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The use of metabolomics to reveal differences in functional substances of milk whey of dairy buffaloes raised at different altitudes†

Jinhui Pu,‡[a] Paramintra Vinitchaikul,‡[b] Zhaobing Gu, *[a] Huaming Mao[a] and Fulan Zhang[a]

Buffalo milk is nutrient-rich and contains less cholesterol than cow milk. Dairy buffaloes are widely distributed at different altitudes in the Yunnan Province, China; however, the impacts of altitude on the whey-derived functional metabolites of buffalo milk whey are not well understood. Here, we used non-targeted and targeted metabolomics to evaluate the differential metabolites in the milk whey of buffaloes raised at low altitudes (LA), medium altitudes (MA), and high altitudes (HA). ANOVA statistical test was performed to acquire differential metabolites using IBM SPSS statistics 22 software. The results showed that LA- and MA-milk whey had higher levels of amino acids (glutamine and pyroglutamic acid) and vitamin B6 than HA-milk whey. LA-milk whey had higher levels of the carbohydrates involved in galactose, amino sugar, and nucleotide sugar metabolism than MA- and HA-milk whey, but HA-milk whey showed significantly higher levels of free fatty acids. In conclusion, owing to the biological functions of their most abundant components, LA-milk is more suitable for the production of functional milk with high levels of amino acids, vitamin B6, and carbohydrates; while HA-milk is suitable as raw milk for the production of dairy products with high free fatty acid content.

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1. Introduction

Milk is rich in the nutrients necessary for a child's cognitive development and health[1] and can be obtained from buffaloes, the second-largest global source of milk.[2] The peroxidase activity of buffalo milk is 2–4 times higher than that of cow milk[2] and contains high percentages of fats, proteins, lactose, and solids, and its whole milk contains water, caseins, whey protein, and milk whey. Whey proteins constitute 20% of total bovine proteins[3–5] and may be used as oxygen scavengers to preserve oxygen-sensitive foods.[6,7] Whey is obtained after the fat and casein have been removed from whole milk and is a nutritious liquid with a complex composition that includes whey proteins, lactose, vitamins, minerals, and enzymes. In addition to nutritional functions, some milk whey components, including butyric acid, lactoperoxidase, and lactoferrin, have health benefits for the heart, bones, immune system, and digestive tract.[8] Thus, milk whey has been used as the raw material for producing functional substances in the pharmaceutical and food industries.[9]

China produces 5% of global buffalo milk, and Dehong, Baoshan, and Dali states are the main producing regions in Yunnan Province.[10] Although the three states are geographically close, they are at low, medium, and high altitudes, respectively. Previous studies have shown that altitude affects the metabolic profiles and composition of milk from dairy cows under grazing conditions[11,12] and also the crude protein and fat in yak milk.[13–16] Additionally, an energy supply deficit at high altitudes might negatively affect milk protein synthesis.[17] Despite these studies, the differences in the whey-derived functional metabolites of dairy buffaloes raised at different altitudes have not been well-characterized. In this study, metabolomics was used to reveal differences in the metabolite profiles of milk whey at different altitudes to identify which type of raw milk is most suitable for producing and processing milk products.

[a]*Faculty of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China. E-mail: zhaobinggu@163.com*
[b]*Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand*

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‡The two authors equally contributed to the study.

2. Materials and methods

2.1. Sample collection and preparation

The dairy buffalo farms were situated at a low altitude (912.0 m, LA, Dehong State), medium altitude (1536.0 m, MA,

Baoshan State), and high altitude (1865.0 m, HA, Dali State). The average daily temperature from April–September varied from 5.9 to 27.2 °C, and the relative air humidity was 41.5–95.3%. The number of buffalo milk samples collected from the LA-, MA-, and HA-farms were 9, 12, and 12, respectively. Thirty-three healthy multiparous (parity = 3.2 ± 1.1) Nili-Ravi × Murrah × Local crossbred buffaloes at mid-lactation (115 ± 10 days) were group-housed in an open-sided barn and fed *ad libitum* using the same total mixed rations (80% whole-plant corn silage *ad libitum*, 12.5% concentrate feeding, 7.5% corn protein powder) in June. The nutritional values of the three feed ingredients are shown in Table S1.†

The experiment lasted for two weeks. On the last day of the experiment, milk was sampled at 06:00 and 20:00 h to obtain morning and evening milk samples, respectively, which were mixed at a ratio of 6 : 4 by volume. Milk compositions (fat percentage, protein percentage, lactose percentage, total solids, somatic cell, and milk urea nitrogen) were measured using Combi Foss FT+ instrument (Foss Electric, Hillerød, Denmark) in Kunming Dairy Herd Improvement Testing Center. Milk yield was standardized by fat corrected milk (FCM) at 4%, and the above milk parameters are shown in Table S2.† According to previous studies,[18,19] fresh raw milk was centrifuged at 3000*g* for 15 min after collection at 4 °C to remove the fat. Skim milk samples were centrifuged at 100 000*g* for 1 h at 4 °C to obtain the whey-containing supernatant and stored at −80 °C until further analysis. SDS-PAGE gel with Coomassie blue staining was used to confirm the extracted buffalo milk whey and the consistency in the whey samples (Fig. S1†).

Aliquots of 100 μL serum (milk whey) were thawed at 4 °C and mixed with 400 μL of cold methanol/acetonitrile (1 : 1, v/v). The mixtures were stored at −20 °C for 60 min and centrifuged (Eppendorf Centrifuge 5430R) for 15 min (14 000*g*, 4 °C). The supernatants were dried in a vacuum centrifuge, and the dried samples were re-dissolved in 100 μL acetonitrile/water (1 : 1, v/v) solvent mixture and then centrifuged for 15 min (14 000*g*, 4 °C).[20]

2.2. UHPLC-QTOF-MS analysis

Analyses were performed using a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (Triple TOF 5600, AB Sciex, Concord, ON, Canada) in Shanghai Applied Protein Technology Co., Ltd. Chromatographic separation was performed on an ACQUITY UPLC BEH Amide column (1.7 μm, 2.1 mm × 100 mm column). A 2 μL sample was injected into a column maintained at 25 °C with a flow velocity of 0.3 mL min^{-1}. The mobile phase consisted of A = 25 mM ammonium hydroxide and 25 mM ammonium acetate (Sigma, 70221) in water; and B = acetonitrile (Merck, 1499230-935). The gradient elution program was 95% B from 0 to 1 min, 95%–65% B from 1 to 14 min, 65%–40% B from 14 to 16 min, 40% B from 16 to 18 min, 40%–95% B from 18 to 18.1 min, and 95% B from 18.1 to 23 min. The autosampler was maintained at 4 °C. To monitor the stability and repeatability of the analysis, quality control samples were prepared by pooling 10 μL of each sample and analyzing them together with other samples.

