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RESEARCH ARTICLE

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Multi-omics analysis reveals alterations of breastmilk metabolites and proteins in Hong Kong lactating mothers

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Abstract

The nutritional contents of breastmilk (BM) directly participate in neonatal metabolism via breastfeeding. Currently, there is limited research on BM metabolites and proteins compositions, and their alterations during the long lactation period in Hong Kong mothers. In this study, liquid chromatography–mass spectrometry-based metabolomics, lipidomics and proteomics studies were applied to compare the compositions in BM of Hong Kong lactating mothers at the 2nd, 6th, and 12th months after delivery. Distinct metabolomics and lipidomics signatures in 6th month versus 2nd month and 12th month versus 2nd month were observed, and a total of 19 differential metabolites and 105 lipids were identified. Metabolomics study showed the significant alterations in key pathways involved in biotin metabolism, amino acid, and fatty acid-associated metabolisms. Lipidomics analysis indicated the accumulation of triglyceride and ceramide during the lactation period. The remodeling of glycerophospholipids was also observed during 12-month period. Moreover, 28 differentially expressed proteins were identified and mainly associated with GO functions and KEGG pathways of ribosome and complement and coagulation cascades, which were validated by network analysis. Our research contributes to the understanding of the BM compositions and differences during the long lactation period in postpartum women of Hong Kong.

KEYWORDS

breastmilk, lipidomics, long lactation period, metabolomics, proteomics

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Abbreviations: 3-NPH, 3-nitrophenylhydrazine; AcCa, Acylcarnitine; ACN, Acetonitrile; ATP6AP1, V-type proton ATPase subunit S1; BM, Breastmilk; CALU, Calumenin; Cer, Ceramide; DG, Diacylglycerol; DIA, Data-independent acquisition; EDC, N-(3-(dimethylamino)propyl)-N′-ethylcarbodiimide; ESI, Electron spray ionization; FA, Formic acid; FAU, Ubiquitin-like FUBI-ribosomal protein eS30 fusion protein; FDR, False discovery rate; FFA, Free fatty acid; GL, Glycerolipid; GO, Gene ontology; GP, Glycerophospholipid; HSPA13, Heatshock 70 kDa protein 13; IGLV6-57, Immunoglobulin lambda variable 6-57; IPA, Isopropyl alcohol; KEGG, Kyoto encyclopedia of genes and genomes; KLKB1, Plasma kallikrein; LC-MS, liquid chromatography-mass spectrometry; NUCB2, Nucleobindin-2; PC, Phosphatidylcholine; PCA, Principal components analysis; PCSK6, Proprotein convertase subtilisin/kexin type 6; PE, Phosphatidylethanolamine; PLS-DA, Partial least squares discrimination analysis; PUFA, Polyunsaturated fatty acid; RPL22, Large ribosomal subunit protein eL22; RPL31, Large ribosomal subunit proteineL31; RPL35A, Large ribosomal subunit protein eL33; RPS4X, Small ribosomal subunit protein eS4, X isoform; RPS5, Small ribosomal subunit protein uS7; RPS7, Small ribosomal subunit protein eS7; SCFAs, Short chain fatty acids; SCP2, Sterol carrier protein 2; SM, Sphingomyelin; SMPDB, The small molecule pathway database; TAG, Triacylglycerol; TCN1, Transcobalamin-1; TG, Triacylglycerol.

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1 | INTRODUCTION

Breastmilk (BM), which contains abundant nutrients, as the optional food for infants in the initial phases of life, can provide dietary energy and a variety of bioactive factors influencing growth and development of infants.[1] BM can be categorized into colostrum milk (about 0–5 days), transitional milk (about 6–15 days), and mature milk (about 16 days or more).[2] Colostrum milk has higher concentrations of protective components including anti-inflammatory cytokines and immunoglobulins compared to mature milk, which plays an important role for infants.[3] The World Health Organization has recommended exclusive breastfeeding for the first 6 months of life, with continued breastfeeding, thereafter while complementary foods are introduced.[4] As many problems, including low BM production, disease, economy, and social environment were encountered,[5] it was reported that only about 35% of newborn to 6-month-old babies could be breastfed over the world.[6] In Hong Kong, the exclusive breastfeeding rate at the sixth month remained low at 26.3%, study indicated that giving birth in a public hospital and breastfeeding support from family and friends predominant breastfeeding were positive factors related to six-month predominant breastfeeding in Hong Kong women.[7] Several studies have investigated the changes of the compositions in the different times of human milk or in other mammals, to contribute to the development of the infant formula. For instance, significantly different lipids were observed between human and ewe colostrum, and DG (19:0/18:0) (Diacylglycerol), TG (10:0/15:0/16:0) (Triacylglycerol), FFA (22:0) (Free fatty acid), and TG (18:1/24:1/18:2) were selected as biomarkers.[8] Zhao et al. analyzed the contents of triacylglycerol (TAG) and FA in human colostrum and mature milk, to facilitate the design of infant formula for Chinese babies.[6]

Multi-omics studies were usually used as powerful phenotyping techniques to detect thousands of features associated with nutrition or disease states.[9–11] Proteomics, lipidomics, and metabolomics analyses have been applied to uncover the significant alternations in BM associated with COVID-19, for which several decreased aromatic amino acids and changed lipids were selected as biomarkers in BM of COVID-19 patients.[12] Some studies have investigated the changes of the metabolites in the BM of the Chinese or western population over lactation stages by omics studies. Li et al. used metabolomic analysis to evaluate the relationship between the alterations in the metabolites of BM over lactation stages (colostrum, transitional, and mature milk) and the dietary intake in Chinese mothers, two metabolites, namely 1,24,25-$(OH)_3$ vitamin D_3 and 11β-hydroxyprogesterone, were identified to be associated with dietary intake.[13] Lipidomics analysis has also been used to assess functional lipid composition in BM.[2] Zhao et al. performed comparative proteomic analysis of human milk pasteurized by hydrostatic high-pressure, and indicated that proteins in milk whey were less affected by holder pasteurization or hydrostatic high-pressure than milk fat globule membrane and casein proteins.[14] In Hong Kong, a recent study detected the concentrations of polyphenols and phenolic metabolites in BM of lactating women, and supported that intake of plant-based foods significantly affected BM phenolic composition 2 months postpartum.[15] Carotenoids and Vitamin A concentrations were determined in BM at 2 months postpartum of Hong Kong mothers, their relationships with maternal diet were further assessed, and served as dietary references for lactating mothers.[16] To date, there are relatively few studies that have applied multi-omics studies to illustrate the features in BM of mothers in Hong Kong region during long lactation period.

In the present study, we aimed to comprehensively identify the metabolites and proteins in BM, and to screen significantly different signatures for distinguishing during 12-month lactation periods, using LC–MS (liquid chromatography–mass spectrometry)-based metabolomics, lipidomics and proteomics analyses. The results are expected to provide vital information for estimating the nutritional value of BM of mothers in Hong Kong, and offer a new idea to develop infant formula for long term nutrition supplementary.

