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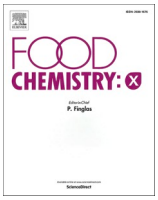

Analytical strategies based on untargeted and targeted metabolomics for the accurate authentication of organic milk from Jersey and Yak

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ABSTRACT

Organic milk has a high risk of food fraud as it can easily be adulterated with non-organic milk. This study aimed to identify metabolite markers for assessing the authenticity of organic milk from Jersey and Yak. In the untargeted strategy, ultra-high performance liquid chromatography-Q Exactive HF-X mass spectrometer coupled with chemometrics analysis was used to screen and identify tentative markers of organic milk from Jersey and Yak. In the targeted strategy, a quick and easy method of ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) was developed to quantify three markers. The peptide of Thr-Ala-Val and D-biotin were determined to be metabolite markers for distinguishing organic and non-organic Jersey milk, whereas trimethylamine *N*-oxide was determined to be a metabolite marker for distinguishing organic and non-organic Yak milk. These findings provide critical information to facilitate assessments of organic milk authenticity.

1. Introduction

Bovine milk, as a natural food with high nutritional value, is favored and strongly recommended as a high-quality protein source in dietary guidelines (Kang et al., 2022a; Nagpal et al., 2012). According to the type of production management system, bovine milk can be divided into organic and traditional milk. Compared to traditional milk, organic milk contains more omega-3 polyunsaturated fatty acids, which are important for the prevention of certain types of malignant diseases and autoimmune disorders (Mie et al., 2017; Mariamenatu, & Abdu, 2021). Organic milk is a highly safe product because the use of synthetic chemical substances, such as chemical fertilizers, pesticides, hormones, growth regulators, feed additives, and food additives, is strictly prohibited in production and processing. In recent years, sales of organic milk products have increased significantly in the market and are up to 50% more expensive than traditional milk (Chung et al., 2018). Driven by economic profits, some traders adulterate organic milk with relatively cheap non-organic milk, and this adulteration phenomenon reduces the product quality and damages the rights of consumers. In China, cow breeds producing organic milk mainly include Holstein, Jersey, and Yak, of which Jersey and Yak are minor dairy animals. Both Jersey and Yak milk are known as premium milk, with lower production levels and higher prices than that of Holstein milk. Moreover, the knowledge of Jersey and Yak organic milk is poor. Therefore, it is urgently required to protect the consumer from wrongly labeled Jersey and Yak organic milk.

To date, authenticity identification technologies for organic milk have mainly focused on stable isotope analysis. Carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) in milk can vary considerably depending on the cow's diet (Molkentin, 2013). The structure of pasture forage directly affects the ratio of C3 to C4 from the photosynthetic plants consumed in the dairy cow diet, resulting in differences of $\delta^{13}C$ value between organic and non-organic milk (Chung et al., 2014; Molkentin, 2013). Compared with manure allowed by organic farms, the $\delta^{15}N$ value of chemical synthetic nitrogen fertilizers is lower. Because organic farms do not use chemical synthetic fertilizers, the $\delta^{15}N$ value of organic milk was significantly different from conventional milk (Chung et al., 2014). Therefore, the $\delta^{13}C$ and $\delta^{15}N$ values were applied to distinguish organic and non-organic milk (Chung et al., 2018; Chung et al., 2014; Molkentin, 2009). However, the isotope ratio mass spectrometer is expensive, and the technology is difficult to translate by conventional instruments, which makes this technology difficult to use in actual detection. Metabolomics have attracted increasing attention because of their high selectivity for characteristic markers and high accuracy in detecting

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adulteration. According to recently published data, metabolomics have been confirmed for the identification of milk from different breeds of animals and different feeding systems based on untargeted metabolomics (Caboni et al., 2019; Ji et al., 2023; Fan et al., 2023; Qin et al., 2022; Rocchetti et al., 2022; Scano et al., 2014; Sen et al., 2021; Yang et al., 2016). However, to date, there is a lack of studies about the use of metabolomic methods to distinguish organic and non-organic milk, especially Jersey and Yak milk. Thus, we aimed to establish an analytical strategy to distinguish organic and non-organic milk (both Jersey and Yak milk) based on metabolic markers, which could fill the gap in the identification of Jersey and Yak organic milk.

In this study, we established untargeted and targeted metabolomics methods to reveal the authenticity of Jersey and Yak organic milk based on liquid chromatography-mass spectrometry (LC-MS). Specifically, high-resolution mass spectrometry combined with chemometrics was used to screen potential markers which are used to distinguish organic and non-organic milk (both Jersey and Yak milk), and a quantitative approach was developed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to verify the potential markers and determine content thresholds of markers.

2. Materials and methods

2.1. Chemicals and solvents

HPLC-grade methanol, acetonitrile, and water were purchased from Fisher Scientific (Pittsburgh, PA), HPLC-grade isopropanol was purchased from Merck (Darmstadt, Germany), and HPLC-grade formic acid was purchased from CNW (Shanghai, China). Trimethylamine *N*-oxide was obtained from Yuanye Bio-technology (Shanghai, China). Biotin was purchased from Solarbio (Beijing, China). Peptide of Thr-Ala-Val was synthesized by TG peptide (Nanjing, China). L-2-chlorophenylalanin was obtained from Adamas-beta (Shanghai, China). All standards had a purity of $\geq$ 95%.

2.2. Samples information

Twenty samples of each variety of milk were collected during July and September 2022, including organic Yak milk (OYM), non-organic Yak milk (NOYM), organic Jersey milk (OJM), and non-organic Jersey milk (NOJM). The OYM samples were collected from Lhasa city (Tibet, China). NOYM samples were collected from Lhasa city (Tibet, China) and Qilian County (Qinghai, China). OJM samples were collected from Hohhot (Inner Mongolia, China). NOJM samples were obtained from Hangzhou city (Zhejiang, China) and Hohhot (Inner Mongolia, China). After ultra-high temperature instant sterilization, all samples were sealed and packaged. Additionally, four different adulteration milk ratios were prepared by mixing 10, 20, 30, and 60% non-organic milk in organic milk (both Yak and Jersey milk). Five adulterated simulants were prepared for each type in Yak and Jersey milk. In total, 80 real samples and 40 adulterated simulants were finally obtained. The liquid samples were vacuum freeze-dried to a loose dry state.

2.3. Untargeted metabolomic analysis

2.3.1. Metabolite extraction

20 mg of milk powder, a 6 mm diameter grinding bead, and 400 μL methanol–water (4:1, v/v) solution with 0.02 mg/mL internal standard of L-2-chlorophenylalanin were added sequentially to a 2 mL centrifuge tube. The mixture was allowed to settle at −10 °C and ground for 6 min, then followed by treatment with 40 kHz ultrasound at 5 °C for 30 min. The extraction solution was placed at −20 °C for 30 min and centrifuged for 15 min (13,000 g, 4 °C). After the supernatant was dried with nitrogen, the obtained extracts were redissolved by 120 μL acetonitrile–-water (1:1, v/v) solution. The solution was vortexed for 30 s and sonicated for 5 min (5 °C, 40 kHz). After centrifugation for 15 min (13,000 g, 4 °C), the supernatant was collected for LC-MS analysis. In addition, 20 μL of supernatant was removed from each sample and mixed as a quality control (QC) sample.

