than in bovine milk (68.927%) and goat milk (51.472%).

Considering the relative content of PC and PE, goat milk was a more suitable milk matrix for milk substitutes for pandas than bovine milk, but supplementation with PLs is still required.

SM, one of the SPs, is the main PL in milk lipid. In the POS ion mode, the most abundant SM-type species in three species milk samples was SM d8:0/26:1, representing more than 28% of all SM lipids present. Its relative content was 31.934% and 35.770% in bovine milk and goat milk, respectively, significantly higher than that in giant panda milk (28.056%) ($P < 0.05$). SM 41:3/3:0 was the second most abundant in giant panda milk (21.539%) and was significantly higher than bovine milk (0.860%) and goat milk (2.472%). In the NEG ion mode, SM d8:1/34:1 (26.727%) was the most abundant SM type in giant panda milk samples; reduced levels of this SM type were observed in bovine milk (1.235%) and goat milk (5.447%) ($P < 0.05$). Conversely, SM d8:1/26:0 was the most prevalent SM type in bovine milk (19.822%) and goat milk (26.137%); while this lipid type constituted only 15.376% of the total SM lipid content of giant panda milk. SMs are the main component of MFGM lipids. In general, SM containing ultra-long-chain fatty acids (LC-SFA) is the main substance in the lipid raft on the MFGM, which helps to reduce the mobility of MFGM and maintain the rigid structure^[15,32]. This unique structure constitutes a specific adhesion site in microorganisms, bacteria, and viruses that is an important source of the bactericidal function of MFGM^[34]. In our study, the proportion of SM-linked LC-SFA in giant panda

milk was higher in both POS and NEG modes (45.78% and 13.27%) than that in bovine milk (29.59% and 4.34%) and goat milk (30.81% and 4.36%) ($P < 0.05$). Therefore, compared with bovine milk and goat milk, the antibacterial properties of giant panda milk may be higher. In addition, SMs and PCs are the main sources of choline for cubs (17% of total choline intake), which is required for the rapid growth of cub organs and the synthesis of biofilms^[35–36].

Cer is the precursor to SM synthesis and the breakdown product of SM; hence, there may be a correlation between the content of SM and Cer in milk, however, there are few studies on the content of Cer in milk^[37]. In the POS ion mode, Cer d18:1/24:1 was the most abundant type of Cer in giant panda milk, and its relative content in giant panda milk (30.637%) was significantly higher than that of bovine milk (1.620%) and goat milk (9.686%) ($P < 0.05$). Cer d18:1/16:0 was the richest type of Cer in bovine milk (18.169%) and goat milk (35.887%), but was low in giant panda milk (16.825%) ($P < 0.05$). In the NEG ion mode, Cer d16:0/26:2 was the predominant Cer in giant panda milk (39.675%), with higher levels than in bovine milk (2.897%) and goat milk (10.348%). Conversely, Cer d16:0/18:1, the most abundant Cer class in bovine milk (30.791%) and goat milk (47.768%), had low levels in giant panda milk (14.813%). The percentage of long-chain Cer in giant panda milk was higher than that in bovine milk and goat milk, which might be consistent with the results for long-chain SM in giant panda milk.

The glycolipids in milk are mainly glycosphingolipids (GSL), and gangliosides are complex GSLs related to MFGM, which prevents pathogens from attaching to neonates and thus prevents infection^[38]. In the NEG ion mode, four HexCers were significantly different in the three types of milk: HexCer d18:1/16:0, HexCer d21:1/19:0, and HexCer d38:1/3:0 at significantly higher levels in bovine milk and goat milk than in giant panda milk, HexCer d22:0/19:1 was significantly higher in giant panda milk (53.551%) than in bovine milk (1.920%) and goat milk (5.496%). Overall, the HexCer content of giant panda milk is lower than that in bovine milk and goat milk. Previous studies have shown that HexCer is an important component of various membrane structures in organisms and plays

a critical role in transmembrane signaling occurring at the cell surface^[39]. This contribution is associated with the immune response, cell development, cell recognition, and differentiation^[40]. Therefore, the high HexCer level in bovine milk and goat milk may be more beneficial to the growth and development of the cubs. In short, in terms of SP lipid composition, goat milk differs less from giant panda milk than bovine milk and is more suitable as a giant panda milk fat substitute.

3.6 Differences in metabolic pathways

Metabolic regulation in organisms is complex, and the analysis of lipid metabolism and regulatory pathways provides a more systematic and comprehensive understanding of the changes in biological processes. The SDL results obtained for bovine milk vs. giant panda milk and goat milk vs. giant panda milk are shown in Tables S3 and S4. There were 223 and 128 SDLs between bovine milk and giant panda milk, and 208 and 126 SDLs between goat milk and giant panda milk in POS and NEG ion modes, respectively. To elucidate the differences in milk lipid metabolism between bovine milk, goat milk, and giant panda milk, SDLs were mapped to the KEGG database. These SDLs are involved in 22 metabolic pathways (Figure 6). Whether the SDLs belong to the bovine milk vs. giant panda milk group or the goat milk vs. giant panda milk group, SP metabolism, GPL metabolism, metabolic pathways, glycerolipid metabolism, and inositol phosphate metabolism were five main differential pathways. It was deduced that the main differences in PC, SM, PE, and other PLs between the three kinds of milk were related to GPL metabolism and SP metabolism. Previous studies have shown that a significant negative correlation has been reported between polar lipids and milk fat globule (MFG) size^[41]. Small MFGs have a larger total surface area, leading to differences in GPL metabolism between samples^[27,42]. Therefore, giant panda milk might contain a higher number and smaller size of MFGs. In addition, significant differences were also observed in the four pathways of fat digestion and absorption, vitamin digestion and absorption, lipolysis regulation of lipolysis in adipocytes, and adipocytokine signaling pathway.

