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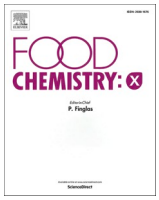

Characterization of lipid composition and nutritional quality of yak ghee at different altitudes: A quantitative lipidomic analysis

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ABSTRACT

Efficient and comprehensive analysis of lipid profiles in yak ghee samples collected from different elevations is crucial for optimal utilization of these resources. Unfortunately, such research is relatively rare. Yak ghee collected from three locations at different altitudes (S2: 2986 m; S5: 3671 m; S6: 4508 m) were analyzed by quantitative lipidomic. Our analysis identified a total of 176 lipids, and 147 s lipid of them were upregulated and 29 lipids were downregulated. These lipids have the potential to serve as biomarkers for distinguishing yak ghee from different altitudes. Notably, S2 exhibited higher levels of fatty acids (21:1) and branched fatty acid esters of hydroxy fatty acids (14:0/18:0), while S5 showed increased levels of phosphatidylserine (O-20:0/19:1) and glycerophosphoric acid (19:0/22:1). S6 displayed higher levels of triacylglycerol (17:0/20:5/22:3), ceramide alpha-hydroxy fatty acid-sphingosine (d17:3/34:2), and acyl glucosylceramides (16:0–18:0–18:1). Yak ghee exhibited a high content of neutralizing glycerophospholipids and various functional lipids, including sphingolipids and 21 newly discovered functional lipids. Our findings provide insights into quantitative changes in yak ghee lipids during different altitudes, development of yak ghee products, and screening of potential biomarkers.

1. Introduction

Yak ghee, a traditional dairy product found on the Qinghai-Tibet Plateau in China, is derived from yak milk and exhibits similarities with regular ghee in terms of its rich content of high-quality fats, proteins, carbohydrates, and various micronutrients that have potential medicinal properties (Liu et al., 2018). Notably, yak ghee is known for its concentrated levels of polyunsaturated fatty acids (PUFAs) such as eicosatetraenoic acid (EPA), docosahexaenoic acid (DHA), and lecithin-cholesterol-(fatty) acyl transferase (LCA) (Chukwunonso et al., 2017). These fatty acids are believed to play a crucial role in maintaining human health (Yi et al., 2020). The abundance of functional lipids, such as conjugated linoleic acid (CLA) and oleic acid, is significantly higher compared to common butter in yak ghee. In fact, the levels of CLA in yak ghee are more than twice as high as those in common butter, while the levels of oleic acid are approximately 8 times higher (Zhou et al., 2014). Currently, ghee is widely utilized in both the food and medical industries, and is used to enhance the nutritional value and flavor of zanba (a traditional Tibetan dish). Meanwhile, ghee is also used to treat infant diarrhea and relieving constipation among elderly populations. Therefore, in order to acquire a more comprehensive and in-depth understanding of ghee lipids, it becomes crucial to study its properties and composition. The investigations will not only contribute to a more comprehensive understanding of the lipid composition in yak ghee, but

Abbreviations: Acronym, Full name; PS, Phosphatidylserine; PG, Phosphatidylglycerol; PA, Glycerophosphoric acid; SQDG, Isothiorhamno glycerindiester; GlcADG, Glucosaldodiacyl glycerindiester; DAT, Dopamine transporter; Cer, Ceramide; NBGly, Neurosporaxanthin beta-D-glucopyranoside; HMcar, 3Beta-hydroxy-4beta-methyl-5alpha-cholest-7-ene-4alpha-carbaldehyde; SP, Sphingosine-1-phosphate; SHexcerd, Sulfatide; BAOGM, Bichanin A 7-O-glucoside-6''-malonate; HydroS, 26-hydroxycholesterol 3-sulfate.

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also serve as a valuable guide for formulating nutritious ghee products and promoting a healthy dietary lipid intake.

Lipids are indispensable constituents of various metabolites and play pivotal roles in numerous biological processes. Yak ghee, as a potentially healthier lipid source, has been extensively investigated for its lipid composition and functional properties such as tyrosine inhibitory activity and antioxidant properties (Silva et al., 2018). Notably, Himalayan alpine yak ghee, derived from yaks inhabiting an altitude of approximately 4300 m, demonstrates a more well-balanced fatty acid composition in comparison to domestic yak ghee from yaks residing at around 3000 m (Or-Rashid et al., 2008). The findings suggest that the nutrient and bioactive substance composition of yak milk lipids undergoes modifications in response to environmental conditions, thereby adapting to meet the nutritional needs of premature infants. Consequently, this leads to variations in the lipid composition of yak ghee. In contrast to regular butter, yak ghee also contains lipids that are comparatively safer due to the natural habitat of yaks in plateau areas (ranging from 3000 m to 5000 m above sea level), while cattle are primarily raised in plain areas (below 200 m above sea level) (Karandikar et al., 2016). However, limited research has been conducted to comprehensively analyze the lipid composition of ghee at various altitudes on a large scale and with absolute quantification.

Lipidomic, an integral part of metabolomics, plays a crucial role in identifying the functional and unique characteristics of lipids in food samples. With advancements in mass spectrometry and related analytical technologies, lipidomics has emerged as a robust tool for the comprehensive analysis and identification of complex food systems and low-abundance lipids of interest, garnering increasing attention from food scientists (Centonze et al., 2019). A lipidomic approach based on UHPLC-QTOF-MS technology was utilized in a study to identify lipid variations in donkey milk across different lactation periods, and to successfully characterize and compare low-abundance lipids present in bovine colostrum and mature milk (Li et al., 2020). Due to the intricate and diverse composition of yak milk components across various altitudinal regions, the production of ghee at different elevations showcases intricacies and disparities. By incorporating state-of-the-art techniques in lipidomic research, it has become feasible to elucidate the variations and functional characteristics of lipids present in yak ghee across different altitudes. However, the distinction and functional characterization of ghee lipids in yak ghee at different elevations are rather little.

Therefore, the aim of this study was to utilize quantitative lipidomic methods to perform a thorough analysis on the lipids found in yak ghee at three different altitudes in Tibet. The findings of this study have the potential to provide valuable theoretical guidance for the development of ghee products, facilitate the promotion of rational dietary lipid intake, and the contribute to the identification of potential biomarkers associated with ghee.

2. Material and methods

2.1. Yak ghee sample collection

The study collected a total of 18 fresh yak ghee samples, divided into three groups of 6, from three distinct elevations in Tibet, China, spanning the period between September and December 2021. Samples got from location S2 [29.61 N, 94.36E, mean sea level is more than 2,968], location S5 [29.67 N, 90.88E, mean sea level is more than 3,671 m (Lhasa)] and location S6 [31.5 N, 92.1E; mean sea level is more than 4,508 m (Naqu)]. Sample of every location were obtained from six different farmers' markets and all ghee samples are mixed for further analysis. All samples were immediately transported to the laboratory chilled on ice and stored at −80 °C until analysis.

2.2. Chemicals and quality control (QC) samples

The chemicals used in this study are as follows: methanol (67–56-1, LC-MS grade, CNW Technologies), acetonitrile (LC-MS grade, CNW Technologies), methyl *tert*-butyl ether (MTBE) (1634–04-4, LC-MS class, CNW Technologies), ammonium formate (540–69-2, LC-MS class, CNW Technologies), ammonium hydroxide (1336–21-6, LC-MS grade, Fisher Chemical), dichloromethane (75–09-2, LC-MS grade, CNW Technologies), isopropanol (67–63-0, LC-MS grade, Fisher Chemical), all other reagents are analytical grade. Six identical QC samples were used to identify lipids in ghee and ensure the reliability of the experimental results.