2 | MATERIALS AND METHODS

2.1 | Characteristics of Hong Kong lactating mothers

BM samples were collected between 2018 and 2019. The inclusion criteria, including (1) Hong Kong residents which have resided in Hong Kong for a continuous period of not less than 18 months; (2) Normal pre-pregnant BMI (i.e., 18.5–22.9) and weight gain during pregnancy (i.e., 11–16.4 kg); (3) Deliver at full term (>37 gestation weeks); (4) Give birth to singleton infant within normal birth weight of >2500 g, and have not participated concurrently in any clinical trial or study; (5) Have not taken any supplements of probiotics during pregnancy and lactation; (6) Have not taken antibiotics for at least 1 month before sample collection; (7) Have not used contraceptive medication after giving birth; (8) With no complicated pregnancy, such as preeclampsia and gestational diabetes; (9) No special dietary restrictions, such as gluten-free diets, vegan, or any restrictions due to food allergies. A private room was allocated to each mother and they were asked to pump out the milk using the sterilized pump and bag provided by our research staff. Prior to the collection, subjects were asked to clean the nipples and hands using the alcohol wipes. The milk was directly collected into the sterile container, the samples were aliquoted to tubes and stored at −80°C for further analysis. Milk was defatted by centrifuge at 6000 rpm for 30 min before use. Total 23 independent samples were selected for the initial metabolomics and lipidomics studies. Ethics approval was obtained for human subjects from the Institutional Review Board (Ref. no. HSEARS20180123009-03) while the biological safety and chemical safety of study were approved by the Health, Safety and Environment Office of the Hong Kong Polytechnic University. All participants were fully informed of the study details and signed the informed consent.

2.2 | Pretreatment of BM samples for metabolomics and lipidomics analyses

For nontargeted metabolomics analysis, three volumes of ice-cold methanol was added to 100 μL of defatted BM, and centrifuged at 12 000 *g* for 10 min at 4°C after 5 min vortex. The supernatant was then centrifuged at 12 000 *g* for another 5 min at 4°C, and the last supernatant was collected for LC–MS analysis, 4-Chloro-phenylalanine was used as internal standard. For lipidomics analysis, 50 μL of thawed defatted BM was added with 600 μL of methanol and 150 μL of water. After vortexing for 2 min, 150 μL of water, and 450 μL of chloroform were added to the mixture, which were vortexed for another 5 min. After a 10-min equilibration at room temperature, the mixture was centrifuged at 12°000 *g* for 15 min at 4°C. The bottom layer was collected and dried with Thermo Scientific SpeedVac Concentrator. The residuals were reconstituted in 100 μL of acetonitrile (ACN), isopropyl alcohol (IPA), and water (65:30:5, *v*/*v*/*v*) containing 16:0–18:2 PC-d31 and 16:0–18:0–16:0 TG-d5 as internal standards, for LC–MS analysis.

Metabolomics profiling and lipidomics analysis were conducted by an Ultimate 3000 separation LC system, coupled with an Orbitrap IQ-X MS (Thermo Scientific). Full scan mode with ESI (Electron Spray Ionization) source was applied for metabolomics study. The ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) was used for metabolites separation. The mobile phase consisted of 0.1% formic acid (FA)-water (v/v; A) and 0.1% FA-ACN (v/v; B) at the flow rate of 0.3 mL/min. The liquid chromatographic gradient was as follows: 0–1 min, 2% B; 19–21 min, 100% B; 21.1–25 min, 2% B. The MS parameters were set as follows: Positive Ion: 3500 V; Negative Ion: 2500 V; Ion Transfer Tube Temp: 300°C; Vaporizer Temp: 350°C; Orbitrap resolution: 120 000. For lipidomics analysis, chromatographic separation was achieved on a Waters CSH C18 analytical column (2.1 mm × 100 mm, 1.7 μm). The mobile phase A was composed of ACN/water (60:40; v/v) containing 10 mM ammonium formate and 0.1% FA, mobile phase B was composed of IPA/ACN (90:10; v/v) with 10 mM ammonium formate and 0.1% FA. Linearity gradient elution was as follows: 30% B at 0–1 min, 45% B at 2 min, 70% B at 7 min, 85% B at 9 min, 100% B at 17–23 min, 30% B at 24–28 min. The flow rate was 0.26 mL/min. MS analysis was performed in a high-resolution full scan mode (resolution: 120 000), followed by DDA-based MS/MS scans mode (resolution: 7500). The spray voltages were set at 3500 and 2500 V for positive and negative ion modes, respectively. Data type was set as profile and centroid in full scan mode and MS/MS mode. Cycle time was set at 1.5 s. Ion transfer tube temperature and vaporizer temperature were set at 300 and 350°C, sheath gas and aux gas were set as 40 Arb and 10 Arb, respectively.

2.3 | Data-independent acquisition (DIA) proteomics analysis

BM samples were prepared based on a previous study with minor modification.[17] The BM was centrifuged at 4000 *g* at 4°C for 30 min. The middle layer was collected, and the protein contents were defined using the bicinchoninic acid assay (Thermo Fisher Scientific). A quantity of 100 μg protein was then used for enzymatic digestion according to the instructions (EasyPep™ MS Sample Prep Kits, Thermo Scientific).

The resuspended peptide mixture (0.1% FA) was analyzed using a Vanquish Neo UHPLC system coupled with an Orbitrap Fusion Lumos (Thermo Fisher Scientific, Milford, MA, USA). Peptide mixtures were separated by a C18 analytic column (Aurora Ultimate; 25 cm × 75 μm, 1.7 μm). Separation was achieved using mobile phase A (0.1% FA) and mobile phase B (0.1% FA in ACN) at a flow rate of 300 nL/min: 0–5 min (2% B), 5–7 min (2%–6% B), 7–92 min (6%–30% B), 92–100 min (30%–90% B), 100–105 min (90% B), and 105.1–115.1 min (2% B). The MS parameters were set with a resolution of 60 000 at 400–1500 *m/z*, and the HCD-MS/MS resolution was 15 000. Normalized HCD collision energy was 32%; the precursor mass range of 500–1000 Da was selected for data acquisition; isolation window: 15 *m/z*; number of scan events: 34; Window overlap: 1 *m/z*.

2.4 | Targeted analysis of fatty acid

The samples were first derivatized as previously described, with minor modification.[18] In brief, 20 μL of BM sample was mixed with 120 μL methanol. After centrifugation at 13 000 g for 10 min at 4°C, a 30 μL aliquot of the supernatant was used for derivatization, 15 μL of 3-NPH (3-nitrophenylhydrazine) and EDC (N-(3-(dimethylamino)propyl)-N′-ethylcarbodiimide) were added and kept at room temperature for 30 min. 4-Chloro-phenylalanine was used as internal standard. The samples were analyzed by an Orbitrap IQ-X MS in the negative mode. The MS parameters were set as previously showed in metabolomics study. Chromatographic separations were conducted on an ACQUITY CSH C18 column (1.7 μm, 100 mm × 2.1 mm). A mixture of water with 0.1% FA (A) and ACN/IPA (7:3) (B) were used as the mobile phases. The gradient elution was as follows: 5% B (0–1 min), 5%–30% B (1–5 min), 30% B–50% B (5–9 min), 50% B–78% B (9–12 min), 78% B–95% B (12–15 min), 95% B–100% B (15–16 min), and 5% B (18.1–20 min).

2.5 | Data analysis of BM metabolites and lipids

Metabolomics and lipidomics data were normalized based on quality control samples according to the reference reported.[10] The Student's *t*-test was used to compare each month with the 2nd month to identify the significantly different metabolites. Partial least squares discrimination analysis (PLS-DA) was performed to identify the discrimination of variables in metabolomics study. Lipids were identified based on the matching of accurate mass to charge ratio (*m/z*) and MS/MS spectra with Lipidsearch. The mass tolerance for precursor and fragment ions was set at 10 ppm. The threshold of m-score was set to 5. Adduct ions in positive and negative ionization modes were set as default. Statistical significances of different groups of lipids were determined by Student's *t*-test. For targeted profiling, the peaks were extracted by Compound Discovery software, the targeted metabolites were validated with the related standards and calculated by paired *t*-test. Proteomics data were collected by Xcalibur (Thermo Fisher Scientific), and the raw data files were imported into Spectronaut. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed using String.