2.3.2. Instrumentation

For untargeted metabolomics analysis, 3 μL of the sample was separated and analyzed by using UHPLC-Q Exactive HF-X Mass Spectrometer of Thermo Fisher Scientific equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm i.d. × 1.8 μm; Waters, Milford, USA). The mobile phases consisted of solvent A (0.1% formic acid in water: acetonitrile = 95:5, v/v) and solvent B (0.1% formic acid in acetonitrile: isopropanol: water = 47.5:47.5:5, v/v). The gradient used was: 0%-20% (B), 0–3 min; 20%-35% (B), 3–4.5 min; 35%-100% (B), 4.5–5 min; 100% (B), 5–6.3 min; 0% (B), 6.3–8 min. The flow rate was 0.4 mL/min, and the column temperature was 40 °C.

The mass spectrometric data was collected using a UHPLC -Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode. The optimal conditions were: sheath gas flow rate, 50 arb; aux gas flow rate, 13 arb; heater temperature, 425 °C; capillary temperature, 325 °C; normalized collision energy, 20–40-60 V rolling for MS/MS; the spray voltage was set at (+) 3500 V and (−) 3500 V, respectively; full MS resolution was 60,000, and MS/MS resolution was 7500; the range of the MS scan was from *m/z* 70 to 1050. Moreover, a quality control (QC) sample was randomly inserted into every 3 analysis samples to evaluate the repeatability and robustness of the analysis.

2.3.3. Data preprocessing and annotation

Raw data of LC-MS was imported into the Progenesis QI software (Waters, Milford, USA) for baseline filtering, peak identification, integration, retention time correction, and peak alignment. Subsequently, the MS and MS/MS mass spectra were matched with the metabolite database (the main databases were the HMDB (https://www.hmdb.ca/), Metlin (https://metlin.scripps.edu/), and Majorbio Database). The MS mass error was set to at least 10 ppm, while the metabolites were identified based on the secondary mass spectra matching score. Metabolites detected at least 80% in any set of samples were retained. At the same time, variables with relative standard deviation (RSD) > 30% of QC samples were discarded and response intensity of the mass spectrum peaks were log-transformed to obtain the final data matrix for subsequent analysis.

2.3.4. Differential metabolites analysis

Statistical analyzes were performed with R and Python packages, such as hierarchical cluster analysis, principal component analysis (PCA), least partial squares discriminant analysis (PLS-DA), student's *t*-test, and fold difference analysis. Differential metabolites were selected based on the variable importance in the projection (VIP) obtained by the PLS-DA model, the *p*-value of the student's *t* test, and fold change (FC). Then, these differential metabolites were summarized through metabolic enrichment and pathway analysis based on database search (KEGG, https://www. genome.jp/kegg/).

2.4. Targeted metabolomic analysis

2.4.1. Metabolite extraction

200.0 mg samples were placed into a 10 mL centrifuge tube containing 1.0 mL of water, vortexed until the milk powder was completely dissolved. After adding 4 mL of methanol/acetonitrile (1:1, v/v), the mixture was vortexed for 30 s, then followed by treated with 40 kHz ultrasound at 5 °C for 40 min. The extraction solution was placed at −20 °C for 30 min and centrifuged for 20 min (8500 rpm, 4 °C). Finally, the supernatant was collected for UPLC-MS/MS analysis.

2.4.2. Instrumentation

Quantification of the selected substances was performed on Agilent

1200 HPLC-6420 Triple Quad LC/MS (Agilent Technologies) equipped with a degasser, binary pump, auto-sampler, and column oven. Separation of the analytes was performed using an Agilent ZORBAX SB-C18 column (150 × 4.6 mm, 5 μm) at 40 °C. The mobile phases consisted of solvent A (0.1% formic acid in water: acetonitrile = 95:5, v/v) and solvent B (0.1% formic acid in acetonitrile: isopropanol: water = 47.5:47.5:5, v/v). The gradient used was: 5%-10% (B), 0–3 min; 10% (B), 3–4 min; 10%-50% (B), 4–5 min; 50%-100% (B), 5–6 min; 100% (B), 6–7.5 min; 100%-35% (B), 7.5–9 min; 35%-5% (B), 9–10 min; 5% (B), 10–12 min. The injection volume was 5 μL.

Detection was carried out in the positive ion mode with a capillary voltage of 4.0 kV. The other instrumental parameters were set as follows: drying gas temperature, 350 °C; drying gas flow, 10 L/min; and nebulizer, 40 psi. Multiple Reaction Monitoring (MRM) parameters for the three target analytes were summarized in Table 1.

2.4.3. Statistical analysis

Statistical analyzes were performed with R packages, while one-way analysis of variance (ANOVA) and box diagram were employed to confirm significant differences and content thresholds of the final markers in organic and non-organic milk with $p < 0.05$.

3. Results and discussion

3.1. Non-targeted metabolic profiling of four cultivars of cow milk

We used the internal standard of L-2-chlorophenylalanin to assess the stability of the experimental procedure and ensure data quality. The z-score values of the internal standard were within two times the standard deviation, which indicated the stable data. Therefore, we did not normalize the data (e.g., sum normalization) and directly used the ion intensity for the subsequent statistical analysis. The QC samples were evaluated to ensure the repeatability and robustness of the whole sample set (Broadhurst et al., 2018): Total ion chromatograms (TICs) of the QC sample in the positive and negative ion scanning modes were shown in Fig. S1, the peak shape and distribution are relatively uniform; the QC samples showed a tight cluster in the PCA plot (Fig. S1). The above assessment results for the QC samples demonstrated the robustness of the analytical procedure and the reliability of the data obtained. Totally, 7465 and 2582 features were detected, respectively, in the positive and negative ion mode, of which 730 and 305 metabolites were annotated, respectively. Since the metabolites identified in the positive ion model are more abundant, we selected the metabolite profiles of the positive ion mode for subsequent analyses.

To characterize the similarity between four cultivars of cow milk, hierarchical cluster analysis of the metabolite ion features was carried out and represented in the form of a heatmap. The color coding from red to blue indicates their content from high to low. In Fig. 1, a significant color distribution is observed, indicating that different milk samples presented differential metabolite profiles. Four milks were clearly clustered into two different sections, revealing significant differences between Yak and Jersey milk. Not surprisingly, organic and non-organic milk from the same breed of cow were clustered into one group for both Jersey and Yak, which meant similarity of their metabolite profiles. However, the significant color distribution between organic and non-organic milk also revealed their differences. It is very meaningful to characterize the differential metabolites between organic and non-organic milk to prevent food fraud.