2.3. Lipid extraction

12.5 mg sample was weighed and placed in an EP tube. Then, 200 μL of water and 480 μL of an extract solution consisting of MTBE (methyl *tert*-butyl ether) and MeOH (methanol) in a ratio of 5:1 was added to the tube sequentially. The tube was vortexed for 30 s to mix the contents, and then the samples were homogenized at a frequency of 35 Hz for 4 min. After homogenization, the samples were sonicated for 5 min in an ice-water bath. This homogenization and sonication cycle was repeated two more times. Following the homogenization and sonication steps, the samples were incubated at −40 ℃ for 1 h to facilitate further extraction. After incubation, the samples were centrifuged at 3000 rpm for 15 min at 4 ℃ to separate the supernatant from the solid components. Next, 300 μL of the supernatant was transferred to a fresh tube, and the solvent was removed by drying the sample using a stream of nitrogen gas. The dried samples were then reconstituted in 200 μL of a solution containing 50 % methanol in dichloromethane through sonication for 10 min in an ice-water bath. The reconstituted samples were centrifuged at 13000 rpm for 15 min at 4 ℃ to remove any remaining particulate matter. Finally, 75 μL of the supernatant was transferred to a fresh glass vial for further analysis using LC/MS. To prepare a quality control (QC) sample, equal aliquots of the supernatants from all the samples were mixed (Dunn et al., 2011).

2.4. Lipid analysis

For the UHPLC separation, an Exion LC Infinity series UHPLC System from AB Sciex was used. It was equipped with a Kinetex C18 column measuring 2.1 * 100 mm with a particle size of 1.7 μm, manufactured by Phenomenex. The mobile phase A was prepared by mixing 40 % water, 60 % acetonitrile, and 10 mmol/L of ammonium format. The mobile phase B was composed of 10 % acetonitrile and 90 % isopropanol, with the addition of 50 mL of 10 mmol/L ammonium format for every 1000 mL of the mixed solvent. The elution gradient for the analysis was as follows: from 0 to 12.0 min, the mobile phase B was increased from 40 % to 100 %; from 12.0 to 13.5 min, the mobile phase B was held at 100 %; from 13.5 to 13.7 min, the mobile phase B was decreased from 100 % to 40 %; and from 13.7 to 18.0 min, the mobile phase B was held at 40 %. The column temperature was maintained at 55 ℃. The auto-sampler temperature was set to 6 ℃, and the injection volume was 2 μL for positive ionization mode or 4 μL for negative ionization mode (Want et al., 2010).

Mass spectrometry analysis was performed using the Triple TOF 5600 mass spectrometer. It allowed for the acquisition of MS/MS spectra on an information-dependent basis (IDA) during the LC/MS experiment. The acquisition software used was Analyst TF 1.7 from AB Sciex. The software continuously evaluated the full scan survey MS data and triggered the acquisition of MS/MS spectra based on preselected criteria. In each cycle, the 12 precursor ions with the highest intensity above 100 were chosen for MS/MS analysis at a collision energy (CE) of 45 eV. Each MS/MS event had an accumulation time of 50 msec. The electrospray ionization (ESI) source conditions were set as follows: Gas 1 at 60 psi, Gas 2 at 60 psi, Curtain Gas at 30 psi, Source Temperature at 600 ℃, DE clustering potential at 100 V, and Ion Spray Voltage Floating (ISVF) at 5000 V for positive ionization mode or −3800 V for negative ionization mode, respectively.

2.5. Data processing and annotation

The original data files in WIFF format were converted to the mzXML format using the MS convert program from ProteoWizard. The first step in data analysis was peak detection on the MS1 data, which was performed using the CentWave algorithm in XCMS. To achieve lipid identification through spectral matching, a spectral comparison was conducted using the Lipid Blast library on the MS/MS spectrum.

2.6. Statistical analysis

To avoid false-positive errors in the multivariate statistical analysis, Student's t-tests and variance analyses curve analyses were employed for SDL screening. The lipids that showed differential contents were identified through statistical methods with the following screening criteria: $p < 0.05$, variable importance in projection (VIP) > 1, fold change (S2, S5 vs S6) > 2.5 or < 0.83. PubChem database (https://pubchem.ncbi.nlm.nih.gov), and LIPID MAPS database (https://www.lipidmaps.org) were used for a qualitative analysis and to search for metabolites in the lipid biosynthesis pathway. Each experiment was conducted at least three times, and the data were analyzed using GraphPad Prism 8 software.

2.7. Bioinformatics analysis

The MultiExperimentViewer 4.9.0 software and SIMCA 14.1 software were used to generate heat maps, VIP value, PCA, OPLS-DA, volcano plots, and perform statistical analysis on the yak ghee samples from different altitudes. This was done to understand the patterns of change in differentially metabolites between the experimental and control groups. In addition, commercial databases such as KEGG pathway database (KEGG, https://www.kegg.jp/kegg/), Human Metabolome Database (HMDB, https://www.hmdb.ca), MetaboAnalyst (MetaboAnalyst, https://www.metaboanalyst.ca) and PubChem database (PubChem, https://pubchem.ncbi.nlm.nih.gov) were employed for qualitative analysis and to search for metabolites involved in the lipid biosynthesis pathway. Finally, a custom Venn diagram was generated using the Calculate and draw custom Venn diagrams online tool (https://bioinformatics.psb.ugent.be) to visualize the identified lipids.

3. Results

3.1. Qualitative analysis of ghee lipids

"A total of 18 samples (6 from S2 loaction, 6 from S5 location, and 6 from S6 location) and 6 QC samples were analyzed in both positive and negative ion modes. After denoising the quartile range of the sample spectrum results, a total of 15,342 positive ion mode peaks and 11,678 negative ion mode peaks were detected (Fig. S1A and Fig. S1B). To fill in missing values in the raw data, half of the minimum value was used (Li et al., 2020). In the differential analysis of lipid abundance, total ion current was employed for normalization purposes. Lipids detected in both positive and negative ionic modes underwent repeated filtration to ensure the precision and consistency of the results. In the positive ion mode, 788 lipids were identified in S5 vs S2, 825 lipids in S6 vs S2, and 142 lipids in S6 vs S5. In the negative ion mode, 1027 lipids were identified in S5 vs S2, 1028 lipids in S6 vs S2, and 364 lipids in S6 vs S5 (Fig. 1A).

Among the identified lipids, glycerophospholipids, fatty acyls, glycerolipids, sphingolipids, and sterol lipids are the major lipid groups (Fig. 1B). Glycerophosphoric acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and liposome (LPIM1) belongs to glycerophospholipids. Fatty acyls have 5 main subclasses [branched fatty acid esters of hydroxy fatty acids (FAHFA), CLA, polyglycerol esters of fatty acids (PGE), the metabolites of 15d-PGJ2 (PGJ2), fatty acids (FA)], Glycerolipids have 5 main subgroups [triglycerides (TG), Glucosaldodiacylglycerindiester (GLcADG), thylakoid lipids monogalactoglycerindiester (MGDG), isothiorhamnoglycerindiester (SQDG), diggalactoglycerindiester (DGDG)]. Sphingolipids have 5 main