3 | RESULTS

3.1 | Relative metabolite abundance measured in BM of lactating mothers

Nontargeted metabolomics analysis was performed to indicate the potential metabolite alteration of BM during the 12-month lactation period. The basic demographics of the study population are presented in Table 1, and the study design was illustrated in Figure 1. PLS-DA revealed the distinct separation of the metabolic profiles among three

TABLE 1 Demographics of study participants.

Characteristics (*N* = 24)	Results
Maternal characteristics	
Receiving higher than secondary school education, *n*/*N* (%)	19/24 (79.2)
Habit of exercise, *n*/*N* (%)	11/24 (45.8)
Paternal characteristics	
Receiving higher than secondary school education, *n*/*N* (%)	17/24 (70.8)
Habit of exercise, *n*/*N* (%)	16/24 (66.7)
Child characteristics	
Male, *n*/*N* (%)	16/24 (66.7)
Gestational age (weeks)	
Birth weight (g)	3124 ± 235
Received the first intake of colostrum, *n*/*N* (%)	18/24 (75.0)
Exposure to household smoking, *n*/*N* (%)	2/24 (8.3)
Breastfed 0–6 months, *n*/*N* (%)	13/24 (54.2)
Breastfed 7–12 months, *n*/*N* (%)	12/24 (50.0)
Exclusively breastfed 0–6 months, *n*/*N* (%)	10/24 (41.7)

Note: Results expressed in number (percentage) or mean ± standard deviation.

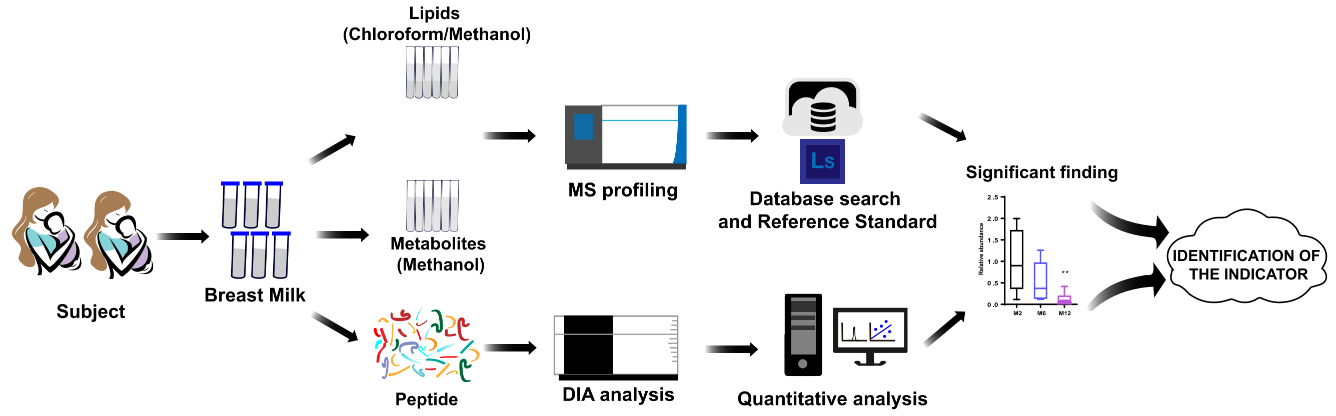


FIGURE 1 Overall study design.

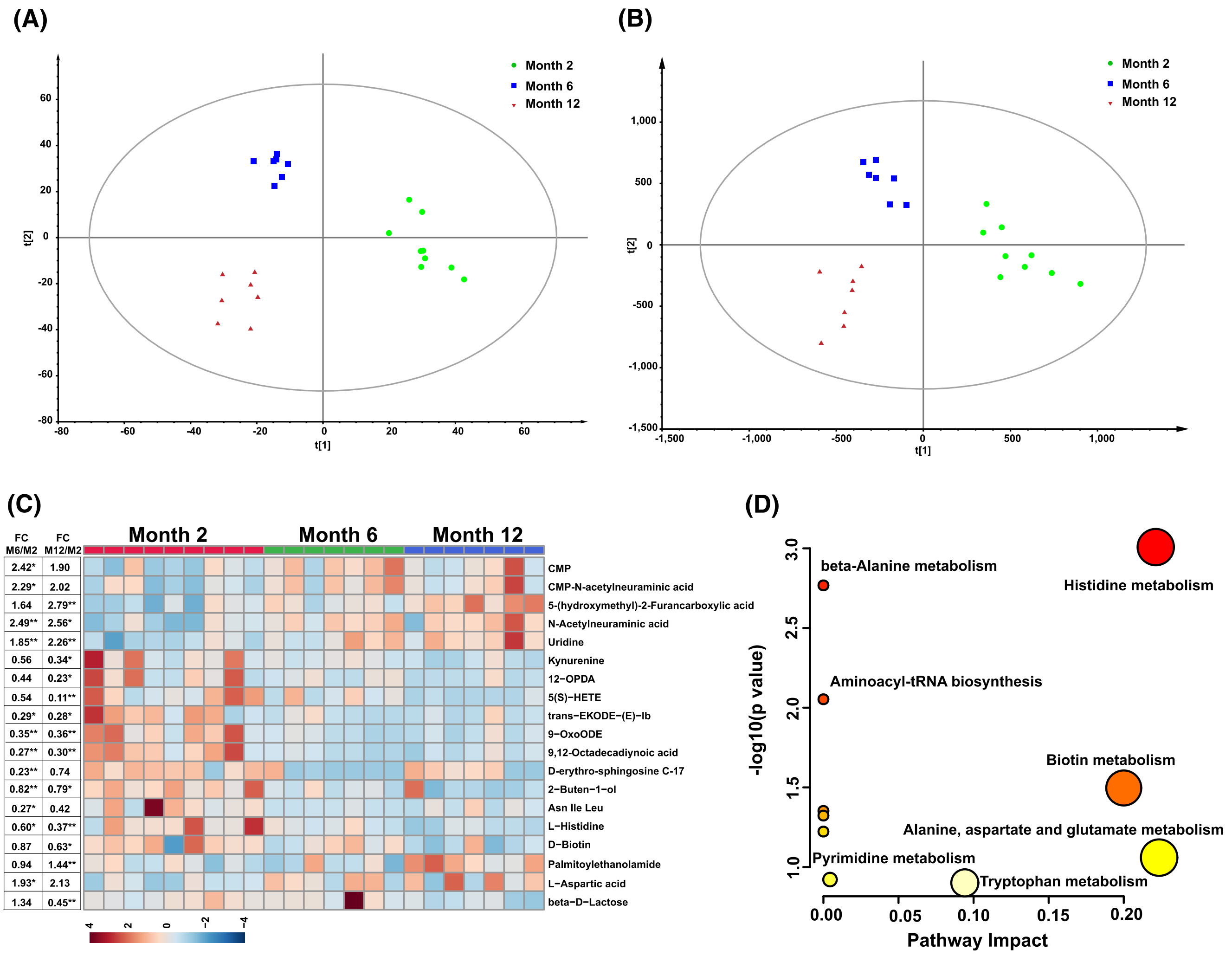


FIGURE 2 Metabolic profiles of BM. PLS-DA score plots from the indicated months in positive (A) and negative (B) modes. (C) Heatmap showed the 19 significantly changed metabolites in the indicated months. (D) The disturbed metabolic pathways showed various metabolic changes when comparing 12th month and 6th month to 2nd month. $^{*}p < .05$, $^{**}p < .01$.

time points (Figure 2A,B). Differential metabolites were screened by using the variable importance in the projection >1, p value <.05, and fold change >1.2 or <0.8. According to this criterion, 19 significantly differentially different metabolites were identified in BM when compared to the 2nd month (7 upregulated and 12 downregulated, Figure 2C).