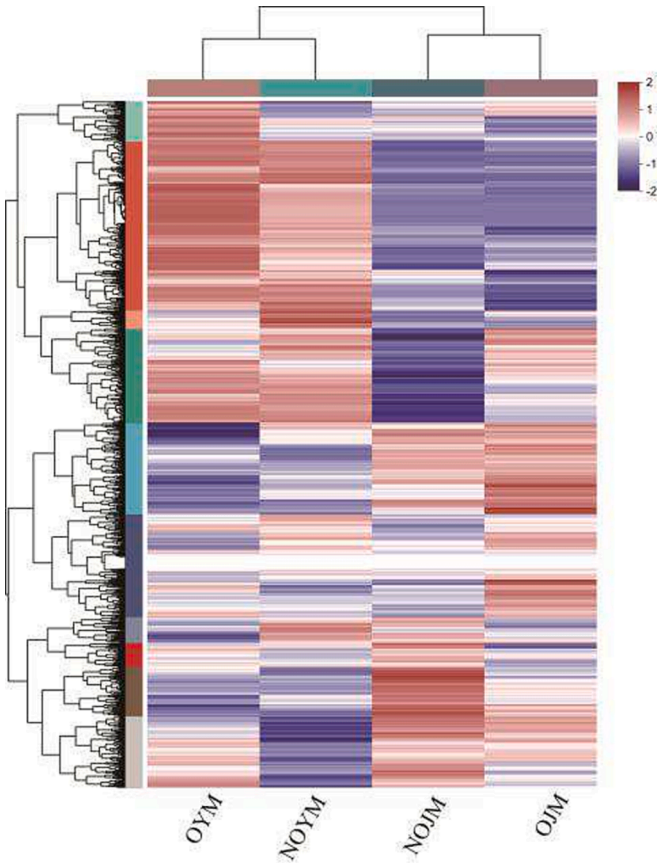

Fig. 1. Heatmap of cluster analysis for metabolite profiles in four cultivars of cow milk (organic Yak milk (OYM), non-organic Yak milk (NOYM), organic Jersey milk (OJM), and non-organic Jersey milk (NOJM)).

3.2. Metabolic characteristics of organic Jersey milk

To dissect the differences in metabolite composition between organic and non-organic Jersey milk (OJM and NOJM), unsupervised PCA and supervised PLS-DA were carried out. The results of PCA were shown in Fig. 2a, OJM and NOJM samples exhibited a distinct separation. The clear clustering indicated that there was a significant difference between the OJM and NOJM. The first two PCs showed 29.8 % and 24.9 % variances, respectively. The results of PLS-DA were similar to PCA

Table 1
Molecular weight and optimized MS parameters for the three potential markers in the ESI + mode.

Compound	Retention time (min)	Precursor ions (*m/z*)	Product ions (*m/z*)	Fragmentor (V)	Collision energy (eV)
Trimethylamine *N*-oxide	3.29	76.1	42.3	35	53
			58.3*	21	
			59.3	9	
Thr-Ala-Val	3.74	290.3	101.1	25	83
			145	13	
			173*	9	
Biotin	9.25	245.3	97.1	33	93
			123	33	
			227.1*	13	

* Quantification ion.

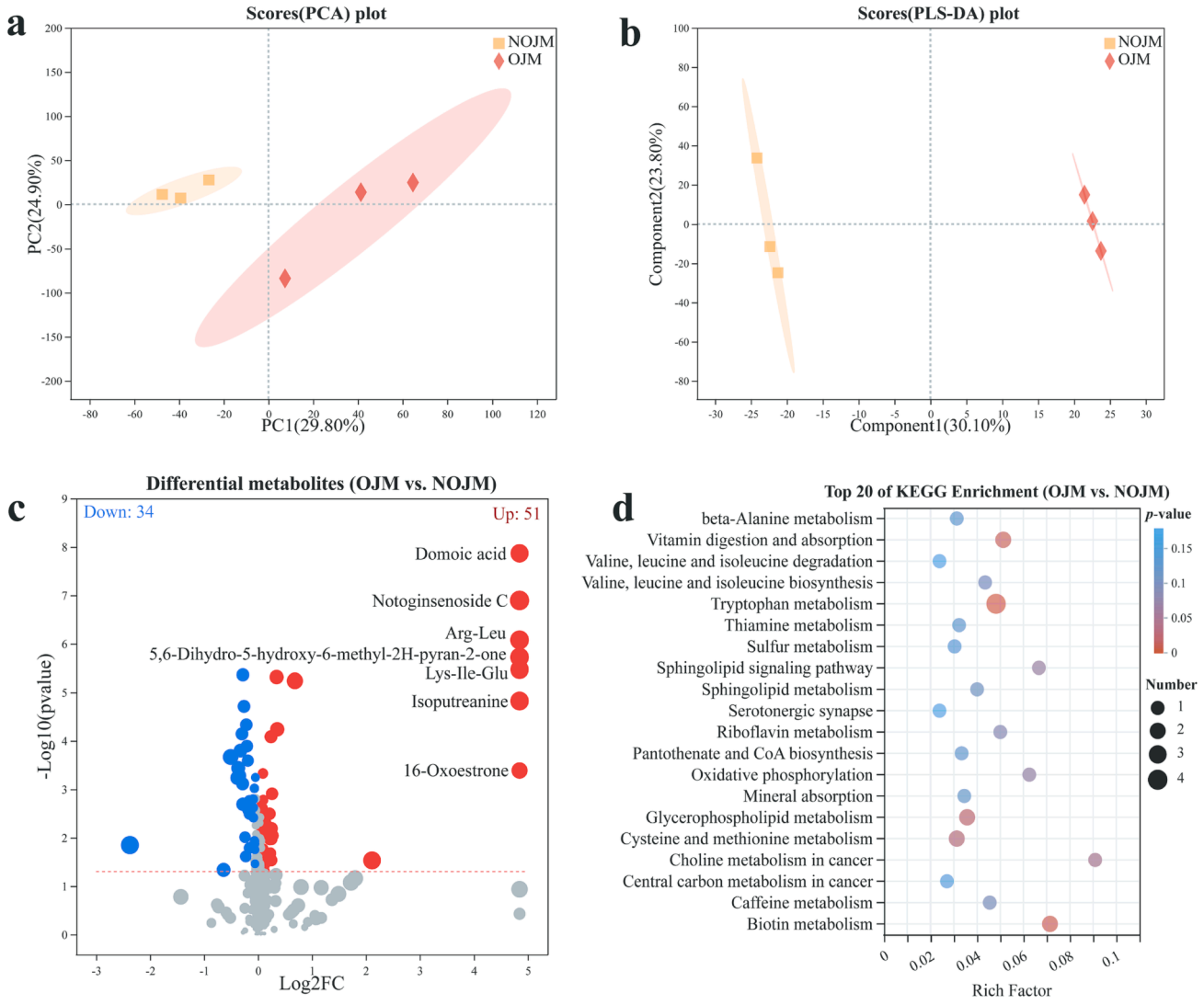

Fig. 2. Multivariate statistical analysis of metabolites in Jersey milk. a. PCA of metabolites from the OJM and NOJM groups; b. supervised PLS-DA of metabolites from the OJM and NOJM groups; c. volcano plots of differential metabolites between OJM and NOJM groups; d. metabolite pathway analysis on differential metabolites from the pairwise comparison "OJM vs. NOJM".