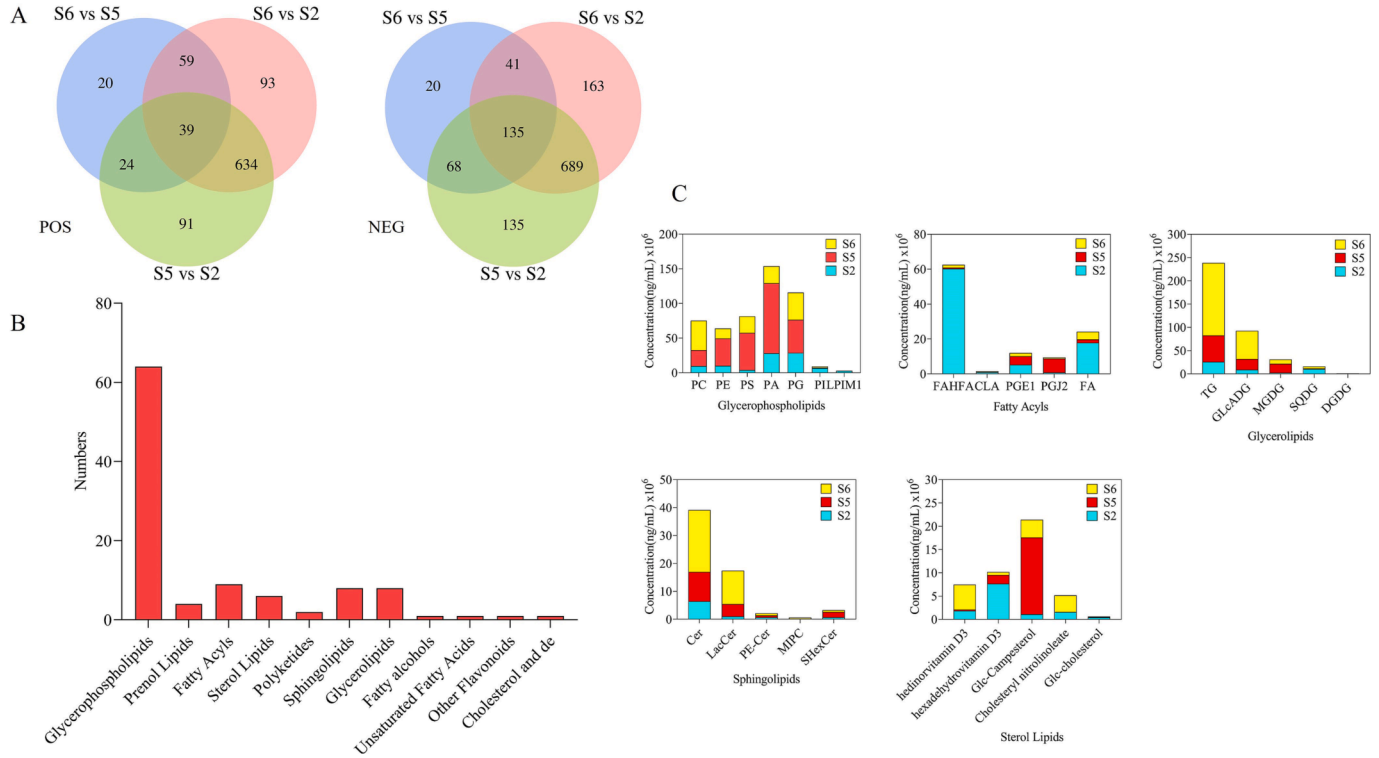

Fig. 1. Lipids identified in yak ghee at different altitudes. Number of crisp oils identified in S2, S5, S6 (A); Classification of crisp oils identified in S2, S5, S6 (B); Sublipids in ghee identified in S2, S5, S6 (C). S2 = Ghee at an altitude of 2,968 m; S5 = Ghee at an altitude of 3,671 m; S6 = Ghee at an altitude of 4,508 m.

subclasses [Ceramide (Cer), Lactose ceramide (LacCer), phosphatidylethanolamine (PE-Cer), Sugar alcohol phosphate ceramide (MIPC), sulfatide (SHexCer)]. Sterol lipids have 5 main subclasses (Hedinorvitamin D3, hexadehydrovitamin D3, Glc-campesterol, cholesterylnitrolinoleate, Glc-cholesterol) (Fig. 1C).

3.2. Quantification of ghee lipids

After loading samples, the total lipid contents were detected in the negative ion mode was 3.33 mg/mL for S2, 4.39 mg/mL for S5, and 5.49 mg/mL for S6. In the positive ion mode, the total lipid content was 0.15 mg/mL for S2, 0.10 mg/mL for S5, and 0.15 mg/mL for S6. The content of each subcategory is estimated using the sum of all lipids detected in the same subcategory. The contents of the identified major lipid subclasses are statistically analyzed and finally presented in Fig. 2C as a bar chart. The results showed that the contents of TG (155.71x10^6 ng/ mL), GlcADG (60.39x10^6 ng/ mL) and Cer (22.16 x10^6 ng/ mL) were the highest in S6 samples ($p < 0.05$). The content of PA (101.14 x10^6 ng/ mL) and PG (47.82 x10^6 ng/mL) was the highest in S5 samples ($p < 0.05$).The FAHFA content was the highest in S2 samples (60.26 x10^6 ng/ mL) ($p < 0.05$). Compared with S2 samples, most sub lipids levels in S5 and S6 samples were significantly increased ($p < 0.05$), such as PC, PE, PS, PA, PG, TG, Cer, etc. Within these subclasses, the subtypes of lipids that showed significant differences in S6 were PC (42.51 x10^6 ng/mL), TG (155.71x10^6 ng/mL), GlcADG (60.39x10^6 ng/mL), Cer (22.16x10^6 ng/mL), LacCer (11.96 x10^6 ng /mL) ($p < 0.05$). Subtypes of fat showed significant differences in S5 were PE (39.49 x10^6 ng/mL), PS (53.70 x10^6 ng/ mL), PA (101.14 x10^6 ng/ mL), PG (47.82 x10^6 ng/mL), PGJ2 (7.98 x10^6 ng/ mL), SHexCer (1.95 x10^6 ng/ mL) ($p < 0.05$). Subtypes of lipids showed significant differences in S2 were FAHFA (60.26 x10^6 ng/ mL), FA (17.84 x10^6 ng/ mL), and hexadehydrovitamin D 3 (7.65 x10^6 ng/ mL) ($p < 0.05$).

3.3. Lipid pattern recognition analysis of ghee samples

Using MultiExperimentViewer4.9.0 software and SIMCA 14.1 software, we obtained data including peak number, sample name and normalized peak area, PCA, OPLS-DA and volcano plot analysis for data sets (Fig. S2). The PCA models are generated based on the principal directions in the data that exhibit the highest variability, which may