The ESI† source conditions were as follows: ion source gas 1 (Gas1) as 60 psi, ion source gas 2 (Gas2) as 60 psi, curtain gas (CUR) as 30 psi, and a source temperature of 600 °C. In MS-only acquisition, the instrument was set to acquire over the *m*/*z* range 60–1000 Da, and the accumulation time for a TOF MS scan was set at 0.20 s per spectra. The capillary voltage was maintained at ±5500 V for positive and negative mode detection, with a source temperature of 600 °C. In auto MS/MS acquisition, the instrument was set to acquire over the *m*/*z* range 25–1000 Da, and the accumulation time for the product ion scan was 0.05 s per spectra. Product ion scans were acquired using information dependent on the acquisition in the high-sensitivity mode. The parameters were as follows: the collision energy was fixed at 35 ± 15 eV; the declustering potential was 60 V (+) and −60 V (−); isotopes were excluded within 4 Da; and 6 candidate ions were monitored per cycle.

2.3. Metabolite identification and pathway analysis

OPLS-DA models with 7-fold cross-validation were also established using SIMCA-P 14.1 software. Prediction parameters of OPLS-DA model are R2X, R2Y and Q2, where R2X and R2Y represent the interpretation rate of the model to X and Y matrix, respectively, and Q2 represents the prediction ability of the model. The closer these three indexes are to 1, the more stable and reliable the model is. When Q2 >0.5, the prediction ability of the model is better. Significantly differential metabolites were screened using the variable importance in the projection (VIP) scores >1 obtained from the orthogonal partial least-squares discriminant analysis (OPLS-DA) model and the *p*-values (<0.05).

Compound identification of metabolites was performed by comparing the accuracy of the *m*/*z* values (<25 ppm) of the MS/MS spectra using an in-house database (Shanghai Applied Protein Technology) established using authentic standards. In the extracted ion features with XCMS software, the variables more than 2/3 of the nonzero measurement values in at least one group were filtered and excluded. A one-way analysis of variance (ANOVA) statistical test was used to calculate the variations within the detected metabolites using IBM SPSS statistics 22 software. If the ANOVA has $p < 0.05$, *Post hoc* tests were used to acquire distinct metabolites. Person correlation coefficient analysis was performed to analyze the metabolite–metabolite interactions among the identified metabolites in milk whey using IBM SPSS statistics 22 software. Differential metabolites were queried in the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway database, and published articles were searched for data relating to global metabolism. A volcano plot of negative log 10-transformed *p*-values against the log 2 fold change was used to visualize the differential metabolites between the three pairwise comparisons by R software. Hierarchical clustering analysis of the differential metabolites was performed using the log 2-transformed expression values and a cutoff of $p < 0.05$.

2.4. Differential metabolites validation by targeted metabolomics

Concentrations of key differential amino acids and fatty acids in milk whey were validated by liquid chromatograph/mass spectrometric (LC/MC) and gas chromatograph/mass spectrometric (GC/MS), respectively. The measurements were conducted by Bionovogene Company (Suzhou, Jiangsu, China). Further details of the LC/MC and GC/MS method are provided as ESI.† Concentrations of metabolites were determined by the ratio between the metabolite peak area or internal standard peak area and the calibration curve.

3. Results

3.1. Metabolic profiles of buffalo milk whey obtained from different altitudes

The OPLS-DA plots of the milk whey metabolomics data show a clear separation, with no overlap under positive and negative ionization mode (Fig. 1A–C), indicating that the milk whey metabolic profiles at different altitudes were distinct. The UHPLC-QTOF-MS metabolomics data of milk whey for these three pairwise comparisons identified one predictive component and two orthogonal components. Satisfactory explanatory and predictive values for the intercepts ($R2$, $Q2$) indicated the validity of the OPLS-DA models (Fig. S2A–C†). Hierarchical clustering analysis and volcano plot, of milk whey metabolites are shown in Fig. 2 and S3.† Person correlation coefficient analysis allowed for the identification of metabolites that related to each other in the three pairwise comparisons. Pearson correlations of milk whey metabolites are shown in Fig. S4.†

3.2. Metabolic differences of milk whey obtained from different altitudes

Based on the individual of fold change (FC), VIP and p-values, the significantly different metabolites with known nutritional functions identified in the milk whey are shown in Table 1. These metabolites were then selected to further evaluate their differences in metabolic profiles. Hierarchical clustering showed that the metabolites could be grouped according to their differential enrichment (Fig. 2).