(Fig. 2b), the well-separated clusters of OJM and NOJM samples exhibited that dairy farming management systems had a strong influence on the metabolite profile of Jersey milk. The quality of PLS-DA model developed was confirmed by a good R^2Y of 1.0 and Q^2 of 0.90 (Wang, Li, Chen, & Zhou, 2022). The variable importance in projection (VIP) analysis based on PLS-DA model was applied to identify the important metabolites for distinguishing OJM and NOJM groups (Kang et al., 2022b), and there were 1517 metabolites with VIP scores > 1.

To obtain differential metabolites of the OJM and NOJM samples, the *p*-value and fold change (FC) of univariate analysis were further performed on these 1517 metabolites (Wang et al., 2022). Selection criteria included VIP scores > 1, *p*-value < 0.05, and FC > 1 or < 1, and the results were visualized using a volcano plot. Finally, a total of 509 metabolites with significant differences were screened and 85 of them were further annotated (Fig. 2c and Table 2), containing primarily 23 amino acids, peptides, and analogues, 3 fatty acids and conjugates, 3 indoles, 2 triterpenoids, 2 glycerophosphocholines, and 2 diterpenoids. It was concluded that amino acids, peptides, and analogues are the largest different chemicals. These 85 differential metabolites were selected as the potential markers to distinguish NOJM from OJM. In Fig. 2c, the red dots represent 51 differential metabolites with higher content in OJM and the blue dots represent 34 differential metabolites with higher content in NOJM. Interestingly, the levels of seven most up-regulated metabolites were above 28 times higher than those in NOJM, namely domoic acid, notoginsenoside C, Lys-Ile-Glu, isoputreanine, 5,6-dihydro-5-hydroxy-6-methyl-2H-pyran-2-one, Arg-Leu, and 16-oxoestrone. Notoginsenoside C is a bioactive saponins from the root of panax notoginseng (Yoshikawa et al., 1997); the 5,6-dihydro-5-hydroxy-6-methyl-2H-pyran-2-one was a natural product from fern species (Gyeltshen et al., 2022); their high content in OJM reflects the characteristics of organic pasture. Most peptides in differential metabolites exhibited up-regulation in OJM, such as kyotorphin, Arg-Asn, Ile-Ile-Asn, Ile-Ile-Val, Val-Leu-Pro-Val-Pro, Thr-Ala-Val, Lys-Ile-Glu, and Arg-Leu. Previous studies have shown that kyotorphin, first isolated from bovine brain in 1979, is an endogenous analgesic neuropeptide with anti-inflammatory and antibacterial activity (De Andrade et al., 2020); Val-Leu-Pro-Val-Pro is an angiotensin converting enzyme (ACE) inhibitory peptide that plays a very important role in blood pressure regulation (Lei et al., 2008). These results provided some basis for confirming that OJM has a better nutritional value than NOJM.

In addition, a pathway enrichment analysis was performed on these differential metabolites to explain the relationship between variation in the chemical composition and dairy farming management systems. The enrichment background in KEGG annotation was all metabolites of dairy

Table 2
The information of all potential differential metabolites from the pairwise comparison "OJM vs. NOJM".