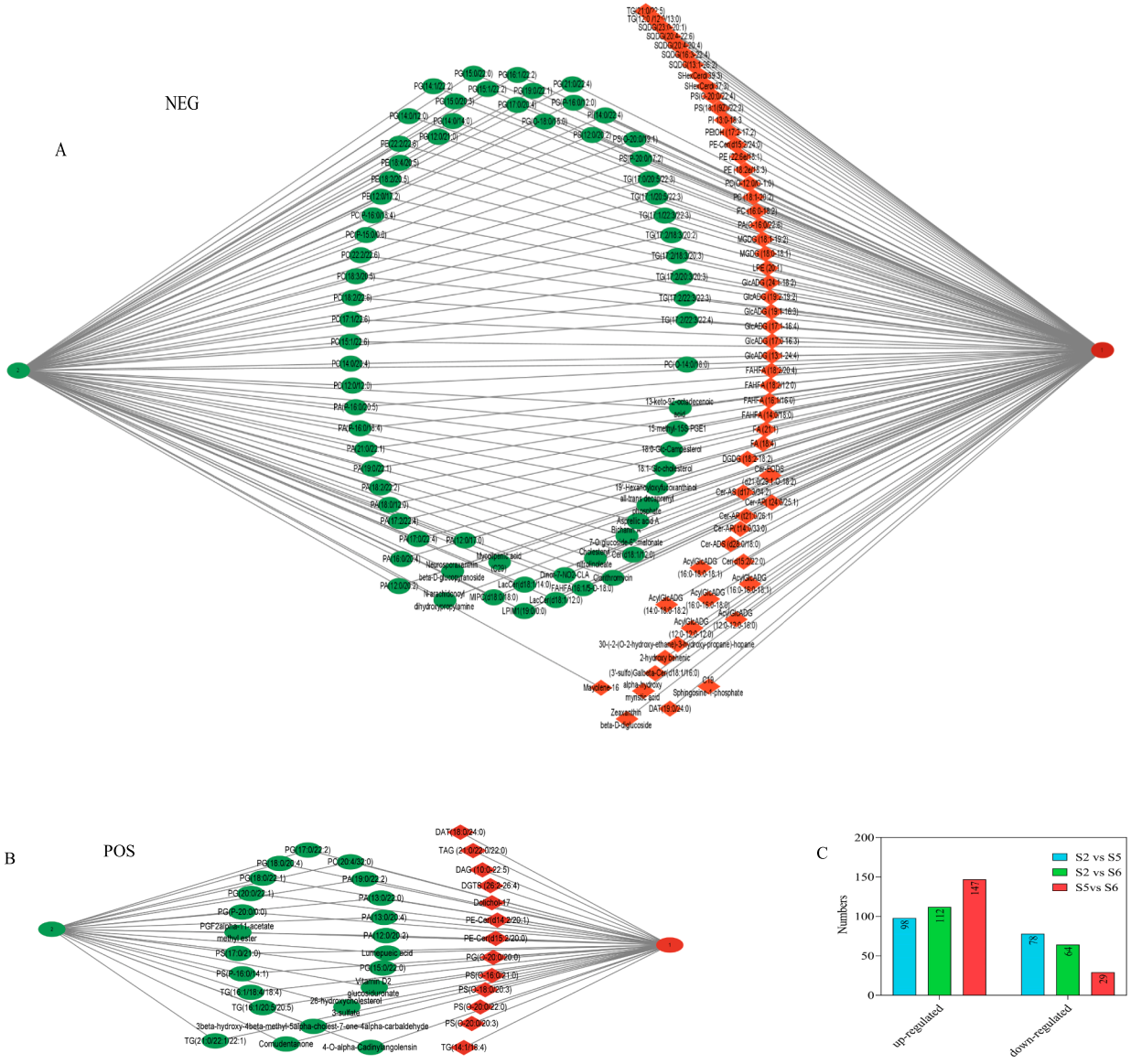

Fig. 2. Differentially expressed lipids were identified in S2, S5 and S6 (A and B); The number of differentially expressed lipids up-regulated/down-regulated were identified in S2, S5, and S6 (C). S2 = Ghee at an altitude of 2,968 m; S5 = Ghee at an altitude of 3,671 m; S6 = Ghee at an altitude of 4,508 m.

differ significantly from the directions of the separated categories (Fig. S2A$_1$, Fig. S2A$_2$). Moreover, the lack of readily available information regarding the differences between classes due to the utilization of multiple models impedes the explanatory power of classification models. To enhance group separation and gain a deeper understanding of the variables responsible for classification, we employed supervised OPLS-DA (Fig. S2B$_1$, Fig. S2B$_2$). The R^2Y values for S5 vs S2, S6 vs S2, and S6 vs S5 are 0.98, 0.98, and 0.99, respectively, in the positive ion mode, and are 0.95, 0.95, and 0.97, respectively, in the negative ion mode. In addition, the Q^2 values of the S5 vs S2, S6 vs S2, and S6 vs S5 are −0.71, −0.72, and −0.55, respectively, in the positive ion mode, while those in the negative ion mode are −0.79, −0.82, and −0.67, respectively (Fig. S2C$_1$, Fig. S2C$_2$). The stability of these two values demonstrates the robustness of our OPLS-DA model, indicating its resistance to overfitting. The low Q^2 intercept values further validate the model's reliability and minimal risk of overfitting, highlighting its strong fitting and predictive capabilities.

The volcano plot analysis (Fig. S2D1 and Fig. S2D2) was used to visualize the changes in the metabolite levels between the experimental and control groups. The plot revealed that the differentially metabolites were distributed across distinct peaks, exhibiting significant variations in abundance between the two groups. This information can be used to further investigate the specific pathways and mechanisms involved in the biosynthesis of yak ghee. The PCA and OPLS-DA results provided a quantitative assessment of the differences between the experimental and control groups, allowing for a more detailed understanding of the metabolic profiles and the factors contributing to them. Overall, these analyses provide valuable insights into the biosynthesis of yak ghee and can be used to guide further research in this area.

3.4. Identification of significantly different lipids between S2, S5 and S6

A total of 176 lipids with significant differences were identified (VIP > 1, $p < 0.05$, Fold change > 2.5 or Fold change < 0.83) (Fig. 2A, Fig. 2B). The S6 exhibited up-regulation of 112 lipids and down-regulation of 64 lipids compared to the S2, whereas the S5 showed up-regulation of 98 lipids and down-regulation of 78 lipids. In comparison to the S5, there was an up-regulation of 147 lipids and a down-regulation of 29 lipids in the S6 (Fig. 2C). These up-regulated lipids included TG, PS, PE, PC, PA, etc., while the down-regulated lipids included PG, SQDG, PI, FAHFA, FA, etc. Log_fold change of PC [18:1–20:2] and FAHFA [14:0–18:0] exhibited the most significant differences among the 176 lipids in S2, S5, and S6, with a maximum value of 5.593 for PC [18:1–20:2] and a minimum value of −12.386 for FAHFA [14:0–18:0]. These significantly different lipids with high expression levels were statistically analyzed (Fig. 3). The results showed that the contents of TG, PS and PA were the highest, the others were PG, PE, PC, FAHFA, FA and GlcADG in order. The highest TG content was found in S6 (10 subtypes), followed by PC (13 subtypes) GlcADG (12 subtypes). TG [17:2/22:3/22:4], TG [17:2/20:3/20:3], TG [17:1/20:5/22:3], TG [17:0/20:5/22:3], PC [18:1–20:2], AcylGlcADG [16:0–18:0–18:1], and AcylGlcADG [16:0–16:0–18:0] are major lipids in S6, and their contents are 19.51 x10^{6}ng/mL, 28.57 x10^6 ng/mL, 21.14 x10^{6}ng/mL, 46.50 x10^6 ng/mL, 16.81 x10^6 ng/mL, 17.48 x10^6 ng/mL, 14.16 x10^6 ng/mL, respectively. The highest content of PS (including five subtypes, mainly PS[O-20:0/19:1], relative content of 43.96x10^6 ng/mL), PA (including 12 subtypes, the most abundant lipids are PA [19:0/22:1] and PA [18:2/22:2], their contents are 34.77x10^6 ng/mL and 24.89x10^6 ng/mL, respectively), and PE (including eight subtypes, mainly PE-CER [d15:2/24:0], which is 19.57x10^6 ng/mL) are exhibited in S5. The highest content of FAHFA (including 2 subtypes such as FAHFA [14:0/18:0] and [18:2/20:4], their contents are 29.36x10^6 ng/mL and 18.07 x10^6 ng/mL, respectively) and FA [21:1] (15.96x10^6 ng/mL) are shown in S2 (Fig. 4).