Figure 2D represents the potential perturbed pathways during the lactation period, including histidine metabolism, biotin metabolism beta-alanine metabolism and aminoacyl-tRNA biosynthesis. Enrichment analysis with SMPDB (The Small Molecule Pathway Database) indicated the metabolic alteration including ammonia recycling, beta-alanine metabolism, methylhistidine metabolism, glutamate metabolism, and biotin metabolism (Figure S1). Disturbance of the biotin metabolism was the significantly perturbed pathway in both two analyses. D-biotin concentrations in BM decreased in a time-dependent manner, and 12th month versus 2nd month had significantly lower abundance by 0.63-fold. L-Histidine, involved in histidine metabolism and beta-alanine metabolism, decreased significantly by 0.60-fold and 0.37-fold at 6th month and 12th month in comparison with 2nd month. Whereas milks at 6th and 12th months had higher concentration of L-aspartic acid by 1.93-fold and 2.13-fold compared to 2nd month. Spearman's correlation analysis showed the positively correlation between D-biotin and L-histidine ($p<.01$). In addition, D-biotin was positively correlated with kynurenine ($p<.05$), which was significantly decreased by 0.34-fold at 12th month compared to 2nd month. L-histidine was positively correlated with metabolites, including 5(S)-HETE ($p<.05$; 0.11-fold reduction, 12th month versus 2nd month, $p=.0050$), BETA-D-Lactose ($p<.05$; 0.45-fold reduction, 12th month versus 2nd month, $p=.0037$) and Asn Ile Leu ($p<.05$; 0.27-fold reduction, 6th month versus 2nd month, $p=.033$; 0.42-fold reduction, 12th month versus 2nd month, $p=.086$), and negatively correlated with four metabolites, including N-acetylneuraminic acid ($p<.05$; 2.49-fold increase, 6th month versus 2nd month, $p=.0021$; 2.56-fold increase, 12th month versus 2nd month, $p=.014$), and uridine ($p<.01$). Uridine and 5′-CMP are the metabolites involved in pyrimidine metabolism, showed increases by 1.85-fold ($p=.0085$) and 2.42-fold ($p=.012$) at 6th month, by 2.26-fold ($p=.0017$) and 1.90-fold ($p=.12$) at 12th month, compared to 2nd month, respectively. And 5′-CMP was positively correlated with uridine ($p<.01$). Additionally, among the downregulated metabolites, 12th month versus 2nd month had significantly lower abundances of 9-OxoODE by 0.36-fold. 9-OxoODE was positively correlated with five metabolites including 5(S)-HETE ($p<.05$) and 12-OPDA ($p<.01$; 0.23-fold reduction, 12th month versus 2nd month, $p=.027$), and negatively correlated with the metabolites, including 5′-CMP ($p<.05$), N-acetylneuraminic acid ($p<.05$) and uridine ($p<.01$). In the upregulated metabolites, CMP-N-acetylneuraminic acid and N-acetylneuraminic acid increased at 6th month (2.29-fold and 2.49-fold, respectively) and 12th month (2.02-fold

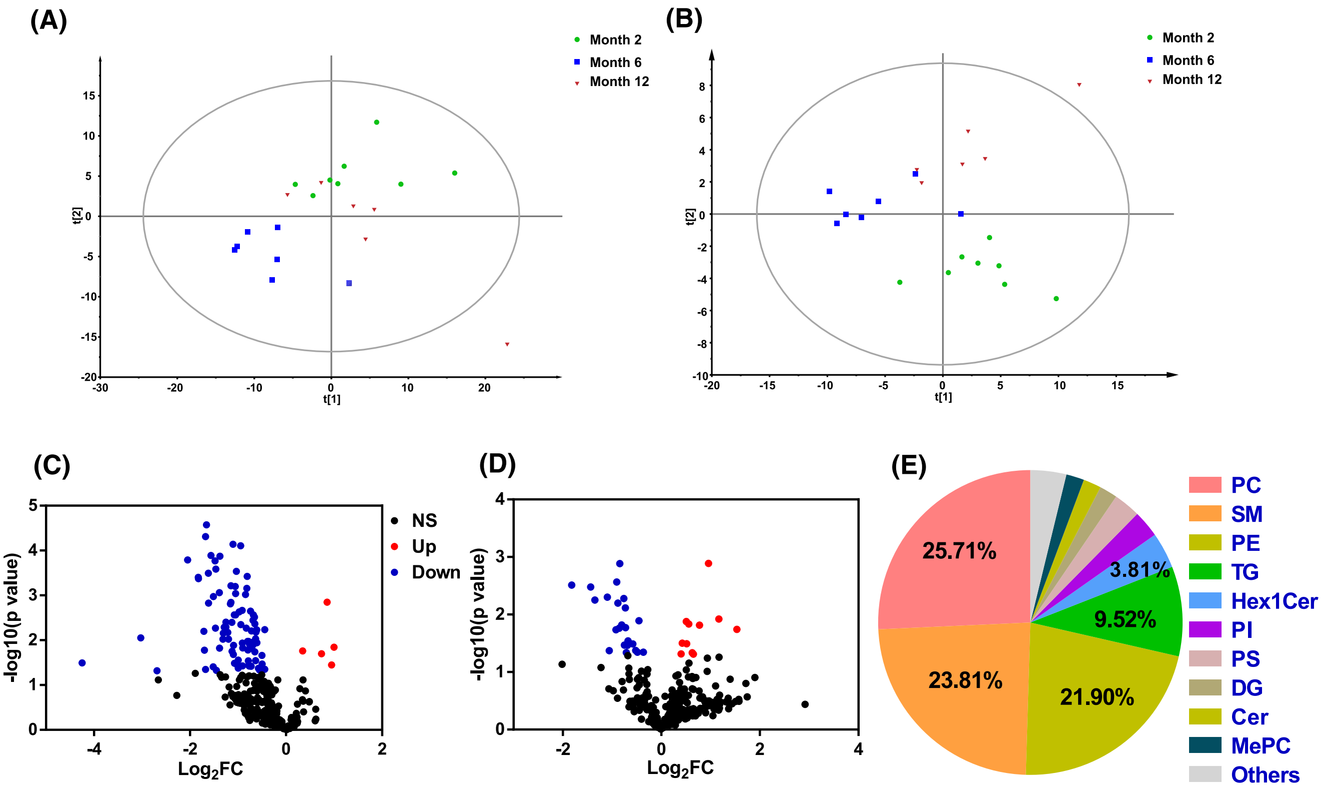


FIGURE 3 Lipidomics profiling analysis for BM. (A) and (B) PLS-DA score plots from the indicated months in positive (A) and negative (B) modes. (C) and (D) Volcano plots showing the relative abundances of lipids compared to the 2nd month (C: 6th month vs. 2nd month; D: 12th month vs. 2nd month). (E) Category of the significantly changed lipids in the BM.