LA- and MA-milk whey had higher levels of glutamine and L-pyroglutamic acid than HA-milk whey. D-Mannose 1-phosphate, alpha-D-galactose 1-phosphate, and alpha-D-glucose 1-phosphate were present at higher levels in LA-milk whey than MA- and HA-milk whey. They are involved in galactose metabolism and amino sugar and nucleotide sugar metab-

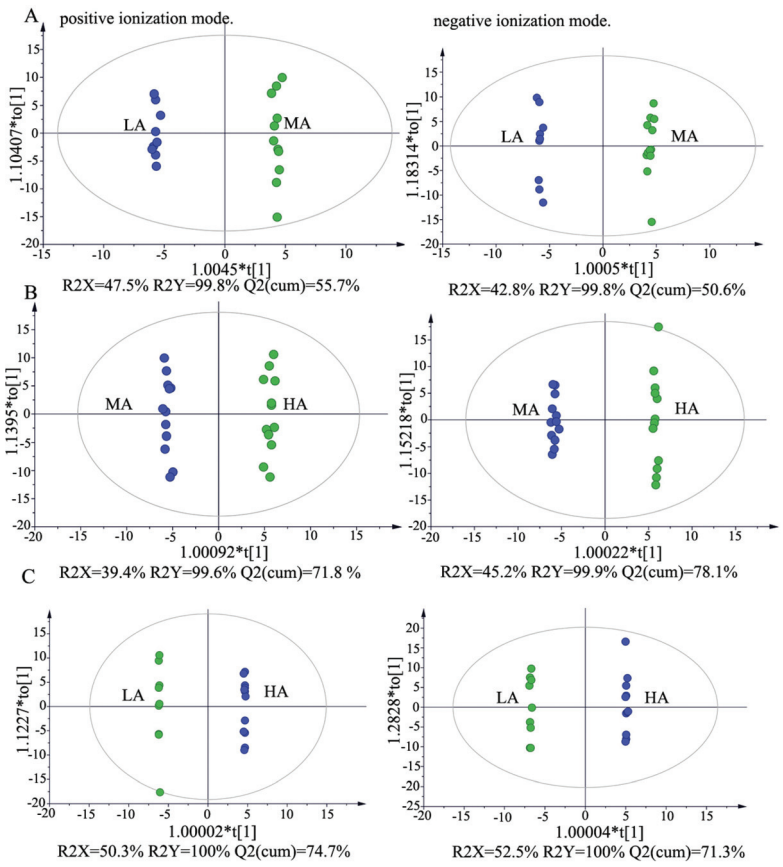

Fig. 1 Pair wise OPLS-DA score plots of the LC-MS data for milk whey metabolome in positive and negative ionization mode. (A) LA *vs.* MA, (B) MA *vs.* HA, (C) LA *vs.* HA. LA, whey at low altitude; MA, whey at medium altitude; HA, whey at high altitude.

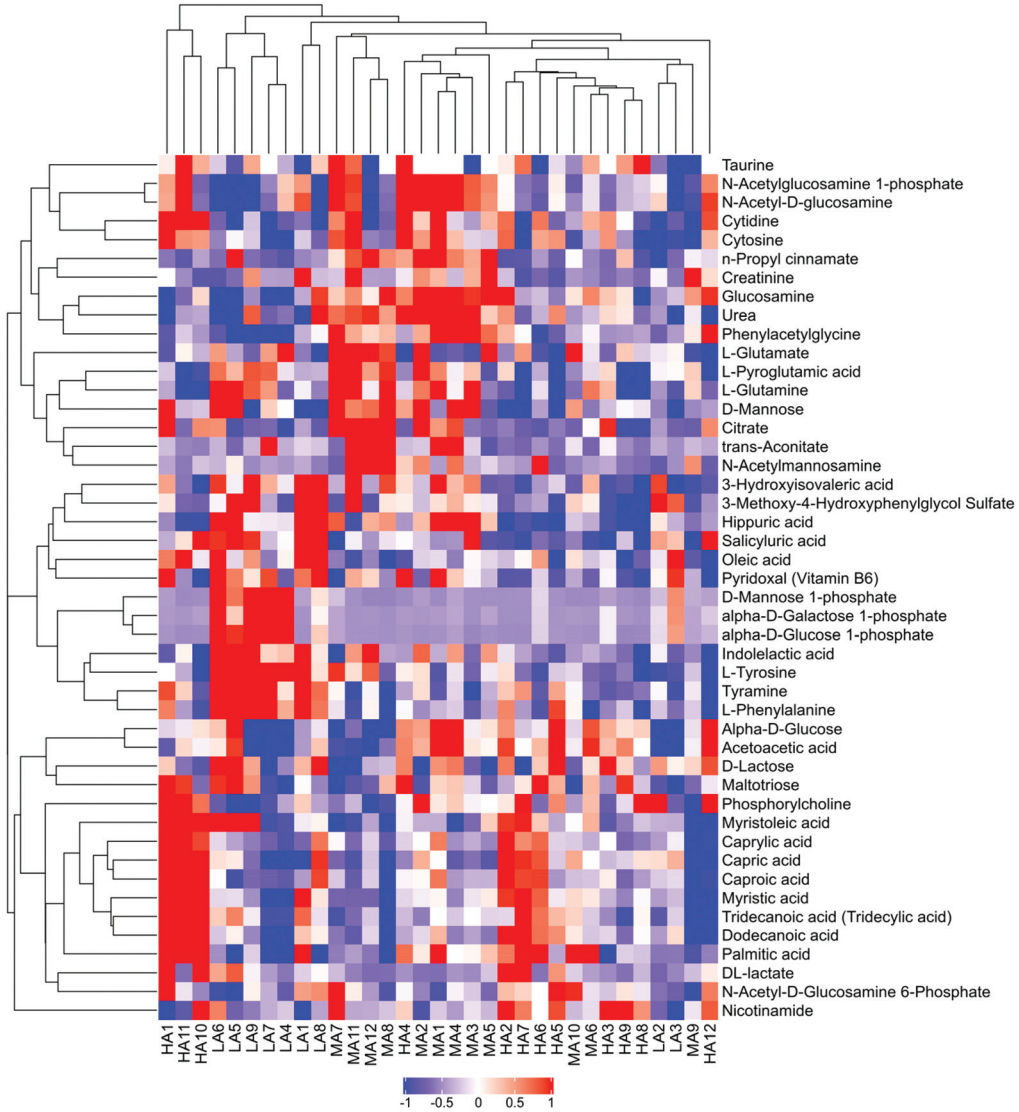

Fig. 2 A hierarchical clustering analysis for the significantly different milk whey metabolites. Red represents increases in metabolite levels, and blue represents decreases in metabolite levels. *X* axis: hierarchical clustering of samples, *Y* axis: the hierarchical clustering of metabolites.