In addition to the lipids mentioned above, the additional 21 functional lipids were also identified in the S2, S5, and S6. Relatively high content of newly identified lipids includes all-trans decaprenyl phosphate, dopamine transporter (DAT), alpha'-trehalose6-mycolate, neurosporaxanthin beta-D-glucopyranoside, alpha-hydroxymyristic acid, and sphingosine-1-phosphate, which were selected for further analysis and discussion (Table 1).

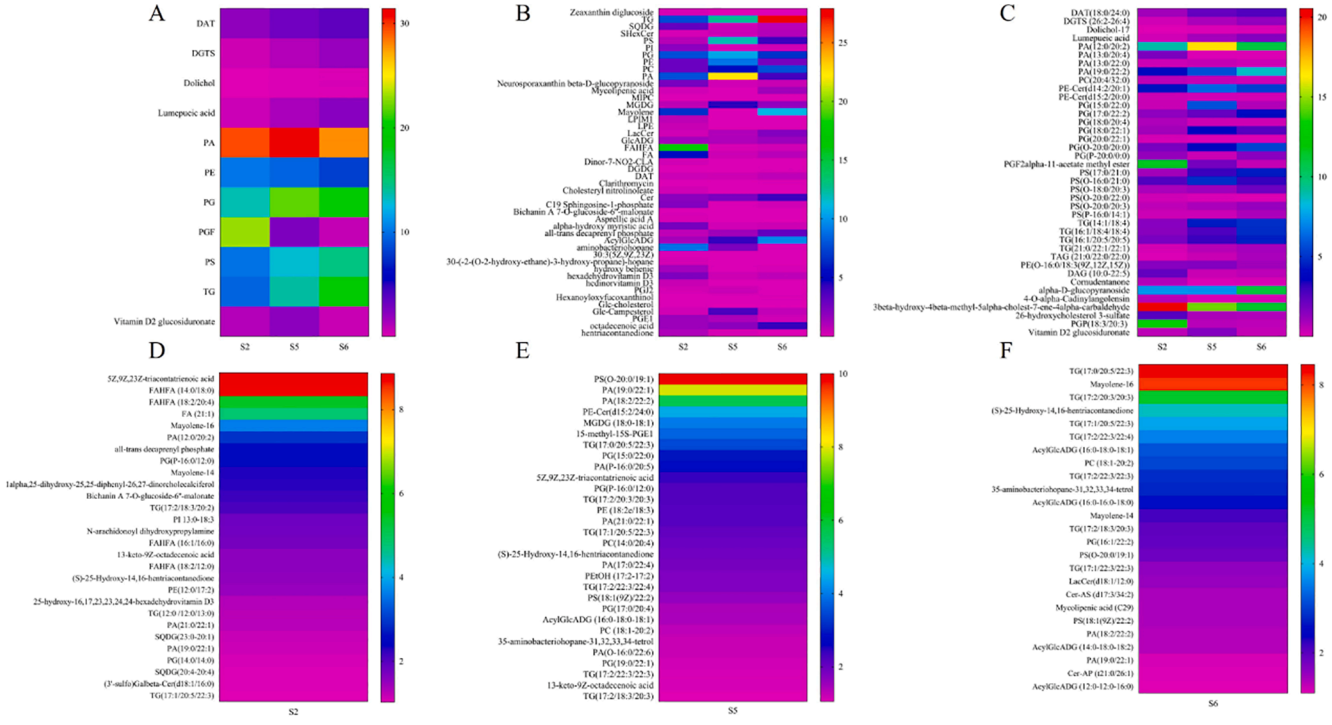

Fig. 3. Heat map analysis of 176 SDLs between S2, S5 and S6. Positive ion mode (A and B), negative ion mode (C–F, Only the top 28 percent). S2 = Ghee at an altitude of 2,968 m; S5 = Ghee at an altitude of 3,671 m; S6 = Ghee at an altitude of 4,508 m.

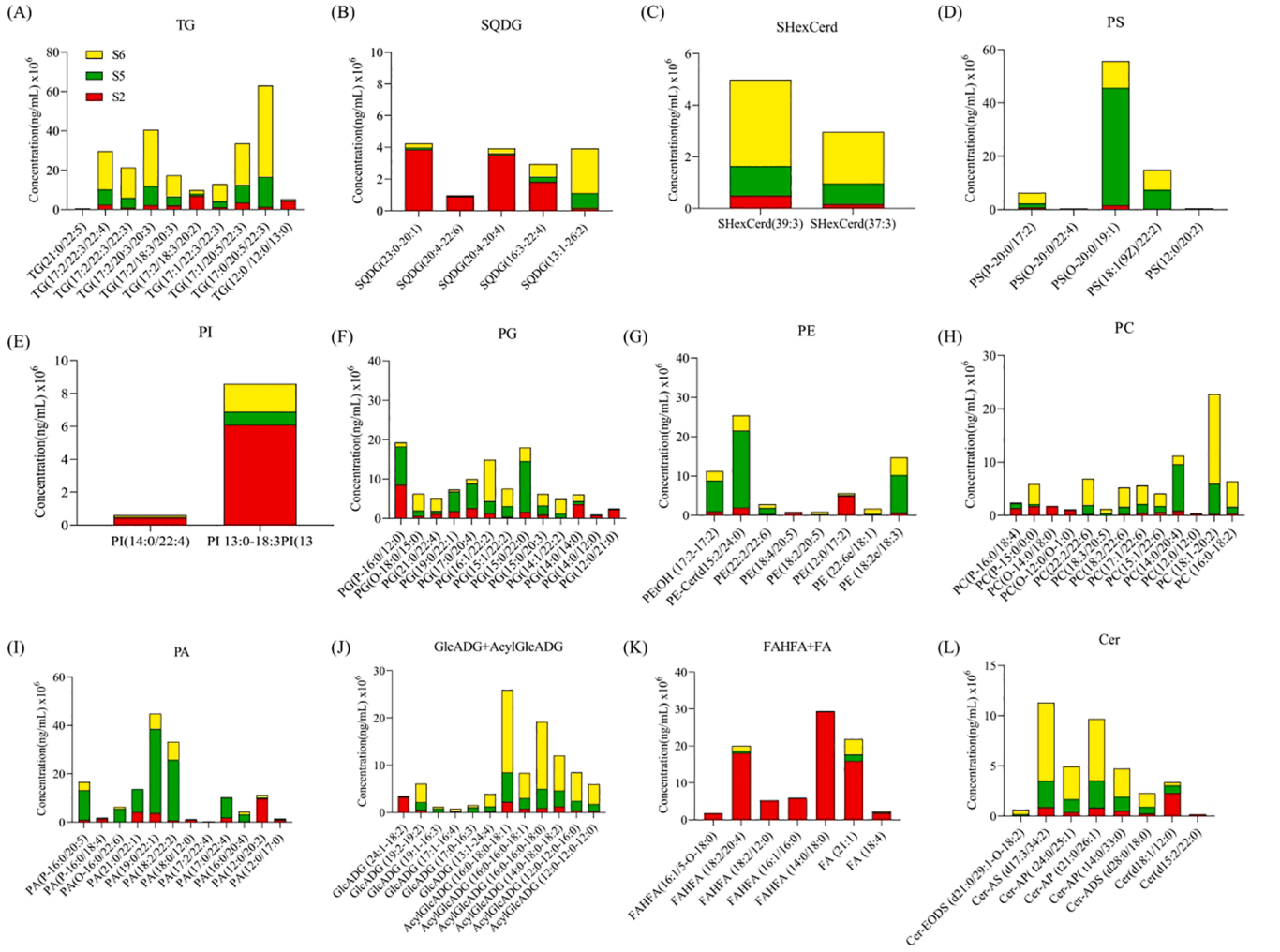

Fig. 4. Comparison of the contents of lipid subclasses between yak ghee S2, S5 and S6 (A-L). S2 = Ghee at an altitude of 2,968 m; S5 = Ghee at an altitude of 3,671 m; S6 = Ghee at an altitude of 4,508 m.