ID	Metabolite	Formula	M/Z	Adducts	Regulate	VIP	FC	*p*-value
1	LysoPC (18:3(6Z,9Z,12Z)/0:0)	C26H48NO7P	518.3224	M + H, M + Na	up	1.59	1.09	0.01
2	Cholesteryl glucoside	C33H56O6	566.4391	M + NH4, M + Na	up	1.08	1.04	0.03
3	L-Serine	C3H7NO3	147.076	M + H-H2O, M + ACN + H	up	1.15	1.05	0.00
4	Isoputreanine	C7H16N2O2	161.1279	M + H	up	4.56	28.76	0.00
5	Nitrilotriacetic acid	C6H9NO6	424.1198	2 M + ACN + H	up	1.09	1.05	0.02
6	Triethylcitrate	C12H20O7	299.1091	M + H, M + NH4, M + Na	down	1.08	0.96	0.02
7	Aminomalonic acid	C3H5NO4	152.0562	M + CH3OH + H	up	1.01	1.03	0.01
8	Triisopropanolamine	C9H21NO3	192.1589	M + H	down	2.28	0.82	0.00
9	Histidylleucine	C12H20N4O3	269.1599	M + H	up	1.55	1.11	0.00
10	*N*-Acetyl-L-Histidine	C8H11N3O3	198.0871	M + H	up	2.05	1.20	0.00
11	6-Sulfanilamidoindazole	C13H12N4O2S	253.0535	M + H-2H2O	up	4.17	4.34	0.03
12	5,6-Dihydro-5-hydroxy-6-methyl-2H-pyran-2-one	C14H12O3	292.0951	M + ACN + Na	up	4.66	28.76	0.00
13	1-[2-Methyl-3-(methylsulfanyl)propanoyl]pyrroli-dine-2-carboxylate	C10H17NO3S	276.0639	M + 2Na-H	up	1.09	1.04	0.00
14	Thr-Ala-Val	C12H23N3O5	290.1699	M + H	up	2.64	1.27	0.00
15	4-Hydroxy-4-(3-pyridyl)-butanoic acid	C9H11NO3	199.107	M + NH4	down	1.07	0.96	0.01
16	Kyotorphin	C15H23N5O4	338.1808	M + H	up	2.17	1.19	0.01
17	Tetrahydroharmol	C12H14N2O	203.1173	M + H	down	2.63	0.79	0.00
18	Domoic acid	C15H21NO6	334.1242	M + Na	up	4.37	28.76	0.00
19	2,3-Methyleneglutaric acid	C7H8O4	121.0283	M + H-2H2O	up	1.11	1.06	0.01
20	Alpha-Methyltryptamine	C11H14N2	175.1225	M + H	up	1.11	1.05	0.00
21	5-Methylthioribose	C6H12O4S	222.0789	M + ACN + H	down	4.34	0.19	0.01
22	Arg-Asn	C10H20N6O4	577.3176	2 M + H	up	2.71	1.28	0.00
23	LysoPS (18:0/0:0)	C24H48NO9P	526.3123	M + H, M + Na, M + 2Na-H	up	1.52	1.07	0.01
24	S-4-Hydroxymephenytoin	C12H14N2O3	199.086	M + H-2H2O	down	2.08	0.87	0.00
25	5-Hydroxy-L-tryptophan	C11H12N2O3	243.0758	M + Na	up	1.44	1.07	0.00
26	Indoleacetic acid	C10H9NO2	217.0966	M + ACN + H	down	2.11	0.86	0.00
27	Nalidixic acid	C12H12N2O3	215.081	M + H-H2O	down	1.89	0.90	0.00
28	D-Biotin	C10H16N2O3S	245.0947	M + H	down	2.49	0.80	0.00
29	Tryptophol	C10H11NO	144.0804	M + H-H2O	down	1.82	0.86	0.02
30	Butyl 4-aminobenzoate	C11H15NO2	158.096	M + H-2H2O	down	1.80	0.90	0.02
31	Alpha-Linolenoyl ethanolamide	C20H35NO2	344.2532	M + Na	up	1.46	1.06	0.00
32	10-Deacetylbaccatin III	C29H36O10	562.2679	M + NH4	up	1.50	1.10	0.04
33	Undecylenic acid	C11H20O2	202.1795	M + NH4	down	2.59	0.64	0.05
34	Panaxydol linoleate	C35H54O3	540.4453	M + NH4	up	1.33	1.06	0.05
35	Glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate	C21H36O9	415.235	M + H-H2O	up	1.93	1.19	0.01
36	Lupeol acetate	C32H52O2	507.364	M + K	down	1.18	0.96	0.00
37	Polypodine B	C27H44O8	535.2686	M + K	up	1.10	1.04	0.01
38	Cer (d17:1/PGJ2)	C37H63NO5	584.4709	M + H-H2O	up	1.55	1.09	0.05
39	Ganoderic acid F	C32H42O9	609.2479	M + K	up	1.05	1.04	0.01
40	16-Oxoestrone	C18H20O3	569.2914	2 M + H	up	3.46	28.76	0.00
41	Ile-Ile-Val	C17H33N3O4	344.2532	M + H, M + K, 2 M + H, M + Na	up	1.58	1.09	0.01
42	1-(7Z,10Z,13Z,16Z-docosatetraenoyl)-*glycero*-3-phosphate	C25H43O7P	528.3067	M + ACN + H	up	1.98	1.19	0.03
43	Cer (d18:2(4E,14Z)/20:5 (7Z,9Z,11E,13E,17Z)–3OH (5,6,15))	C38H63NO6	630.4767	M + H	down	1.06	0.97	0.00
44	KAPA	C9H17NO3	188.1276	M + H, M + Na, M + H-H2O, M + H-2H2O	up	1.22	1.05	0.03
45	Ser-Phe	C12H16N2O4	217.0968	M + H-2H2O, M + K	down	2.20	0.84	0.00
46	PC (10:0/0:0)	C18H38NO7P	412.2443	M + H	down	1.02	0.96	0.03
47	Xanthosine	C10H12N4O6	267.0731	M + H-H2O	up	1.19	1.06	0.00
48	PGP (PGE2/18:3 (9Z,12Z,15Z))	C44H74O16P2	472.2226	M + H + Na, M + 2Na	up	1.07	1.04	0.04
49	Cyclo (aspartylleucylthreonylvalyltyrosylphenylalanylglycyl)	C39H53N7O11	809.3983	2 M + 3H2O + 2H	up	1.08	1.04	0.03
50	Val-Leu-Pro-Val-Pro	C31H53N7O8	652.4009	M + 2H, M + H	up	1.89	1.17	0.02
51	Diphenylamine	C12H11N	187.1223	M + H, M + NH4	down	1.95	0.90	0.00
52	2-(4-(Piperidin-3-yl) phenyl)–2H-indazole-7-carboxamide	C19H20N4O	663.3153	2 M + Na	up	1.99	1.16	0.00
53	(R)-beta-Aminoisobutyric acid	C4H9NO2	245.0914	2 M + K	up	1.40	1.07	0.01
54	*N*-Acetyl-D-tryptophan	C13H14N2O3	247.107	M + H	up	1.38	1.07	0.02
55	3-Methyl-*N*-phenylaniline	C13H13N	201.138	M + H, M + NH4	down	2.33	0.82	0.00
56	Notoginsenoside C	C54H92O25	571.3022	M + 2H	up	4.95	28.76	0.00
57	Methyl 4-amino-5-ethyl-3-thiophenecarboxylate	C8H11NO2S	186.0577	M + H	down	1.24	0.94	0.00
58	Quinaldic acid	C10H7NO2	215.081	M + ACN + H	down	1.95	0.88	0.00
59	Carbazole	C12H9N	185.1068	M + NH4	down	1.88	0.88	0.00
60	5-Hydroxyindoleacetaldehyde	C10H9NO2	217.0967	M + ACN + H	down	3.27	0.70	0.00
61	1,5-Naphthalene diisocyanate	C12H6N2O2	243.0758	M + CH3OH + H	up	1.32	1.07	0.01
62	Taxine B	C33H45NO8	584.3174	M + H	up	2.13	1.20	0.01
63	Flavin Mononucleotide	C17H21N4O9P	457.1105	M + H	down	1.32	0.94	0.00
64	Riboflavin cyclic-4′,5′-phosphate	C17H19N4O8P	439.1	M + H	up	1.14	1.05	0.03
65	Fusaric Acid	C10H13NO2	197.1279	M + NH4	up	2.21	1.18	0.00
66	Ile-Ile-Asn	C16H30N4O5	359.2277	M + H	up	1.18	1.06	0.03
67	Harmalol	C12H12N2O	201.1017	M + H	down	2.60	0.78	0.00
68	1,2,5,6-Tetrahydro-4H-pyrrolo[3,2,1-*ij*]quinolin-4-one	C11H11NO	174.0909	M + H	down	2.62	0.77	0.00
69	3-Indolebutyric acid	C12H13NO2	186.0908	M + H-H2O	down	2.22	0.82	0.00
70	Methylene bisacrylamide	C7H10N2O2	326.181	2 M + NH4	up	1.38	1.07	0.00

(*continued on next page*)

Table 2 (*continued*)

ID	Metabolite	Formula	M/Z	Adducts	Regulate	VIP	FC	*p*-value
71	2-(1-Naphthyl) acetamide	C12H11NO	203.1172	M + NH4	down	2.18	0.81	0.00
72	Norvaline	C5H11NO2	100.0758	M + H-H2O	down	1.12	0.95	0.02
73	4-Methyl-5-Thiazoleethanol	C6H9NOS	144.0474	M + H	up	1.12	1.06	0.05
74	Tetrahydrouridine	C9H16N2O6	271.0914	M + Na	down	1.11	0.94	0.01
75	2-Isopropyl-3-oxosuccinate	C7H10O5	157.0493	M + H-H2O	down	1.78	0.85	0.01
76	Butenylcarnitine	C11H19NO4	230.138	M + H	up	1.23	1.06	0.00
77	Arg-Leu	C12H25N5O3	288.2019	M + H	up	4.69	28.76	0.00
78	5′-Deoxyadenosine	C10H13N5O3	252.1083	M + H	up	3.51	1.61	0.00
79	Lys-Ile-Glu	C17H32N4O6	389.238	M + H	up	4.50	28.76	0.00
80	D-4′-Phosphopantothenate	C9H18NO8P	300.0832	M + H	down	1.09	0.96	0.01
81	4-Guanidinobutanoic Acid	C5H11N3O2	146.092	M + H	up	1.51	1.10	0.02
82	Mimosine	C8H10N2O4	216.0973	M + NH4	up	1.98	1.17	0.01
83	Prenyl arabinosyl-(1->6)-glucoside	C16H28O10	403.1545	M + Na	down	1.12	0.96	0.01
84	Calcium pantothenate	C18H32CaN2O10	477.174	M + H, M + Na	down	1.01	0.96	0.01
85	Methyl methacrylate	C5H8O2	218.138	2 M + NH4	down	1.22	0.94	0.00

and the results were represented by a bubble plot (Fig. 2d), in which the abscissa is rich factor (x/y, the number of differential metabolites in the corresponding metabolic pathway / the number of total metabolites in this pathway); the color and size of the bubble indicate the *p*-value and the number of differential metabolites. We found that differential metabolites between OJM and NOJM were assigned in 31 metabolic pathways, of which eight main pathways with significant enrichment were determined ($p < 0.05$): biosynthesis of cofactors, tryptophan