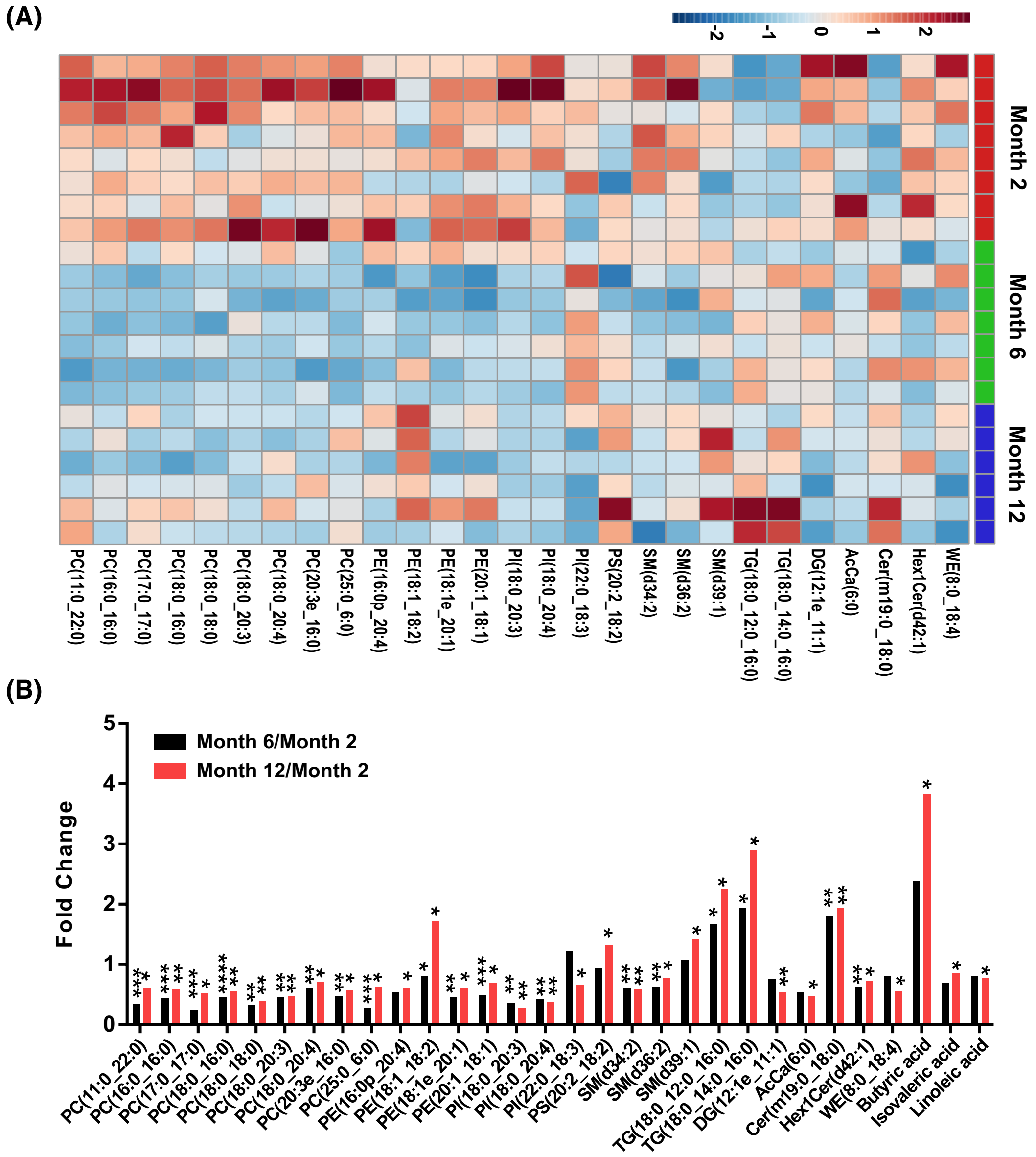


FIGURE 4 Alternations of lipids and fatty acids in BM. (A) Heatmap showed the lipids in BM associated with the lactation period. (B) Fold changes of the related lipids and fatty acid corresponding to the 2nd month. $n=9$ for fatty acid analysis between the 6th and 2nd months, $n=4$ for fatty acid analysis between the 12th and 2nd months. $^*p<.05$, $^{**}p<.01$, $^{***}p<.001$, $^{****}p<.0001$.

and 2.56-fold, respectively), as compare with 2nd month, with a positive correlation ($p<.01$). These two metabolites were all positively correlated with 5′-CMP ($p<.01$) and uridine ($p<.01$) (Figure S2).

3.2 | Lipids abundance during the lactation period

To assess the lipid composition of BM in different months, we further performed lipidomics study of BM. The hydrophobic constituents were extracted from the BM samples and analyzed using LC–MS. In total, about 2837 and 520 species of lipids in positive and negative modes were identified. The PLS-DA score plots based on identified lipids are shown in Figure 3A,B, while the 6th and 12th months groups could be distinguished from the 2nd month, indicating the potential alteration in lipid metabolism during the lactation period. Volcano plots also indicated the relative abundances of lipids in the BM (Figure 3C,D).

In this analysis, we found about 105 lipids that were significantly changed during the lactation period. The differential lipids screened in lipidomics mainly included phosphatidylcholine (PC), sphingomyelin (SM), Phosphatidylethanolamine (PE), and TG (Figures 3E and S3A–D). Several important lipids showed similar trends during the lactation period, the majority of them belongs to glycerophospholipids (GPs), and most of them were decreased at the 6th and 12th months compared to the 2nd month (Figures 4A,B and S4A). Whereas12th month versus 2nd month showed significantly decreases in PCs, PEs and PIs including PC(11:0_22:0), PC(16:0_16:0), PC(17:0_17:0), PC(18:0_16:0), PC(18:0_18:0), PC(18:0_20:3), PC(18:0_20:4), PC(20:3e_16:0), PC(25:0_6:0), PE(16:0p_20:4), PE(18:1e_20:1), PE(20:1_18:1), PI(18:0_20:3), and PI(18:0_20:4). Whereas PE(18:1_18:2) and PS(20:2_18:2) exhibited an upward trend in the later of lactation period. Changes of sphingolipid categories were also found to be correlated to lactation period. In brief, SM(d34:2) and SM(d36:2) were downregulated, while SM(d39:1) and Cer(m19:0_18:0) (Ceramide) increased significantly compared to the 2nd month. As the results showed, the levels of glycerolipids (GLs), including TG(18:0_12:0_16:0) and TG(18:0_14:0_16:0) were elevated obviously at 6th and 12th months, and DG(12:1e_11:1) reduced significantly at 12th month when compared with the 2nd month. These data suggested the TGs accumulation during the lactation period.

Spearman correlation analysis was further performed to reveal the important lipids and relationships between the significantly changed lipids (Figure S4B). PC was correlated with the majority of lipids including PC, PE, TG, SM, and Cer. PI(18:0_20:4) and PI(18:0_20:4) were positively correlated with PCs, AcCa (Acylcarnitine) and SM including PC(18:0_20:4) (all $p < .001$), PC(16:0_16:0) (all $p < .001$), PC(11:0_22:0) (all $p < .01$), AcCa(6:0) (all $p < .01$), SM(d34:2), ($p < .001$ and .0001, respectively), and negatively correlated with Cer(m19:0_18:0) (all $p < .01$), TG(18:0_12:0_16:0) (all $p < .01$). TG(18:0_12:0_16:0) was positively correlated with TG(18:0_14:0_16:0) ($p < .001$), and was negatively correlated with PCs, including PC(18:0_20:3) ($p < .01$), PC(20:3e_16:0) ($p < .05$), PC(18:0_18:0) ($p < .05$), PC(18:0_16:0) ($p < .05$), PC(16:0_16:0) ($p < .01$), and PC(25:0_6:0) ($p < .05$).