3.5. Correlation analysis of ghee lipids among the three altitudes

Various lipids share certain similarities in their physiological and molecular properties, and in this study, it was also found that lipids exhibit similar pattern in yak ghee samples collected in different altitude places. Thus, we assume that these lipids are correlated/co-regulated. To validate this hypothesis, we conducted an unweighted correlation network analysis on the differential representations of lipids obtained, which could unveil the interplay among lipids at varying altitudes. The correlation between most lipids, as demonstrated in Fig. 5A-7C, is highly significant ($p < 0.05$). Moreover, the correlation among differential lipids of high-altitude ghee is notably stronger compared to that of low-altitude ghee. In total, 395 and 410 correlations were identified in the S2 group and S5 group, respectively, but 817 correlations were detected in the S6 group. The effect of altitude is evident in the correlation networks. There are an extremely strong association among TG, AcylGlcADG, SQDG, PS, PE, PC, PA, SHexCerd, all-trans phosphodiesterase, LacCer, DAT, clarithromycin, 15-methyl-15S-PGE1, and others, particularly in the S6 group. It also reflects changes in the homeostasis of glycerol lipids, phospholipids, sphingolipids, macrolipids, and hormonal lipids in high-altitude ghee (S6:4,508 m). In the S2 group, PC, PG, PA, SQDG, and TG formed dense clusters with additional differential lipids, reflecting changes in the steady-state of glycerides in low altitude ghee (S2: 2,968 m). In S5 group, 18C mono unsaturated fatty acid, clarithromycin, LacCer, PA, PC, PE, PG, PS, TG, and SQDG form dense clusters with other differential lipids, reflecting the changes in the stability of glycerol lipids, 18C mono unsaturated fatty acid, and lipids in the macrocycle in ghee (S5) at an altitude of 3,671 m.Our findings suggest a correlation between different lipids and indicate that altitude may influence the composition and functional properties of numerous additional lipids present in yak ghee.

3.6. Metabolic pathways and functional analysis of different ghee lipids among the three altitudes

To further investigate the potential impact of altitude on ghee lipid metabolism, a comparative analysis was conducted by mapping the different content lipids identified onto the KEGG, HMDB, and PubChem databases. Path analysis was conducted using MetaboAnalyst 4.0, wherein p-values and path effects were computed via path topology analysis. The study unveiled significant correlations between altitude and five key pathways, namely linoleic acid metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, glycerolipid metabolism, glycerophospholipid metabolism, and steroid hormone biosynthesis. Among these pathways, glycerophospholipid metabolism exhibited the most significant correlation with altitude, followed by glycerolipid metabolism (Fig. 5D). These findings suggest that alterations in lipid metabolism may play a pivotal role in the response of yak ghee to variations in altitude. Further investigation is warranted to elucidate the specific mechanisms underlying these alterations and their

Table 1
Special differential lipid function of yak ghee.

Members	S2 x 10^6 ng/mL	S5 x 10^6 ng/mL	S6 x 10^6 ng/mL	Functions	References
Octadecenoic acid	5.1200	8.3400	0.1300	Anti-inflammatory, inhibit microglia cell activation, Antibacterial, enhance energy metabolism	(Xie et al., 2022)
All-trans decaprenyl phosphate	2.2900	6.1800	23.0276	Antibacterial (Bacillus divergences)	(Grover et al., 2014)
DAT	1.4400	2.0700	8.0600	Activation neuron regulation, prevention of Parkinson's disease and related diseases	(Brücke and Brücke, 2022)
Neurosporaxanthin beta-D-glucopyranoside	8.4400	1.3000	3.8209	Participate in the synthesis of carotenoids, with the prevention of aging, cardiovascular and cerebrovascular diseases, tumors, and cataracts	(Von-Lintig et al., 2020)
Alpha-hydroxymyristic acid	8.5400	1.1900	3.1428	Inhibits varicella-zoster virus (VZV) replication in tissue and controls actin cytoskeleton rearrangement required for FCgammarii-mediated movement.	(Kwiatkowska et al., 2003)
Sphingosine-1-phosphate	7.3100	0.7500	2.4285	Regulate embryonic development, postnatal organ function, and disease, anti - inflammation, prevention of atherosclerosis and ischemic diseases (such as myocardial infarction and ischemic stroke).	(Hodun et al., 2021)
Hydroxybehenic acid	4.3800	0.5500	1.5845	Inhibiting prostaglandin D2 production in bone marrow-derived mast cells (inhibiting prostate cancer)	(Jung et al., 2013)
Hexanoyloxyfucoxanthinol	0.0900	0.4200	1.1848	Anti - inflammation, enhance immunity, analgesia and so on	(Chakraborty and Joy, 2020)
LPE	1.9000	0.2200	0.5974	Inhibition of esophageal cancer is a biomarker for predicting diabetic nephropathy (DN)	(Wang et al., 2017)
Asprellic acid A	0.8100	0.0800	0.2263	Antitumor	(Kashiwada et al., 1993)
Bichanin A 7-O-glucoside-6''-malonate	0.3800	0.0400	0.1439	Prevent cardiovascular disease, senile dementia, delay aging and so on	(Hinderer et al., 1986)
Zeaxanthin diglucoside	0.8200	0.0200	0.0771	antioxidation	(Fidan and Zhan, 2019)
TAG	0.0085	0.0380	0.1277	The emergence of TAG as a reserve compound is widespread in eukaryotes and regulates cell membrane fluidity	(Alvarez and Steinbüchel, 2002)
DAG	0.3462	0.0418	0.0875	Reduce visceral fat, inhibit weight gain, reduce blood lipids, anti-cancer, etc	(Cooke and Kazanietz, 2022)
DGTS	0.0323	0.0845	0.2010	Antioxidant, anti-inflammatory, and anti-atherosclerosis	(Khattib et al., 2020)
Dolichol-17	0.0009	0.0049	0.0204	Regulation of congenital glycosylation Disorder (CDG)	(Buczkowska et al., 2015)
Cornudentanone	0.1154	0.0107	0.0221	Anti-tuberculosis, Carbamates therapeutic release agents as amidase inhibitors	(Chang et al., 2011)
Alpha'-Trehalose 6-mycolate	1.1013	0.7472	1.6830	Antioxidant, can act as a protective agent against physiological stress	(Furuki et al., 2009)
3beta-hydroxy-4beta-methyl-5alpha-cholest-7-ene-4alpha-carbaldehyde	3.1024	1.4986	1.7336	Anti-depressant, anti-fungal, anti-viral	(Mohammadi et al., 2022)
26-hydroxycholesterol 3-sulfate	0.3856	0.0873	0.1164	Regulation of Hepatocyte Lipid Metabolism and Inflammatory Response	(Vallejo et al., 2019)
1b,3a,7b-Trihydroxy-5b-cholanoic acid	2.0130	0.0606	0.0744	Adipose tissue function and plasticity orchestrate nutritional adaptation	(Kim, et al., 2017)

implications for the quality and functional properties of yak ghee.