olism, and their presence indicates that LA-milk whey has a higher nutritive value with more carbohydrates. HA-milk whey showed higher levels of short- and medium-chain saturated FFAs (free fatty acids) than that of MA-milk whey. Meanwhile, HA-milk whey also contained higher levels of unsaturated FFA (myristoleic acid C14:1). The levels of medium-chain saturated FFAs (caproic acid C6:0, caprylic acid C8:0, and capric acid C10:0) were significantly higher in HA-milk whey than that of the LA- and MA-milk whey. Saturated FFAs (dodecanoic acid C12:0, tridecanoic acid C13:0, myristic acid C14:0, palmitic acid C16:0) were present at higher levels in HA-milk whey than LA- and MA-milk whey. The levels of all five metabolites (dodecanoic acid, capric acid, myristic acid, palmitic acid, and caprylic acid) were involved in the FFA biosynthesis pathway in two pairwise comparisons (MA *vs.* HA, and LA *vs.* HA).

3.3. Differential metabolites validation by targeted metabolomics

Because amino acids and fatty acids are important milk components for their functional and nutritional properties, tyrosine, glutamine, caproic acid C6:0, caprylic acid C8:0, capric acid C10:0, dodecanoic acid C12:0, tridecanoic acid C13:0, myristic acid C14:0, myristoleic acid C14:1, and oleic acid C18:1 were quantified with targeted metabolomics. The results showed the similar trends in contents of the three fatty acids (capric acid C10:0, dodecanoic acid C12:0, oleic acid 18:1) and tyrosine (Fig. 3). The contents of these fatty acids in LA- and HA-whey showed no significant difference, but the contents of the three fatty acids in LA- and HA-whey were significant higher as compared to MA-whey. In terms of amino acids, LA- and MA-whey had a significant higher content of tyrosine than that of the HA-whey.

Table 1 Distinct metabolites in liquid whey identified by UHPLC-QTOF-MS and comparison of their levels at different altitudes

Metabolite	Adduct	*m/z*	VIP	rt	MS2 values of metabolites			Fold change		
					LA	MA	HA	LA/MA	MA/HA	LA/HA
Phenylacetylglycine	(M + H)+	194.0798	3.62	328.94	18 965.3^{a}	54 508.0^{b}	34 196.2^{a}	0.35	1.59	0.55
L-Phenylalanine	(M + H)+	166.0848	1.45	472.29	12 399.9^{a}	7390.2^{b}	7793.7^{b}	1.68	0.95	1.59
D-Mannose 1-phosphate	(M + H-H_2O)+	243.0249	3.11	646.25	27 281.3^{a}	1773.1^{b}	1907.1^{b}	15.39	0.93	14.31
Alpha-D-glucose 1-phosphate	(M + H-H_2O)+	243.0248	1.81	620.17	9459.9^{a}	719.7^{b}	964.0^{b}	13.14	0.75	9.81
Salicyluric acid	(M + H)+	196.0591	1.07	401.00	5086.5^{a}	2308.4^{b}	2844.4^{b}	2.20	0.81	1.79
Tyramine	(M + H-H_2O)+	120.0790	1.17	474.26	8830.7^{a}	5214.0^{b}	5606.9^{b}	1.69	0.93	1.57
Urea	(M + H)+	61.0384	2.74	176.03	43 305.0^{b}	71 075.2^{a}	51 834.2^{b}	0.61	1.37	0.84
Oleic acid C18:1	(M + H)+	283.2618	2.23	78.29	25 725.6^{a}	12 352.8^{b}	14 125.1^{b}	2.08	0.87	1.82
Citrate	(M + H)+	193.0327	2.50	958.18	10 849.6^{b}	32 884.5^{a}	17 242.6^{b}	0.33	1.91	0.63
Pyridoxal (vitamin B6)	(M + H)+	168.0639	1.28	187.68	13 806.1^{a}	8847.7^{b}	7449.2^{b}	1.56	1.19	1.85
N-Acetyl-D-glucosamine	(M + H-H_2O)+	204.0854	6.76	831.14	175 435.0^{b}	347 830.8^{a}	289 370.0^{ab}	0.50	1.20	0.61
D-Mannose	(M + NH_4)+	198.0956	1.14	572.15	24 871.5^{b}	34 801.1^{a}	24 067.2^{b}	0.71	1.45	1.03
Hippuric acid	(M + H)+	180.0643	7.62	342.87	336 903.7^{a}	300 019.3^{a}	108 134.8^{b}	1.12	2.77	3.12
L-Glutamate	(M + H)+	148.0589	3.19	757.17	92 264.0^{ab}	114 857.4^{a}	59 292.5^{b}	0.80	1.94	1.56
Creatinine	(M + H)+	114.0646	4.61	351.24	127 503.3^{ab}	185 078.1^{a}	78 587.4^{b}	0.69	2.36	1.62
trans-Aconitate	(M + H)+	175.0225	1.54	961.86	7910.2^{ab}	14 606.9^{a}	4163.1^{b}	0.54	3.51	1.90
D-Lactose	(M + NH_4)+	360.1484	1.78	869.55	46 446.5^{a}	33 857.9^{b}	42 629.6^{ab}	1.37	0.79	1.09
Nicotinamide (vitamin B3)	(M + H)+	123.0539	2.05	84.33	63 870.8^{b}	73 680.1^{b}	96 889.2^{a}	0.87	0.76	0.66
Indolelactic acid	(M + H-H_2O)+	188.0693	1.61	474.23	10 382.8^{a}	7067.9^{b}	4891.8^{b}	1.47	1.44	2.12
L-Pyroglutamic acid	(M + H)+	130.0483	1.60	706.81	20 372.1^{a}	23 249.4^{a}	13 480.7^{b}	0.88	1.72	1.51
3-Hydroxyisovaleric acid	(M + H-H_2O)+	101.0581	1.14	735.48	10 502.4^{a}	8919.4^{a}	7068.9^{b}	1.18	1.26	1.49
L-Tyrosine	(M + H)+	182.0795	1.08	558.27	7237.8^{a}	4629.8^{b}	3567.8^{b}	1.56	1.30	2.03
Cytosine	(M + H)+	112.0489	1.14	443.82	7980.8^{b}	11 395.5^{ab}	12 788.1^{a}	0.70	0.89	0.62
N-Acetyl-D-glucosamine 6-phosphate	(M + H-H_2O)+	284.0515	2.13	846.16	28 590.3^{b}	34 005.3^{ab}	49 283.8^{a}	0.84	0.69	0.58
Glucosamine	M-	179.0785	1.63	187.36	8153.4^{c}	15 293.1^{a}	11 445.2^{b}	0.53	1.34	0.71
Alpha-D-galactose 1-phosphate	(M − H_2O-H)−	241.0118	2.21	626.33	19 323.8^{a}	1355.5^{b}	1959.4^{b}	14.26	0.69	9.86
Cytidine	(M − H)−	242.0780	1.11	446.01	8081.4^{b}	12 517.1^{a}	14 842.9^{a}	0.65	0.84	0.54
Myristoleic acid C14:1	(M − H)−	225.1857	2.69	85.52	98 299.5^{ab}	56 240.6^{b}	105 597.9^{a}	1.75	0.53	0.93
L-Glutamine	(M − H)−	145.0617	2.62	707.36	43 128.9^{a}	49 644.8^{a}	25 518.2^{b}	0.87	1.95	1.69
Dodecanoic acid C12:0	(M − H)−	199.1703	5.96	87.28	253 303.2^{bc}	231 132.0^{c}	402 799.2^{a}	1.10	0.57	0.63
Capric acid C10:0	(M − H)−	171.1390	6.62	88.20	284 442.8^{ab}	237 614.0^{b}	452 059.2^{a}	1.20	0.53	0.63
Maltotriose	(M − H)−	503.1607	1.77	893.49	46 562.2^{ab}	38 121.8^{b}	60 839.6^{a}	1.22	0.63	0.77
Myristic acid C14:0	(M − H)−	227.2016	8.36	84.92	644 479.6^{ab}	559 598.9^{b}	895 693.3^{a}	1.15	0.62	0.72
Phosphorylcholine	(M − H)−	182.0587	1.44	909.36	17 294.7^{b}	21 747.0^{b}	34 374.0^{a}	0.80	0.63	0.50
Tridecanoic acid (Tridecylic acid) C13:0	(M − H)−	213.1858	1.21	86.06	14 261.0^{ab}	12 251.4^{b}	19 673.5^{a}	1.16	0.62	0.72
Caproic acid C6:0	(M − H)−	115.0763	6.91	159.01	284 164.0^{b}	278 087.7^{b}	516 402.6^{a}	1.02	0.54	0.55
DL-Lactate	(M − H)−	89.0242	2.01	427.01	16 762.9^{ab}	9708.3^{b}	32 089.6^{a}	1.73	0.30	0.52
Caprylic acid C8:0	(M − H)−	143.1074	3.73	89.31	86 366.3^{b}	89 044.6^{b}	163 940.5^{a}	0.97	0.54	0.53
N-Acetylmannosamine	(M − H)−	220.0826	2.81	523.94	18 728.6^{b}	63 384.4^{a}	24 717.2^{b}	0.30	2.56	0.76
Taurine	(M − H)−	124.0072	2.67	551.04	94 581.1^{b}	110 103.4^{a}	137 428.6^{a}	0.86	0.80	0.69
Alpha-D-glucose	(M − H)−	179.0557	1.02	144.28	7339.8^{b}	10 825.9^{ab}	12 084.8^{a}	0.68	0.90	0.61
Palmitic acid C16:0	(M − H)−	255.2327	2.64	103.25	48 049.7^{b}	69 425.9^{ab}	82 895.5^{a}	0.69	0.84	0.58