3.3 | Targeted fatty acids profiles

Nontargeted metabolomics profiling has indicated the important contributions of fatty acid and the relevant derivations. The targeted metabolomic profiling of the relevant fatty acids in the BM from different times was further conducted, by using the selected paired BM samples. Several types of short chain fatty acids (SCFAs) in the BM were measured in the indicated months. The concentrations of butyric acid increased significantly by 3.83-fold at the 12th month, and the levels of isovaleric acid decreased significantly by 0.86-fold at the 12th month when compared with the 2nd month. Whereas the concentrations of the other SCFAs were not changed with significant difference (Figures 4B and S5).

For the long chain polyunsaturated fatty acid (PUFA) analysis, the results indicated that when compared with the 2nd month, the concentration of linoleic acid was significantly decreased by 0.77-fold at the 12th month, while

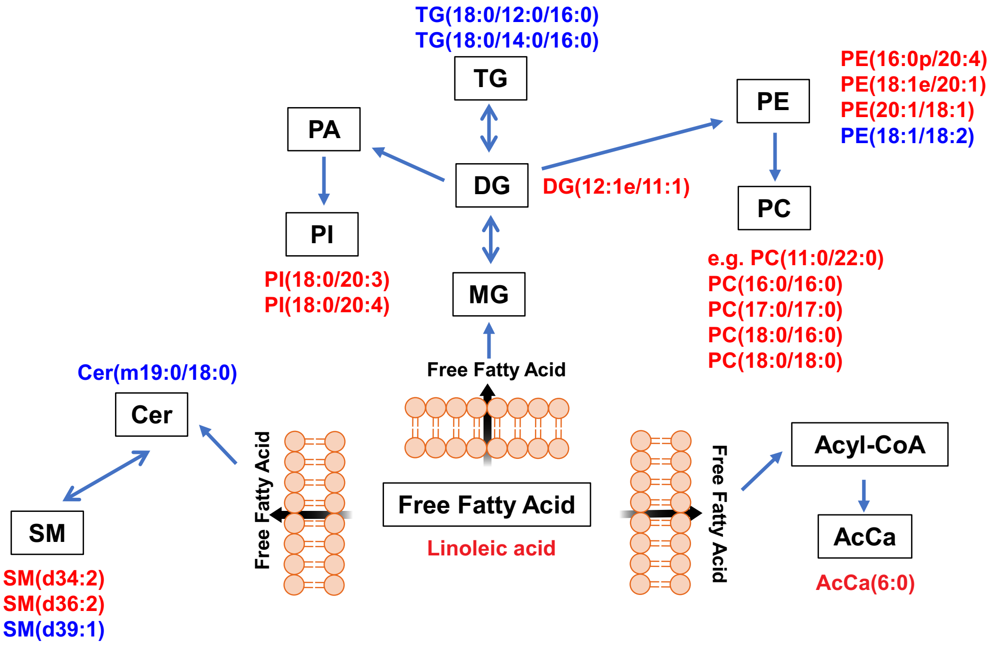
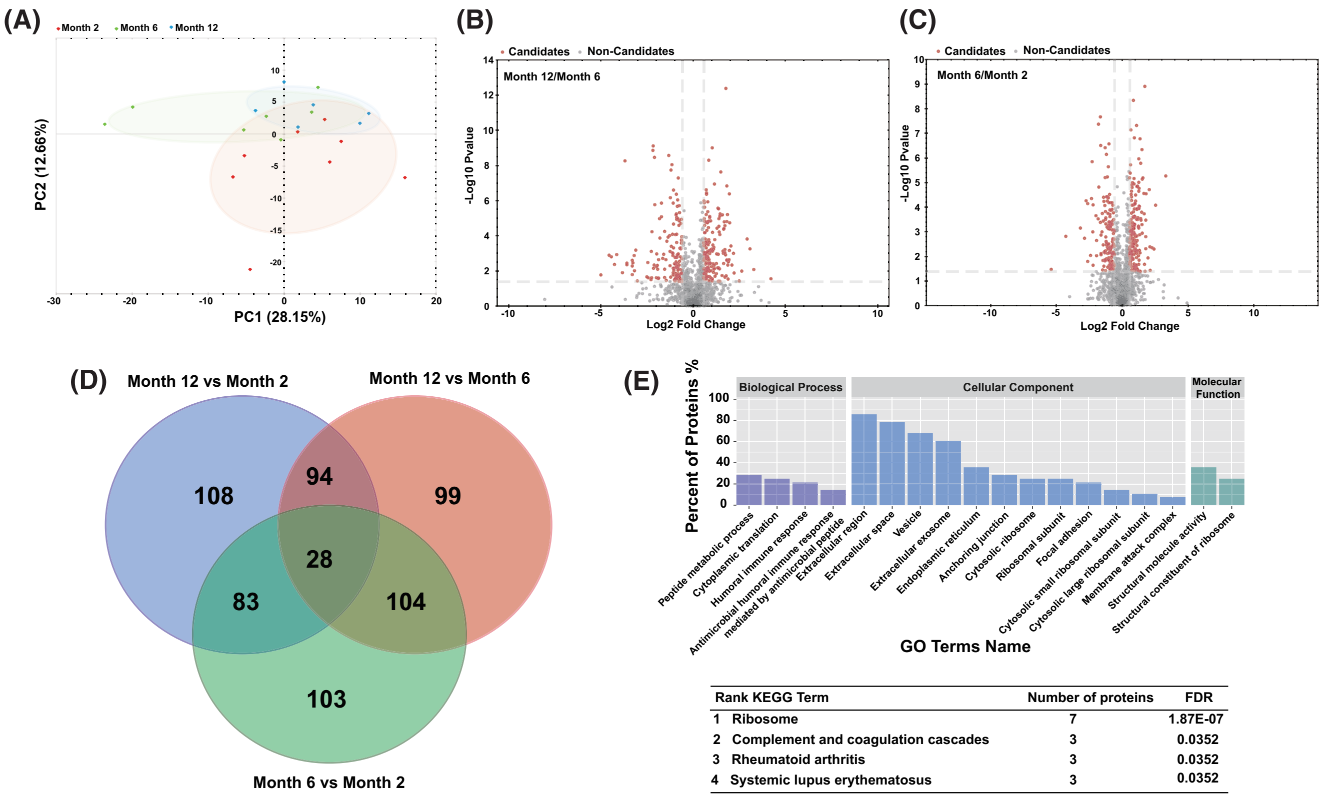


FIGURE 5 Proposed lipid alternations in BM during the lactation period. The downregulated lipids were marked in red color and upregulated ones were marked in blue color.



Rank	KEGG Term	Number of proteins	FDR
1	Ribosome	7	1.87E-07
2	Complement and coagulation cascades	3	0.0352
3	Rheumatoid arthritis	3	0.0352
4	Systemic lupus erythematosus	3	0.0352

FIGURE 6 Proteomics results from different lactation periods. (A) Principal components analysis (PCA) score plots for discriminating proteins from 2nd, 6th, and 12th months. (B) Volcano plots showing the relative abundances of proteins in the comparison between 12th and 2nd months. (C) Volcano plots showing the relative abundances of proteins in the comparison between 6th and 2nd months. (D) 28 shared proteins were measured by different comparisons. (E) GO annotation of differentially expressed proteins (upper) and rank KEGG pathways analysis (lower).

the BM level of arachidonic acid decreased by 0.65-fold at 6th month, and 0.70-fold at 12th month, with no significant difference (Figures 4B and S5). Taken together, the fatty acid-related metabolism might be altered during the lactation period, thereby induced the increases in lipids such as TG and the remodeling of lipids like GPs (Figure 5).