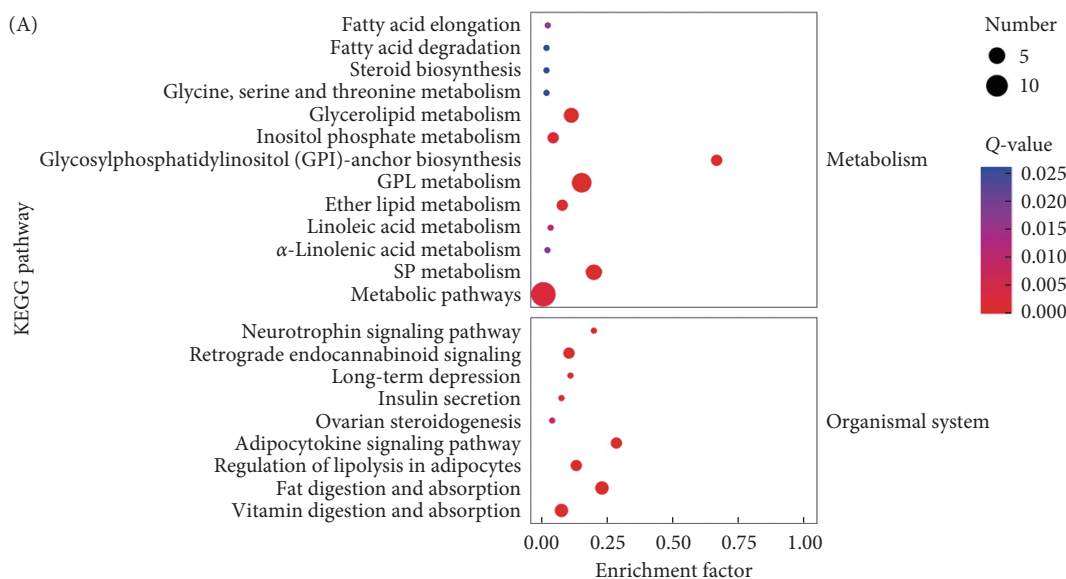


Figure 6 KEGG pathway analysis of SDLs in (A) bovine milk vs. giant panda milk and (B) goat milk vs. giant panda milk.

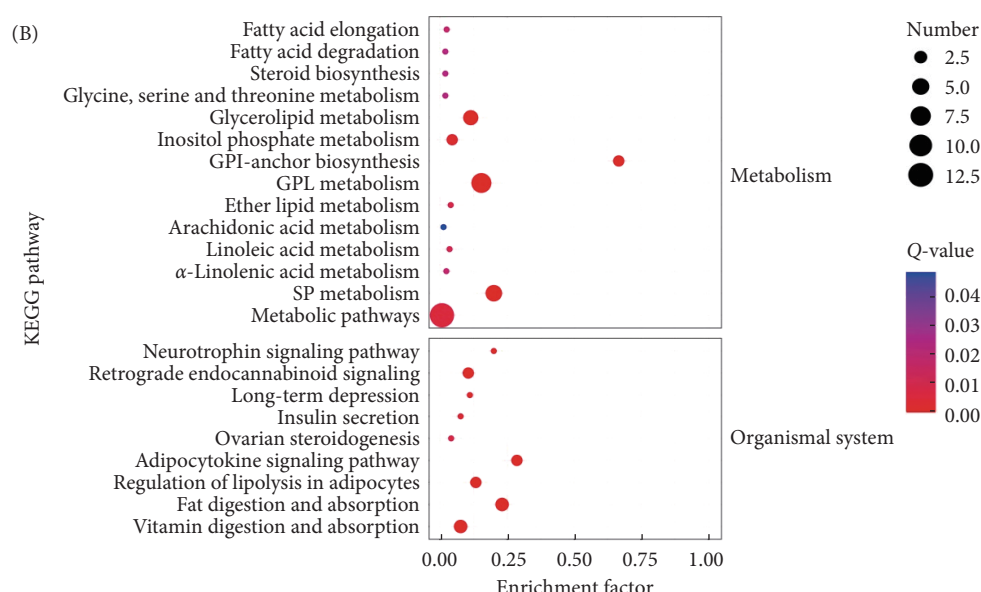


Figure 6 (Continued)

4 Conclusion

In the current study, we used a relatively quantitative method to analyze the lipids of bovine milk, goat milk, and giant panda milk. A total of 18 lipid subclasses and 860 lipid molecules were identified in three types of milk samples, and we found some interesting results on neutral and polar lipids. In general, giant panda milk had lower levels of neutral lipids than bovine milk and goat milk, but giant panda milk contained more PA, which was more favorable for the growth and development of the cubs. In polar lipids, giant panda milk contained much higher levels of PLs than bovine milk and goat milk, and the distribution of major GPLs in the three milk types differed significantly. The main differential lipid analysis results of bovine milk, goat milk, and giant panda milk showed that goat milk was closer to the lipid composition of giant panda milk than bovine milk. In addition, SP metabolism, GPL metabolism, metabolic pathways, glycerolipid metabolism, and inositol phosphate metabolism were identified as the five main differential pathways in the three species' milk. This study further analyzed the differences in biological characteristics of bovine milk, goat milk, and giant panda milk and provided practical information for using bovine milk and goat milk as the basic milk matrix for milk substitutes for giant pandas.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statements

Milk samples from the giant panda were carried out under ethical approval from the Qinling Giant Panda Research Center where the animals are held. All the giant panda mothers and their cubs were captive bred and maintained. Handling of cubs for health, normal growth, and veterinary checks is carried out daily at the facility of Qinling Giant Panda Research Center.

Appendix A. supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.26599/FSAP.2025.9240136>.

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