Data are the MS2 peak area of metabolites. ANOVA statistical test was used to acquire distinct metabolites. Row values with different lowercase superscripts are significantly different at $p < 0.05$. LA, whey at low altitude; MA, whey at medium altitude; HA, whey at high altitude. rt, retention time. VIP, variable importance in the projection.

3.4. Characterization and functional analysis of metabolic pathways in pairwise comparisons of LA- and MA-milk whey

Twenty-eight pathways were observed when significantly different metabolites were imported into KEGG, and 11 pathways showed an impact value at the comprehensive level (Table 2). After performing enrichment and pathway topology analysis on the 11 identified pathways, 3 significantly different metabolism pathways were revealed: the first one for amino sugars and nucleotide sugars, the second one for galactose metabolism, and the third one for phenylalanine metabolism (Fig. 4A). For the amino sugar and nucleotide sugar metabolism pathway, 5 significantly different metabolites were found: D-mannose 1-phosphate (FC = 15.39, $p < 0.05$), alpha-D-glucose 1-phosphate (FC = 13.14, $p < 0.001$), alpha-D-galactose 1-phosphate (FC = 14.26, $p < 0.05$), glucosamine (FC = 0.53, $p < 0.05$), and *N*-acetyl-D-glucosamine (FC = 0.50, $p < 0.05$). Alpha-D-galactose 1-phosphate, alpha-D-glucose 1-phosphate, D-mannose, and D-lactose were involved in the galactose metabolism pathway.