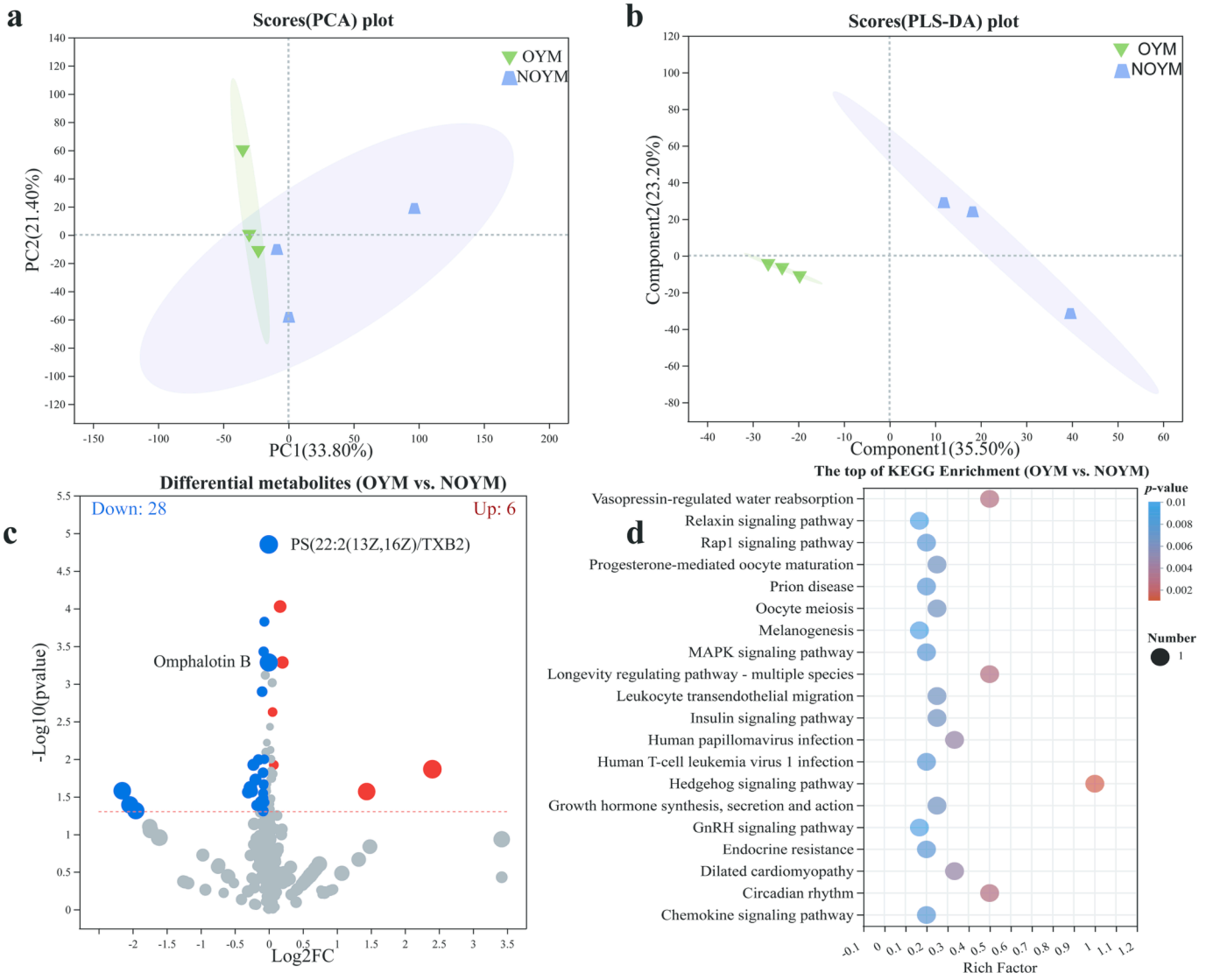

Fig. 3. Multivariate statistical analysis of metabolites in Yak milk. a. PCA of metabolites from the OYM and NOYM groups; b. supervised PLS-DA of metabolites from the OYM and NOYM groups; c. volcano plots of differential metabolites between OYM and NOYM groups; d. metabolite pathway analysis on differential metabolites from the pairwise comparison "OYM vs. NOYM".

metabolism, cysteine and methionine metabolism, ABC transporters, glycerophospholipid metabolism, vitamin digestion and absorption, biotin metabolism, and choline metabolism in cancer. These eight pathways contributed to the metabolic differences between OJM and NOJM. It was also concluded that the organic feeding management system was found to possess a high impact on these metabolic pathways of Jersey. It is noteworthy that tryptophan metabolism was greatly affected and four differential metabolites, including tryptophol, indoleacetic acid, 5-hydroxyindoleacetaldehyde, and 5-hydroxy-L-tryptophan, were enriched. Amino acid metabolism pathways composed of valine, leucine, isoleucine, glycine, serine, threonine, arginine, proline, cysteine, methionine, and tryptophan may be a possible biological process for the different chemicals relating to the forage structure. Similarly, a previous study has shown that the arginine biosynthesis and some amino acid metabolisms of dairy cow are significantly affected by different feeding strategies (based on corn silage as the main ingredient) (Rocchetti et al., 2022). As mentioned above, these results could provide new insights into the effects of organic production systems on metabolism of Jersey.

3.3. Metabolic characteristics of organic Yak milk

To dissect the differences in metabolite composition between organic and non-organic Yak milk (OYM and NOYM), unsupervised PCA and supervised PLS-DA were carried out. The results of PCA were shown in Fig. 3a, OYM and NOYM samples exhibited a large degree of overlapping, which was indicative of their similarity. But the two groups had a tendency to separate, and the first two PCs showed 33.8 % and 21.4 % variances, respectively. To further determine the metabolites to distinguish NOYM from NOYM samples, the PLS-DA model was established (Fig. 3b), which showed well repeatability of samples within the same group and significant separation degree between OYM and NOYM. The quality of PLS-DA model developed was confirmed by a good R^2Y of 0.996 and Q^2 of 0.918 (Shi et al., 2022). The VIP analysis based on PLS-DA model was applied to identify the important metabolites for distinguishing OYM and NOYM groups (Kang et al., 2022a), and there were 1537 metabolites with VIP scores > 1.