Functional annotation analysis of the significantly different lipids revealed the 20 most relevant enrichment functions, including metabolic processes, response to xenobiotic stimuli, and cellular modified amino acid metabolic processes, among others. In addition, we also analyze the function of other differentially expressed lipids (Functional lipids with relatively high content in ghee) through literature database (E.G.P used, Web of science and sciencedirect) (Table1). These lipids exhibit a range of functions, including anti-inflammatory, antibacterial, cardiovascular and cerebrovascular disease prevention, lipid-lowering effects, prevention of Parkinson's disease and related conditions, as well as anti-aging properties. In addition to these common physiological functions, ghee lipids also demonstrate stress resistance (alpha'-trehalose 6-mycolate), analgesic effects (hexanoyloxyfucoxanthinol), enhanced energy metabolism (octadecenoic acid) and other functions (Table 1). Interestingly, the contents of most of these functional lipids were significantly correlated with altitude (increasing/decreasing with altitude) (Table S1).

4. Discussion

The utilization of UHPLC-QTOF-MS in lipidomic has significantly improved the coverage and sensitivity of lipid detection in ghee. Our research significantly contributes by providing, for the first time, a comprehensive understanding of the composition and functional characteristics of lipids in yak ghee at different altitudes through high-throughput quantitative lipidomic analysis. The high-throughput quantitative lipidomic method enables the simultaneous qualitative and quantitative determination of numerous polar and non-polar lipids, particularly in trace amounts, surpassing traditional analytical methods. Previous studies have identified a range of polar and non-polar lipids in milk using various techniques (Liu et al., 2015; Chiofalo et al., 2011). In our study, yak ghee, a complex lipid mixture derived from yak milk, contains a diverse range of functional lipids. Utilizing the quantitative lipid method, we successfully identified over 1000 lipids from S2, S5, and S6 samples and classified them into 5 major lipid classes.

This extensive lipid identification greatly enhances our understanding of the composition of yak ghee, providing a basis for the better utilization of its lipids. We comparison of lipid content in the S2, S5, and S6 groups revealed significantly elevated levels of various lipids in the S5 and S6 groups, with the exception of FAHFA and FA ($p < 0.05$) (Fig. 1C). This result aligns with previous findings that Himalayan alpine yak ghee contains higher amounts of fatty acids compared to domestic yak ghee (Or-Rashid et al., 2008). Variations in lipid metabolism among yaks, cattle, and buffaloes may be attributed to differences in their living environments (such as altitudes, extreme cold/heat, and low pressure)

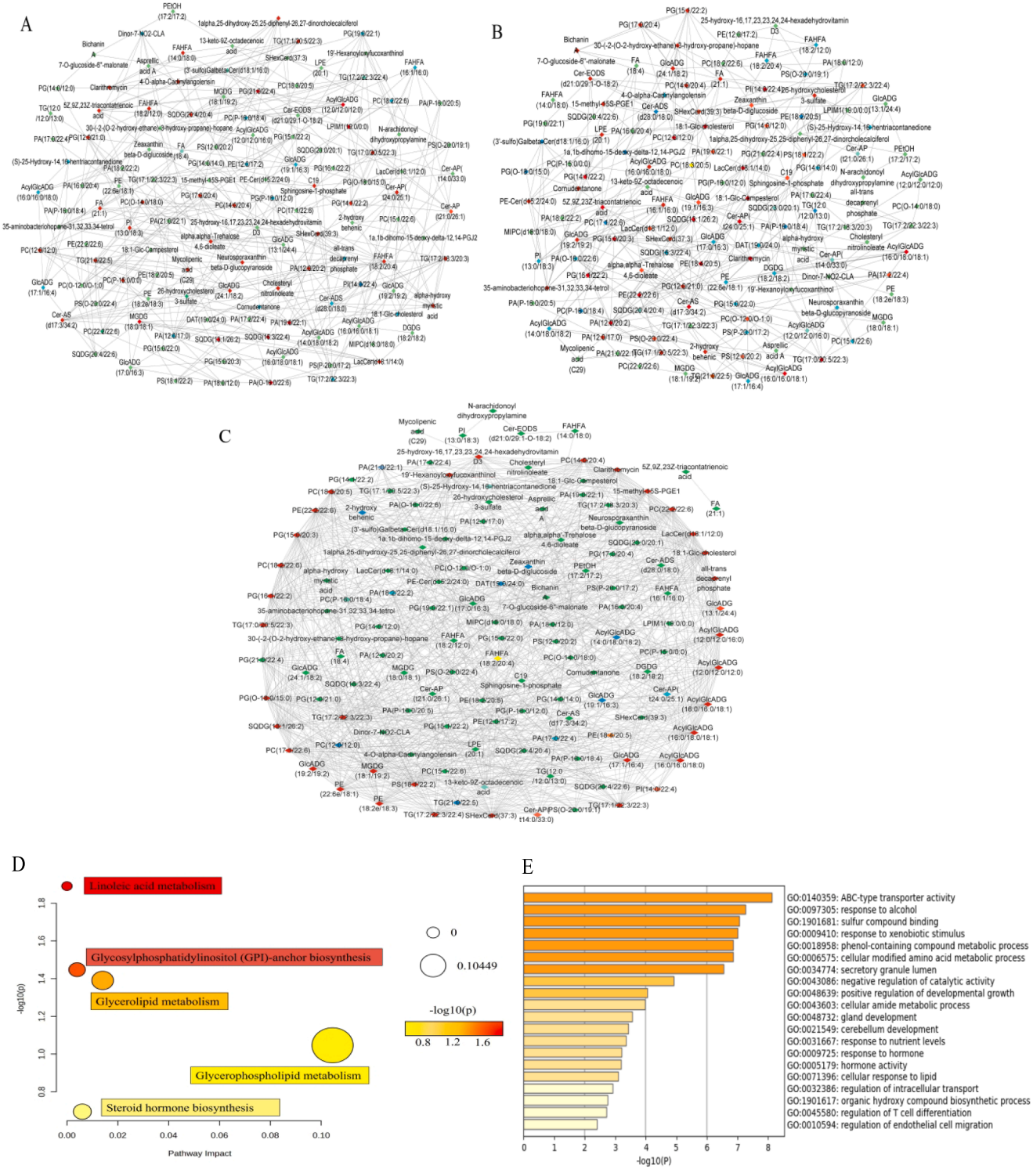

Fig. 5. Spearman's correlation network ($p < 0.05$) of 176 differential lipids were analyzed in S2 (A), S5 (B), and S6 (C), KEGG pathway analysis of significantly different lipids were carried out between S2, S5, and S6 (D) and metabolomic view map of the significant metabolic pathways of yak ghee lipids were exhibited between S2, S5, and S6 (E). Red represents the most correlated lipids, followed by blue, and green represents the least correlated lipids. Significantly changed pathways based on the enrichment and topology analysis are shown. The X-axis represents pathway enrichment, and the Y-axis represents the pathway impact. Large sizes and dark colors represent the major pathway enrichment and high pathway impact values, respectively. S2 = Ghee at an altitude of 2,968 m; S5 = Ghee at an altitude of 3,671 m; S6 = Ghee at an altitude of 4,508 m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and lifestyles. These variations could potentially be regulated by intestinal microorganisms through the regulation of lipid metabolism and gene expression (Zhang et al., 2022, He et al., 2020). Additionally, ruminants rely on microbial production of acetate and β-Hydroxybutyrate, which are key precursors for C4-C16 lipids in their milk (Fidan and Zhan, 2019). Gut microbiota diversity of free-range yaks (at 4000 m-5000 m) has been found to be significantly higher compared to captive yaks (around 3000 m), thereby providing further substantiation for our hypothesis (Wang et al., 2022). These differences in composition, nutrition, and function of ghee lipids at different altitudes lay the foundation for precision processing and the development of nutritional products containing functional lipids derived from ghee.