3.5. Characterization and functional analysis of key metabolic pathways in pairwise comparisons of MA- and HA-milk whey

Thirty-six pathways were obtained when significantly different metabolites were imported into KEGG. Ten pathways showed an impact value at the comprehensive level after enrichment and pathway topology analysis (Table 3). A comprehensive analysis of the *p*-value and pathway impact showed that 4 path-

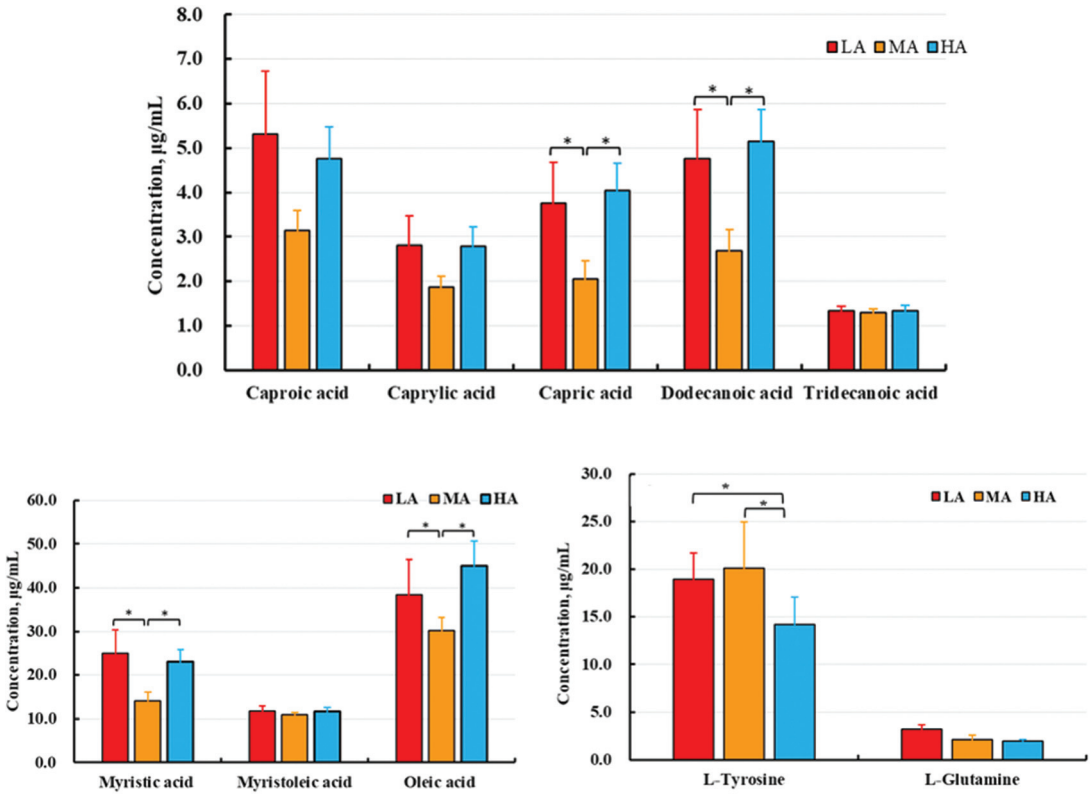

Fig. 3 Concentrations of metabolites in milk whey at different altitudes. *$p < 0.05$. LA, whey at low altitude; MA, whey at medium altitude; HA, whey at high altitude. LA, whey at low altitude; MA, whey at medium altitude; HA, whey at high altitude.

Table 2 KEGG pathway enrichment analysis of differential metabolites in pairwise comparisons of LA- and MA-whey

Pathway	Hits[a]	*p*-Value	Holm P[b]	Impact value
Amino sugar and nucleotide sugar metabolism	6	6.67×10^{-5}	0.006	0.224
Galactose metabolism	3	0.002	0.152	0.066
Phenylalanine metabolism	2	0.024	1	0.357
Fructose and mannose metabolism	2	0.061	1	0.124
Citrate cycle (TCA cycle)	2	0.061	1	0.140
Phenylalanine, tyrosine and tryptophan biosynthesis	1	0.080	1	0.500
Glyoxylate and dicarboxylate metabolism	1	0.138	1	0.056
Pyrimidine metabolism	1	0.182	1	0.114
Nicotinate and nicotinamide metabolism	1	0.235	1	0.194
Phosphatidylinositol signaling system	1	0.443	1	0.037
Tyrosine metabolism	1	0.586	1	0.025

[a] Hits represents the number of metabolites in one pathway. [b] Holm P indicates the statistical *P* values that were further adjusted using the Holm–Bonferroni method for multiple tests. LA, whey at low altitude; MA, whey at medium altitude.

ways were significantly different: amino sugar and nucleotide sugar metabolism, D-glutamine and D-glutamate metabolism, FFAs biosynthesis, and pyrimidine metabolism. The integrated key pathways are shown in Fig. 4B. Two significantly different metabolites were characterized in the amino sugar and nucleotide sugar metabolism pathways: D-mannose (FC = 1.45, $p < 0.05$), and glucosamine (FC = 1.34, $p < 0.05$). Glutamine (FC = 1.95, $p < 0.05$) were involved in the glutamate and glutamine metabolism pathways. The galactose metabolism pathway was significantly enriched with differential metabolite: D-mannose (FC = 1.45, $p < 0.05$).

3.6. Characterization and functional analysis of the key metabolic pathways in pairwise comparisons of LA- and HA-milk whey

Thirty-seven pathways were obtained when significantly different metabolites were imported into KEGG. After performing enrichment and pathway topology analysis of the identified pathways, nine pathways showed an impact value at the comprehensive level (Table 4). A comprehensive analysis of the *p*-value and pathway impact showed that 6 metabolic pathways were significantly different (Fig. 4C): amino sugar and nucleo-