3.4 | Differentially expressed BM proteins during the lactation period

To clarify protein alternations during the lactation period, DIA-based label free proteomics study was performed by LC–MS/MS. A total of 1629 ions were detected in BM. Principal components analysis (PCA) showed the variation in protein abundance among different months (Figure 6A). In this analysis, differentially abundant proteins were defined as |fold change| ≥1.5 and *q* value <.05. Identified proteins were accepted with at least one unique peptide. As shown in Figure 6B,C, there were 956 significant differentially expressed proteins in different stages. Venn diagram analysis was used to illustrate the identified proteins, and to demonstrate the overlap of proteins between milk samples from different months. Twenty-eight proteins were enriched from different comparisons (Figure 6D). The main downregulated proteins in milk of 12th and 6th months compared to milk from 2nd month were RPS7 (Small ribosomal subunit protein eS7, decreased by 0.25-fold and 0.11-fold at 12th month and 6th month compared to 2nd month), RPL31 (Large ribosomal subunit protein eL31, decreased by 0.53-fold and 0.15-fold at 12th month and 6th month compared to 2nd month), RPL35A (Large ribosomal subunit protein eL33, decreased by 0.54-fold and 0.32-fold at 12th month and 6th month compared to 2nd month), RPL22 (Large ribosomal subunit protein eL22, decreased by 0.57-fold and 0.32-fold at 12th month and 6th month compared to 2nd month), FAU (Ubiquitin-like FUBI-ribosomal protein eS30 fusion protein, decreased by 0.65-fold and 0.21-fold at 12th month and 6th month compared to 2nd month), RPS4X (Small ribosomal subunit protein eS4, X isoform, decreased by 0.65-fold and 0.35-fold at 12th month and 6th month compared to 2nd month) and RPS5 (Small ribosomal subunit protein uS7, decreased by 0.62-fold and 0.39-fold at 12th month and 6th month compared to 2nd month) (Figure S6). About eight proteins significantly increased at both 12th and 6th months. In comparison with 2nd month, 12th and 6th months had significant higher levels of CALU (Calumenin, 3.97-and 1.65-fold), TCN1 (Transcobalamin-1, 3.64- and 2.18-fold), IGLV6-57 (Immunoglobulin lambda variable 6-57, 3.22- and 1.78-fold), SCP2 (Sterol carrier protein 2, 3.13- and 1.56-fold), HSPA13 (Heat shock 70 kDa protein 13, 2.64- and 4.22-fold), ATP6AP1 (V-type proton ATPase subunit S1, 1.80- and 3.13-fold), NUCB2 (Nucleobindin-2, 1.64- and 2.67-fold), and PCSK6 (Proprotein convertase subtilisin/kexin type 6, 1.54- and 2.72-fold). In contrast, proteins including complement component C6, complement component C9 and KLKB1 (Plasma kallikrein), which were involved in complement and coagulation cascades, were all increased at the 6th month milk sample, whereas decreased at 12th month compared with BM of 2nd month (Figure S6).

Further GO analysis showed that in the biological process group, differentially expressed proteins were mainly distributed in the process of the peptide metabolic (eight proteins). For the category of cellular component, most proteins were assigned to extracellular region (24 proteins) while proteins were involved mostly in terms of structural molecule activity (10 proteins) under the branch of molecular function (Figures 6E and S6). The most significant

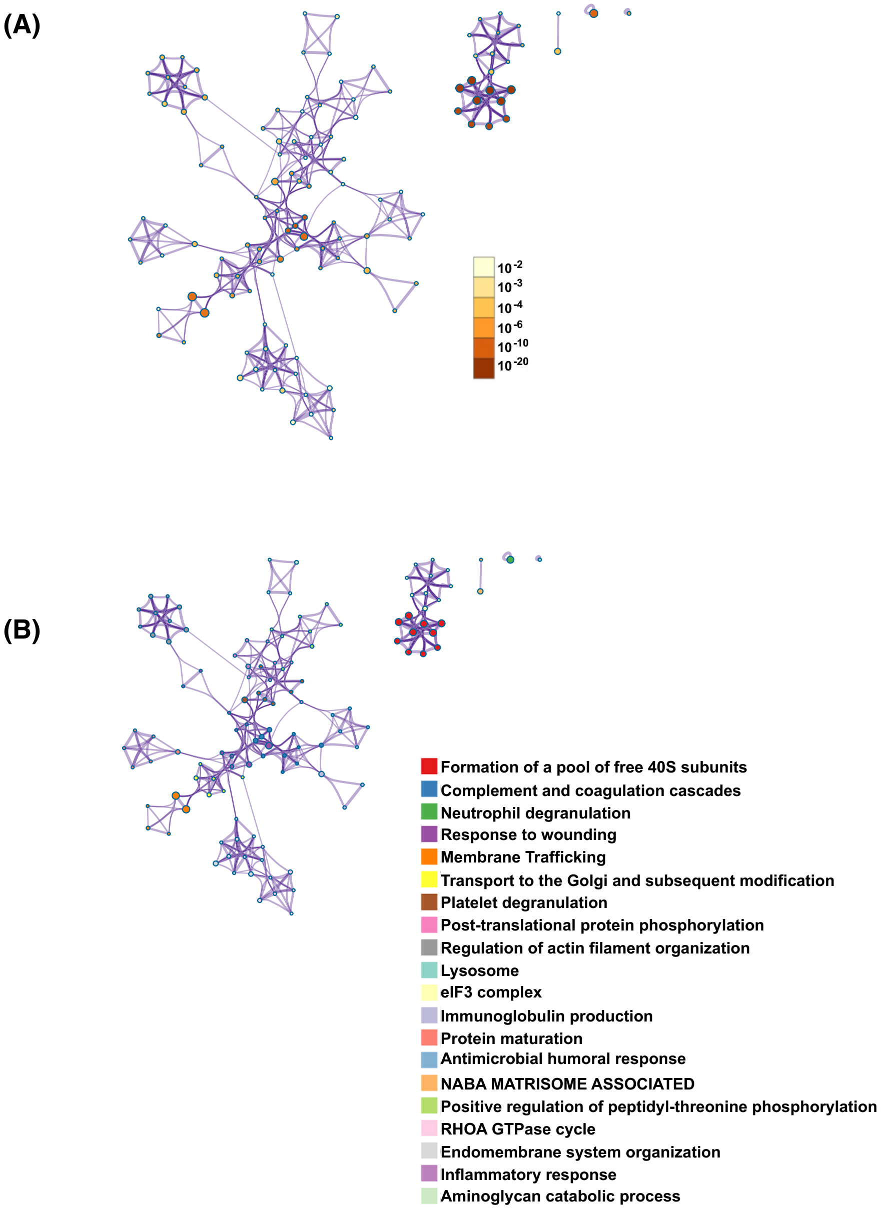


FIGURE 7 Proteins–proteins networks of overexpressed proteins during the lactation period. (A) Enrichment network with nodes colored by *p*-value. (B) Enrichment network with nodes colored by cluster.

items in the three categories were cytoplasmic translation (Biological process, false discovery rate (FDR) = 5.73E-06), structural constituent of ribosome (Molecular function, FDR = 1.50E-05), and extracellular region (Cellular component, FDR = 2.15E-10). KEGG pathways annotation implied that ribosome and complement and coagulation cascades ranked as the top 2 pathways with involved protein numbers of 7 and 3 (Figures 6E and S6). These results indicated that the different proteins in the human BM were mainly located in the extracellular space and were involved in the translation process.