To obtain the differential metabolites of OYM and NOYM samples, the *p*-value and FC of univariate analysis were further performed on these 1537 metabolites. Selection criteria included VIP scores > 1, *p*-value < 0.05, and FC > 1 or < 1, and the results were visualized using a volcano plot. Finally, a total of 156 metabolites with significant differences were screened and 34 of them were annotated (Fig. 3c and Table 3), mainly including 8 amino acids, peptides, and analogues, 3 carbohydrates and carbohydrate conjugates, and 2 carbonyl compounds. It was concluded that amino acids, peptides, and analogues were the largest different chemicals between OYM and NOYM, which is similar to Jersey milk. In Fig. 3c, the red dots represent 6 differential metabolites with higher content in OYM, and the blue dots represent 28 differential metabolites with higher content in NOYM. The top 2 most up-regulated metabolites in OYM were 2′-deoxyadenosine 5′-phosphate and hydroxykynurenine, their FC values are 5.29 and 2.71, respectively. Interestingly, the FC values of omphalotin B and PS (22:2(13Z,16Z)/TXB2) are 0 because their response intensity in OYM is lower than the instrument detection limit. Omphalotin B, a bioactive ingredient, is a nematicidal cyclic dodecapeptides which contain oxidised glycine, valine, isoleucine, and tryptophan, and Omphalotin B was successfully isolated from the basidiomycete omphalotus olearius in the early phase (Büchel et al., 1998). All peptides in differential metabolites exhibited down-regulation in OYM, such as Lys-Leu, Pro-Ile-Phe, Arg-Leu,

Table 3
The information of all potential differential metabolites from the pairwise comparison "OYM vs. NOYM".

ID	Metabolite	Formula	M/Z	Adducts	Regulate	VIP	FC	*p*-value
1	Trimethylamine *N*-oxide	C3H9NO	76.07601	M + H	down	1.69	0.86	0.01
2	Dihydroxyacetone	C3H6O3	244.0785	2 M + ACN + Na	down	1.10	0.96	0.01
3	Deoxycytidine	C9H13N3O4	228.0971	M + H	down	1.18	0.93	0.05
4	2′-Deoxyadenosine 5′-phosphate	C10H14N5O6P	332.0741	M + H	up	3.95	5.29	0.01
5	Capsicoside C1	C44H72O18	445.2426	M + 2H	down	3.40	0.26	0.05
6	Alpha-Methyltryptamine	C11H14N2	175.1225	M + H	up	1.79	1.15	0.00
7	Hydroxykynurenine	C10H12N2O4	207.0758	M + H-H2O	up	3.50	2.71	0.03
8	Tiglylglycine	C7H11NO3	158.0807	M + H	up	1.10	1.05	0.01
9	PS (22:2 (13Z,16Z)/TXB2)	C48H84NO14P	487.7691	M + 2Na	down	3.94	0.00	0.00
10	PG (i-12:0/a-13:0)	C31H61O10P	324.1963	M + H + Na	down	1.02	0.95	0.05
11	Leu-Pro	C11H20N2O3	211.1435	M + H-H2O	down	1.17	0.94	0.03
12	CDP-DG (a-15:0/20:5 (6E,8Z,11Z,14Z,17Z)–OH (5))	C47H77N3O16P2	1015.495	2 M + 3H2O + 2H	down	1.31	0.94	0.02
13	L-Pyridosine	C12H18N2O4	255.1331	M + H, M + Na, M + H-H2O, M + H-2H2O	up	1.83	1.12	0.00
14	Cyclic adenylic acid	C10H12N5O6P	330.0585	M + H, M + Na	up	1.06	1.04	0.00
15	Cer (d16:1/20:5 (7Z,9Z,11E,13E,17Z)–3OH (5,6,15))	C36H61NO6	586.4505	M + H-H2O	down	1.57	0.91	0.04
16	Cer (d18:2(4E,14Z)/20:5 (7Z,9Z,11E,13E,17Z)–3OH (5,6,15))	C38H63NO6	630.4767	M + H	down	2.13	0.83	0.02
17	Hericenone D	C37H58O6	616.4607	M + NH4	down	1.22	0.95	0.03
18	4-Oxo-1-(3-pyridyl)-1-butanone	C9H9NO2	164.0701	M + H, M + NH4	down	1.33	0.93	0.00
19	PE (LTE4/P-18:1 (11Z))	C46H81N2O10PS	898.5479	2 M + 3H2O + 2H	down	1.69	0.87	0.02
20	PS (18:1 (12Z)–2OH (9,10)/14:0)	C38H72NO12P	405.7277	M + 2Na	down	1.45	0.89	0.04
21	Pro-Ile-Phe	C20H29N3O4	376.2217	M + H	down	3.44	0.24	0.04
22	[6]-Gingerdiol 4′-O-beta-D-glucopyranoside	C23H38O9	491.2849	M + CH3OH + H	down	1.39	0.92	0.04
23	Methyl 4-amino-5-ethyl-3-thiophenecarboxylate	C8H11NO2S	186.0577	M + H	down	1.25	0.92	0.04
24	PS (14:0/20:4 (8Z,11Z,14Z,17Z)–2OH (5S,6R))	C40H70NO12P	405.7277	M + H + Na	down	1.83	0.84	0.03
25	Phe-Pro	C14H18N2O3	263.1381	M + H	down	1.06	0.95	0.02
26	Salsolinol	C10H13NO2	162.0923	M + H-H2O	down	1.43	0.90	0.01
27	Indole-3-Carboxylic Acid	C9H7NO2	162.0545	M + H	down	1.25	0.95	0.00
28	CHEBI: 69,439	C10H16N2O2	197.1279	M + H	down	1.21	0.95	0.05
29	Omphalotin B	C74H123N13O18	752.9563	M + H + Na, M + 2Na	down	3.78	0.00	0.00
30	DOPA sulfate	C9H11NO7S	242.011	M + H-2H2O	down	1.06	0.95	0.02
31	Pyridine *N*-oxide glucuronide	C11H14NO7+	314.1084	M + ACN + H	down	1.05	0.96	0.04
32	Arg-Leu	C12H25N5O3	288.2019	M + H	down	1.83	0.81	0.03
33	Lys-Leu	C12H25N3O3	260.196	M + H	down	3.56	0.22	0.03
34	Draflazine	C30H33Cl2F2N5O2	586.1934	M + H-H2O	down	1.15	0.96	0.00

omphalotin B; additionally, no significant advantage was observed in nutrient substances in OYM. According to the above results, we can not confirm whether organic yak milk has more nutritional value than non-organic yak milk. Differences in metabolites may be produced by different genetics and different regional environments, and the above results show that the metabolite compositions of OYM and NOYM are more similar compared to "OJM vs. NOJM", which can be explained by the fact that both organic and non-organic feeding pastures for Yaks are located in highlands with harsh environments.

In addition, a pathway enrichment analysis was performed on these differential metabolites to explain the relationship between variation in the chemical composition and dairy farming management systems. The enrichment background in KEGG annotation was all metabolites of Yak. We found that the differential metabolites between OYM and NOYM were involved in 80 metabolic pathways, of which 69 pathways were significantly enriched ($p < 0.05$) and the top 20 are shown in Fig. 3d. Most pathways involved the endocrine systems. It was revealed that four pathways with maximum enrichment degree, namely hedgehog signaling pathway, vasopressin - regulated water reabsorption, longevity regulating pathway-multiple species, and circadian rhythm. These results suggested that the organic feeding management system was found to possess a high impact on these metabolic pathways of Yak. Previous research has shown that the metabolic pathways of nucleic acid derivatives (purines and pyrimidines) of cow are significantly affected by feed composition (Rocchetti et al., 2022). In this study, we found that purine and pyrimidine metabolism in Yak are also affected by the organic feeding system. Furthermore, we found that some metabolic pathways affected by organic feeding strategies are shared in Yak and Jersey, such as serotonergic synapse, pyrimidine metabolism, chemical carcinogenesis - DNA adducts, ABC transporters, purine metabolism, metabolism of xenobiotics by cytochrome P450, and glycerophospholipid metabolism. There is currently a lack of focus on the metabolomics of Yak milk, especially organic yak milk, and the results of this study could provide new insights into the metabolite composition of Yak milk.