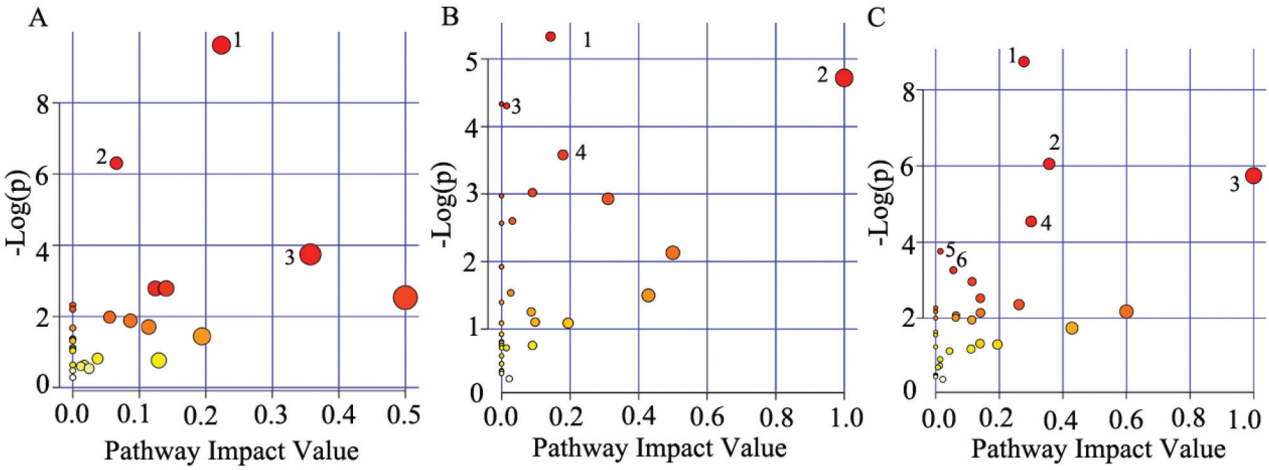

Fig. 4 The metabolome view map of significant metabolic pathways characterized in milk whey. This figure aims to find pathways significant changed based on enrichment and topology analysis. The *x*-axis represents pathway enrichment, and the *y*-axis represents pathway impact. Larger sizes and darker colors represent greater pathway enrichment and higher pathway impact values, respectively. (A) LA *vs.* MA: 1. Amino sugar and nucleotide sugar metabolism; 2. Galactose metabolism; 3. Phenylalanine metabolism. (B) MA *vs.* HA: 1-Amino sugar and nucleotide sugar metabolism; 2. D-Glutamine and D-glutamate metabolism; 3. Fatty acid biosynthesis; 4. Pyrimidine metabolism. (C) LA *vs.* HA: 1. Amino sugar and nucleotide sugar metabolism; 2. Phenylalanine metabolism; 3. Phenylalanine, tyrosine and tryptophan biosynthesis; 4. Fructose and mannose metabolism; 5. Fatty acid biosynthesis; 6. Glyoxylate and dicarboxylate metabolism. LA, whey at low altitude; MA, whey at medium altitude; HA, whey at high altitude.

Table 3 KEGG pathway enrichment analysis of differential metabolites in pairwise comparisons of MA- and HA-whey

Pathway	Hits[a]	*p*-Value	Holm P[b]	Impact value
Amino sugar and nucleotide sugar metabolism	3	0.005	0.406	0.143
Arginine biosynthesis	3	0.008	0.669	0.117
D-Glutamine and D-glutamate metabolism	2	0.009	0.731	1
Fatty acid biosynthesis	5	0.013	1	0.015
Pyrimidine metabolism	3	0.028	1	0.179
Alanine, aspartate and glutamate metabolism	2	0.053	1	0.311
Glyoxylate and dicarboxylate metabolism	2	0.074	1	0.032
Glutathione metabolism	2	0.215	1	0.028
Arginine and proline metabolism	3	0.332	1	0.098
Nicotinate and nicotinamide metabolism	1	0.338	1	0.194

[a] Hits represents the number of metabolites in one pathway. [b] Holm P indicates the statistical *P*-values that were further adjusted using the Holm–Bonferroni method for multiple tests. MA, whey at medium altitude; HA, whey at high altitude.

Table 4 KEGG pathway enrichment analysis of differential metabolites in pairwise comparisons of LA- and HA-whey

Pathway	Hits[a]	*p*-Value	Holm P[b]	Impact value
Amino sugar and nucleotide sugar metabolism	6	0.0001	0.014	0.277
Phenylalanine metabolism	3	0.002	0.195	0.357
Phenylalanine, tyrosine and tryptophan biosynthesis	2	0.003	0.263	1.000
Fructose and mannose metabolism	2	0.011	0.867	0.300
Fatty acid biosynthesis	3	0.023	1	0.015
Glycolysis/gluconeogenesis	2	0.125	1	0.064
Galactose metabolism	3	0.133	1	0.063
Alanine, aspartate and glutamate metabolism	1	0.141	1	0.114
Nicotinate and nicotinamide metabolism	1	0.270	1	0.194

[a] Hits represents the number of metabolites in one pathway. [b] Holm P indicates the statistical *P* values that were further adjusted using the Holm–Bonferroni method for multiple tests. LA, whey at low altitude; HA, whey at high altitude.

tide sugar metabolism; phenylalanine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; fructose and mannose metabolism; fatty acid biosynthesis; glyoxylate and dicarboxylate metabolism. Of these, the amino sugar and nucleotide sugar metabolism pathway contained 6 differential metabolites: D-mannose 1-phosphate (FC = 14.31, $p < 0.05$),

alpha-D-glucose 1-phosphate (FC = 9.81, $p < 0.05$), alpha-D-galactose 1-phosphate (FC = 9.86, $p < 0.05$), *N*-acetyl-D-glucosamine 6-phosphate (FC = 0.58, $p < 0.05$), alpha-D-glucose (FC = 0.61, $p < 0.05$), and *N*-acetyl-D-glucosamine (FC = 0.61, $p = 0.06$). Alpha-D-glucose 1-phosphate, alpha-D-galactose 1-phosphate, and alpha-D-glucose were also involved in galactose metabolism. Tyrosine (FC = 2.03, $p < 0.05$) and phenylalanine (FC = 1.59, $p < 0.05$) were involved in tyrosine, phenylalanine, and tryptophan biosynthesis.