The protein–protein interaction (PPI) was analyzed according to the reference reported,[19] by using the shared proteins between month 12 versus month 2 and month 6 versus month 2 (Figure S7A). The network included clusters with significant differences including formation of a pool of free 40S subunits and complement and coagulation cascades (Figure 7). The integrative multi-omics analysis was further performed with metabolomics, lipidomics, and proteomics datasets consisted of 19, 29, and 28 molecules, respectively (Figure S7B). Four clusters were detected, among them, cluster 3 consisted of 10 proteins, 10 lipids, and six metabolites, and was related to peptide chain elongation and formation of a pool of free 40S subunits (Reactome Pathway Database); cluster 4 consisted of 10 proteins, four lipids, and two metabolites, and was related to terminal pathway of complement (Reactome Pathway Database). These findings highlighted the altered molecular pathway during the long term lactation of Hong Kong mothers and facilitated the formula or functional proteins development for the infants in Hong Kong.

4 | DISCUSSION

BM was considered as the sole source of nutrition in the first 6 months of life with World Health Organization recommendation, and could be influenced by lactation stage. With the hope to facilitate our understanding of the metabolites and proteins profiles in BM of Hong Kong mothers during 12-month lactating period, the global abundance of BM metabolites and proteins were quantified through metabolomics, lipidomics, and proteomics approaches for the first time. Our findings demonstrated a significant alteration in multiple metabolites during the lactation period. As compared with 2nd month, a significant difference in the level of biotin was observed in the BM of 12th month. Biotin is a B vitamin that plays roles in skin symptoms and brain functions of infants.[20] Previous study indicated a statistical difference in the average concentrations of biotin in BM of four Asian countries with different climate zones.[21] Li et al. found that biotin content significantly increased over 42-day lactation stages.[13] In our research results, BM at 12th month was less in biotin, this might be associated with the dietary biotin intake[22] and the detection time, which requires further investigation. Previous studies revealed that reduction of amino acids in the BM of overweight/obese women might be a metabolic risk factor in offspring.[23] In the present study, the majority of the amino acid including kynurenine and L-histidine were all lower than those in the later lactation time. Kynurenic acid, the downstream metabolites of kynurenine, was reported to be decreased in the BM of overweight-obese mothers.[23] In contrast, the conflicting results were observed in the changes of L-histidine in BM, which could be attributed to different lactation stages or different populations.[13] We also identified a tripeptide significantly decreasing during lactation period. Further explorations are needed to illustrate the function of the related peptides over lactation stages in infant development. These metabolomic signatures might indicate the maternal health status, and further investigation regarding correlation between maternal/infant nutritional status and milk composition is warranted.

Specifically, we observed the reductions of several fatty acids that associated with linoleic acid metabolism and arachidonic acid metabolism. Linoleic acid is the precursor of long chain PUFAs (e.g., arachidonic acid).[2] Studies have indicated that linolenic acid acted as a growth promoter in infants.[2] High concentration long chain PUFAs such as arachidonic acid in the early period of lactation were beneficial for the brains and retinas development of infants.[24] From the targeted experimental results, linoleic acid at 12th month was significantly lower than that at 2nd month. Meanwhile, our targeted profiles displayed the significantly increasing of butyric acid and decreasing of isovaleric acid in the later lactation stage. SCFAs were derived from the fermentation of dietary components, which have been recognized as an important regulator of the host homeostasis.[25] Butyric acid is an important microbial metabolite in the BM that may play a vital role in the reduction of the excessive inflammation. Butyric acid and isovaleric acid were also associated with the effective approach for the prevention and treatment of hyperglycemia.[26] Further studies are needed to explore the role of SCFAs and PUFAs during the long lactation period in the metabolisms of mother and infant.

Fatty acid is a useful indicator of lipid metabolism. The lipidomic data in the present study revealed that abundant GPs, including the PC, PE, and PI, were dysregulated during the lactation period. Previous research indicated the role of the variations of GP species in the membrane fusion, fission and vesicle transport.[27] The GP remodeling during the lactation period suggested the potential influence on cell homeostasis in lactating mothers. GLs including TG or DG play a vital role in the energy metabolism. GLs, such as TG was also reported to be positively related to the risks of cardiovascular diseases.[28] Our results suggested the upregulation of the TG during the lactation period. DG, which was considered as a substrate or a metabolite of TG metabolism, was downregulated in the later stage of lactation. The results indicated the potential perturbation of energy homeostasis in the lactating mothers. We also found the alterations of the SM, Cer, and AcCa in BM of the lactating mothers. The elevation of the SM and the decline of the Cer indicating the trigger of the hydrolysis of SM and the overproductions of Cer in the BM of the lactating mothers. Cer has been reported to play role in multiple signaling pathways, such as apoptosis and inflammation,[29] implying the potential inflammatory and apoptotic responses during the long lactation period.

We further quantified the proteome and identified differential expressed proteins during the long-term lactation period. Several pathways were enriched, the pathways of ribosome and complement and coagulation cascades which included 10 differentially decreased proteins were worth discussing. Ribosomes are usually described as a molecular machine with significant roles in protein synthesis and mRNA translation.[30] Ribosomal protein was reported to be decreased in porcine colostrum compared to porcine mature milk. Complement components were critical component of the innate immune system, which could supply passive immunity to infants during their growth period.[31] Our experimental results presented an inverted "V"-shaped change of complement component. In addition, we also identified some other indicators in BM associated with the host or infant homeostasis. TCN1 was documented to be involved in cobalamin transport and metabolism, which was significantly increased during the 12-month lactation period. Cobalamin is a key cofactor in folate metabolism and DNA synthesis and is crucial for infant development.[32] HLA-DRB5 showed a decreasing trend during the lactation period, which was related to the phagosome, and was crucial to host immunity and tissue homeostasis maintenance and was also the most represented pathway in colostrum and mature milk.[31] The detailed functions of all these changed proteins during the long lactation time in the infant development need to be investigated further.

5 | CONCLUSION

In summary, the main purpose of this study was to reveal the composition of the BM of Hong Kong lactating mother by using LC–MS-based metabolomics, lipidomics, and proteomics analyses. Alterations of metabolites, lipids, and proteins in later stage of lactation period (6th and 12th months) were noted compared to early stage (2nd month). To the best of our knowledge, this is the first multi-omics study of human milk in the lactating mothers of Hong Kong. The results of this study improve the understanding of the compositions of BM and have important significance for further study in human milk of Hong Kong. However, the present results may not be a perfect representation considering the small sample size was used. Large-scale of deep multi-layer analysis is needed for future validation, and the bias in the sample collection cannot be ignored. Second, it would be important to investigate the lipid and protein compositions of milk fat globules. Last, analysis involved with the information of the mother's diet, and the condition of the infant is warranted to investigate the correlation between the health of the mother, the infant and the metabolite.

AUTHOR CONTRIBUTIONS

Lilong Jiang performed the research, acquired the data and drafted the manuscript; Oi-Yee Yeung, Wing-Wa Ho, Tim-Fat Shum, Man-Sau Wong, and Carly S. Y. Lam provided assistance with sample collection. Jiachi Chiou was responsible for the supervision and the revision of the manuscript.

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DATA AVAILABILITY STATEMENT

Data will be available upon request to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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