Previous studies have primarily focused on the identification of trace amounts of lipids, such as fatty acids and conjugated linoleic acids, in yak ghee. In contrast, our study utilizing the UHPLC-QTOF/MS technique identified 176 significantly different lipids in yak ghee, providing insights into five associated metabolic pathways based on the differentially expressed ghee lipids among the three altitudes. These lipids include 13 PG, 13 PC, 12 PA, 11 TG, 8 PE, 5 SQDG, 5 PS, and various others, which were found to be significantly different ($p < 0.05$) in S2, S5, and S6 (Fig. 4 and table S1). Among these 176 differential lipids, the contents of TGs, PA, PG, PC, PS, and others significantly increased with increasing altitude, indicating that altitude is a crucial factor influencing the composition and content of ghee lipids. Differential lipids in the same subclass show similar trends, supporting correlations among lipid levels. As depicted in Fig. 5, differential lipids in S2, S5, and S6 groups are highly correlated. Our results suggest that changes in one or more specific lipids may have an impact on changes in other specific lipids, regardless of whether they belong to the same subclass. Further analysis revealed that the S6 group exhibited more significant clustering than the S5 and S2 groups, forming a tight single cluster (817 correlations) compared to the S2 group (395 correlations) and the S5 group (410 correlations), respectively (Fig. 5A-7C). Notably, certain lipids in the S5 and S6 groups exhibited stronger correlations with additional differential lipids compared to those in the S2 group, such as TG, PC, and SQDG. This could be attributed to the higher abundance of glycerolipids in the S5 and S6 groups, which is associated with increased energy demands of Tibetan individuals residing in high-altitude cold regions (Li et al., 2020). Furthermore, PA, PG, PS, and PE (red dots), are deeply correlated among different lipid subclasses in the S2, S5, and S6 groups, particularly in the S6 group, probably owing to the role of PA, PG, PS, and PE as the precursor substance and intermediate for lipid biosynthesis (Tong et al., 2019). By identifying a greater number of lipids, we have deepened our understanding of the composition of ghee. The analysis of distinct lipids and their interactions can serve as a foundation for constructing potential sets of biomarkers and evaluating the quality of ghee at different altitudes, as well as for the development of functional food applications.

The predominant lipids present in yak ghee are glycerophospholipids, accompanied by abundant sub-lipids such as PA, PS, TG, and PG. Furthermore, their contents are increased with rising altitude (Fig. 3). These lipids are rich in functional lipids with various bioactive functions, including increasing cell membrane fluidity, enhancing cognitive ability, and delaying aging. Previous studies have also explored the biological functions of several lipid subclasses, including TG, PI, PE, PS, PC, SM, DG, PA, CL, Cer, Hex2Cer, and Hex3Cer. In our study, the additional lipids identified including all-trans decaprenyl phosphate, DAT, alpha'-Trehalose6-mycolate rise gradually with increasing elevation, except for Neurosporaxanthin beta-D-glucopyranoside (Table1).Interestingly, these lipids are the first to be found in higher animals, and they are reported only in plants, microorganisms and marine Mollusca. All-transdecaprenylphosphate, a crucial constituent of biological cell membranes, has been demonstrated to facilitate cellular proliferation and individual development during mammalian ontogeny, and it is also known to have anti-tuberculosis physiological effects (Grover *et al.*, 2014). DAT, which has been identified as another crucial component, plays a pivotal role in the regulation of dopamine transmission within the brain and is intricately involved in the metabolic processes of sugars and fats. Tibetan nomads, who habitually consume a high-fat diet, have been found to have good cardiovascular and cerebrovascular health, which may be attributed to the presence of DAT in yak milk and its dairy products such as ghee (Guo et al., 2014). DAT has also been shown to activate neuronal regulation to prevent Parkinson's and related diseases (Brücke and Brücke, 2022). The identified lipid, alpha'-trehalose6-mycolate, functions as a protective agent against physiological stress and potentially contributes to the adaptation of Tibetans to high altitude, low temperature, hypoxia, and low pressure, however, its mechanism of action is still unknown (Furuki et al., 2009). The neurosporaxanthin beta-D-glucopyranoside is primarily found in marine microorganisms and has demonstrated anti-aging, cardiovascular and cerebrovascular disease prevention, anti-inflammatory, immune-enhancing, and analgesic properties. These attributes may play a crucial role in enhancing the immunity of Tibetan herdsmen, preventing aging, as well as alleviating injuries and pain caused by extreme conditions (Chakraborty and Joy, 2020). These findings presented in this study offer a comprehensive framework for comprehending the distinct ingredient and biological functionalities of lipids in the S2, S5, and S6 samples.

Furthermore, significantly different lipids among the S2, S5, and S6 were not only identified in the study, metabolic pathways involved to these lipids were but also investigated. TG, SQDG, PS, PG, PC, PA, and GLcADG were implicated in both glycerophospholipid metabolism and glycerolipid metabolism. In terms of glycerophospholipid metabolism, the levels of TGs, PCs, and GLcADGs were higher in S6 compared to S2 and S5. Additionally, SQDGs exhibited elevated abundance in S2 during glycerophospholipid metabolism when compared to S5 and S6. Otherwise, both PC and PA levels in glycerophospholipid metabolism were higher in S5 compared to S2 and S6. These findings suggest that ghee obtained at higher altitudes is enriched with higher levels of phosphatidylcholines (PCs), phosphatidylserines (PSs), and glycolipids containing alkyl diacylglycerols (GLcADGs). These components may contribute to the provision of essential lipid nutrition for Tibetan herdsmen residing in high-altitude regions, thereby facilitating their cognitive development and offering protection against cardiovascular and cerebrovascular diseases as well as stress-induced damage caused by plateau environments. Unfortunately, based on the existing database and bioinformatics, we have not been able to determine the specific metabolic pathways involved in the newly identified lipids, although we have some information on their content and function. Further exploration is needed to understand the specific metabolic pathways and the effects of these lipids.

5. Conclusions

In this study, we used quantitative lipidomics analysis to investigate the lipid composition of ghee at different altitudes. We identified ten lipid subclasses in S2, S5, and S6, with the highest proportions of glycerophospholipids, sphingolipids, and glycerolipids among macromolecular lipids. The content of glycerophospholipids, sphingolipids, and glycerolipids was found to be higher at high altitudes compared to lower altitudes. Furthermore, we conducted bioinformatics analysis on 176 distinct lipids to explore their metabolic pathways and physiological functions. In future research, we plan to investigate the impact of different altitudes on mammalian phospholipids in yak and their role in neural development. This study provides valuable insights into the lipid composition and functionality of yak ghee at different altitudes, which can be utilized for the development of new products with specific nutritional properties and targeted biological activity. Additionally, the identification of potential biomarkers using these lipids can aid in distinguishing yak ghee from other dairy products based on its unique composition. Overall, this research contributes to our understanding of the lipids in yak ghee and their potential uses in functional food

products.

CRediT authorship contribution statement

Feiyan Yang: Formal analysis, Investigation, Writing – original draft. **Xin Wen:** Software, Visualization. **Siwei Xie:** Investigation. **Xudong He:** Visualization. **Guangfan Qu:** Data curation. **Xueying Zhang:** Funding acquisition, Resources. **Shuguo Sun:** Conceptualization, Funding acquisition. **Zhang Luo:** Funding acquisition, Resources. **Zhendong Liu:** Validation, Visualization. **Qinlu Lin:** Project administration, Supervision.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101166.

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