4. Discussion

4.1. Free fatty acids in milk whey at different altitudes

The main dairy buffalo raising regions in the Yunnan Province, China have very different altitudes, and our study showed that HA-milk whey showed higher levels of free fatty acids than that of LA- and MA-milk whey. Previous research has also shown that the FFA profiles of cows vary by altitude, and it was speculated that animal hypoxia occurs in high-altitude regions.[21] Animal milk are the main sources of medium-chain saturated FFAs.[22] Milk FFAs have beneficial effects on human health and disease prevention. Caprylic acid C8:0 and capric acid C10:0 have antiviral activities, and C8:0 can delay tumor growth.[23] Dodecanoic acid 12:0 is the most antiviral and antibacterial FFAs.[24]

Milk saturated FFAs have favorable effects on health, but high intake of C14:0 and C16:0 has been reported to increase blood cholesterol levels, resulting in heart disease and obesity.[25,26] Myristoleic acid C14:1, a unsaturated FFA, was present at high levels in HA-milk whey, which is consistent with a previous report that milk fat from low-altitude regions contained lower levels of unsaturated FFAs.[27] Oleic acid C18:1 is the most important monounsaturated FFA in milk and decreases plasma cholesterol, LDL-cholesterol, and triacylglycerol concentrations in humans.[28,29] Hippuric acid, a key metabolite, is associated with milk protein yield,[30] which also plays an important role in milk fermentation to produce benzoic acid.[31]

4.2. Amino acids in milk whey from different altitudes

Amino acids are the major nutritional components of milk. The level of the essential amino acids tyrosine in buffalo milk was similar to those of cow milk.[32] Tyrosine can eliminate free radicals and inhibit lipid peroxidation,[33,34] and were present in higher levels in LA-milk whey than in HA-milk whey, but no significant difference was observed between MA- and HA-milk whey. Tyramine is a biogenic trace amine that is synthesized from tyrosine and suppresses bacterial growth to protect milk protein.[35,36] It was present in greater amounts in LA-milk whey than HA-milk whey, but no significant difference was observed between MA- and HA-milk whey. Milk is abundant in the amino acid glutamine—an antioxidant that can increase immune performance—while the methionine content decreased as the amount of glutamine increased.[37] A previous study reported that a high glutamine content in cow milk improves the growth performance of pre-weaning piglets.[38]

4.3. Vitamins in milk whey from different altitudes

Buffalo's milk contains a higher content of vitamins but lower cholesterol than cow's milk.[39,40] Vitamin B6 is involved in many metabolic processes, such as protein metabolism, energy production, and normal nervous system function, by synthesizing the neurotransmitters: gamma-aminobutyric acid, dopamine, and serotonin.[41] Vitamin B6 is essential for normal growth and red blood cell synthesis and is enriched in vitamin digestion and absorption metabolic pathways.[42] Buffalo milk contains 10-fold higher amounts of vitamin B6 and 2-fold higher amounts of vitamin B3 than cow milk.[32] LA-milk whey contained higher levels of vitamin B6, but it did not significantly differ between MA- and HA-milk whey. Vitamin B3 mainly functions in the coenzyme forms of NAD and NADP, and enzymes containing NAD and NADP are involved in many biochemical reactions of carbohydrates, proteins, and lipids.[43,44] Vitamin B3 is also a key metabolite associated with milk protein yield,[30] which is involved in the metabolism of FFAs and the release of energy from carbohydrates, and it helps decrease the plasma levels of cholesterol and triglycerides.[45] Previous research has indicated that rumen fermentation and basal metabolism rate in Holstein dairy cow are changed by altitude,[46] and the differences in vitamin contents may also be caused by altitude. However, few studies have focused on the relationship between milk vitamin content and rumen fermentation in dairy buffaloes raised at different altitudes.

4.4. Carbohydrates in milk whey from different altitudes

D-Lactose is the most important carbohydrate in cow's milk, and it is constituted by glucose and galactose to supply the body with energy. Normally, cow's milk contains about 4.7% D-lactose, which provides 30% of newborn energy requirement.[47] The D-lactose content is nearly constant in buffalo milk and regulates the water content in milk during osmosis.[48,49] In our study, D-lactose was present at higher level in LA-milk whey than MA- and HA-milk whey, but few studies have reported the effect of altitude on the D-lactose content in buffalo milk.

4.5. Metabolic pathway analysis of differential metabolites

Amino sugar and nucleotide sugar metabolism, involved in carbohydrate energy metabolism,[50] was enriched in the three pairwise comparisons (LA-, MA-, and HA-milk whey). Eight significantly enriched metabolic pathways (galactose metabolism; phenylalanine metabolism; fructose and mannose metabolism; citrate cycle; phenylalanine, tyrosine and tryptophan biosynthesis; glyoxylate and dicarboxylate metabolism; tyrosine metabolism; glycerophospholipid metabolism) were detected in the two pairwise comparisons (LA *vs.* MA, LA *vs.* HA). Apart from glutamine, higher levels of cytidine and cytosine were observed in HA-milk whey that is responsible for the pyrimidine synthesis. These are involved in the metabolism of

pyrimidine to urea by gastrointestinal microorganisms. Cytidine is involved in complex lipids synthesis in milk secretion[51] and is the only exogenous cytosine-containing metabolite used to increase internal cytidine triphosphate.[52]

Cow reduced milk yield and protein content in high altitude region.[53] Previous researches have analyzed the composition of yak milk from different altitudes,[54] but few researches have focused on the impact of altitude on functional substances in buffalo milk. Dairy buffaloes are fed with the same rations and management in similar housing types, but the different geographic situations of LA-, MA- and HA-farms have different types of regional climates (subtropical monsoon, tropical monsoon, and north subtropical plateau monsoon, respectively). The different types of regional climates may affect the milk yield and composition, so it should be explored further for better understanding the effects of climatic conditions on functional substances in buffalo milk in future research.

5. Conclusion

In conclusion, a combination of non-targeted and targeted metabolomics approach was used to detect different metabolites in whey as a function of altitude. Owing to the biological functions of their most abundant components and nutritional function, HA-milk is available as the raw milk of the production of dairy products with high FFAs. While LA-milk is more suitable for the production of functional milk with high levels of amino acids, vitamin B6, and carbohydrates.

Author contributions

Jinhui Pu and Paramintra Vinitchaikul: Writing – original draft. Fulan Zhang: Formal analysis. Zhaobing Gu: Writing – review & editing. Huaming Mao: Project administration. All authors participated in reviewing the manuscript.

Conflicts of interest

All authors declared no conflict of interest.

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