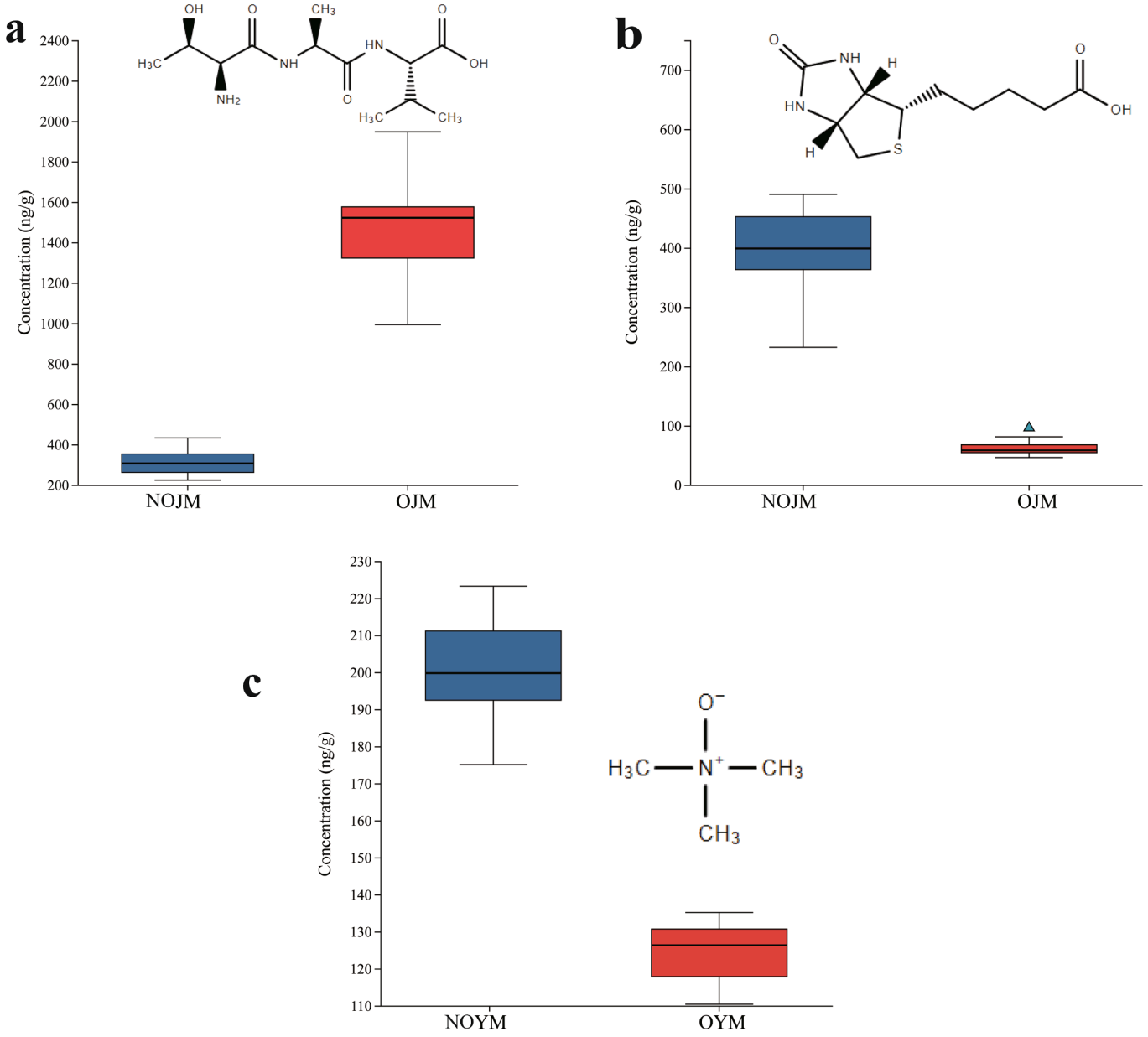

Fig. 4. Quantitative results for markers in authentic NOJM, OJM, NOYM, and OYM samples. a. Peptide of Thr-Ala-Val, b. D-biotin, and c. trimethylamine *N*-oxide.

3.4. Verification of markers using real milk samples

The utility of potential markers was further determined using authentic OJM, NOJM, OYM, and NOYM samples. In this study, the content thresholds of three candidate markers were quantified. The peptide of Thr-Ala-Val had contents of 223.78–432.38 ng/g and 993.06–2137.86 ng/g in NOJM and OJM samples, respectively. Furthermore, D-biotin contents ranged from 232.16 to 676.08 ng/g in NOJM samples and from 46.17 to 96.29 ng/g in OJM samples. It is very apparent that peptide of Thr-Ala-Val was determined to be a marker of OJM, while D-biotin was determined to be a marker of NOJM (Fig. 4a, 4b). D-Biotin, also known as coenzyme R and vitamin H or B7, has been recognized as an essential nutrient; it participates as a cofactor in gluconeogenesis, fatty acid synthesis and branched chain amino acid catabolism (Gravel & Narang, 2005). Previous research demonstrated that biotin was abundant in milk and our finding further indicated biotin was more abundant in NOJM than OJM. To further validate the detection sensitivity of markers, the adulterated simulants were prepared by mixing 10, 20, 30, and 60% NOJM in OJM. The content of D-biotin in the adulterated simulants did not fall in the range of that in OJM when the percentage of NOJM was up to 20%. Thus, as little as 20% adulteration of NOJM could be identified using D-biotin as the judgment index. If the percentage of NOJM in adulterated simulants exceeded 60%, the content of Thr-Ala-Val in adulterated simulants fell outside the scope of that in OJM.

NOYM and OYM had a trimethylamine *N*-oxide of 175.07–223.21 and 110.41–135.17 ng/g, respectively (Fig. 4c). In dairy cows, dietary choline, betaine, and levocarnitine are all degraded by rumen microorganisms to trimethylamine, which is then endogenously biosynthesized by trimethylamine *N*-oxide (Myers et al., 2021). Previous studies have linked trimethylamine *n*-oxide to the progression of cardiovascular disease, chronic kidney disease, fatty liver, and insulin resistance (Janeiro, Ramírez, Milagro, Martínez, & Solas, 2018). Our results showed that trimethylamine *N*-oxide was more abundant in NOYM samples. To further validate the detection sensitivity of markers, the adulterated simulants were prepared by mixing 10, 20, 30, and 60% NOYM in OYM. The content of trimethylamine *N*-oxide in adulterated simulants did not fall in the range of that in OYM when the percentage of NOYM was up to 30%. Thus, as little as 30% adulteration of NOYM could be identified using trimethylamine *N*-oxide as the judgment index.

In brief, the D-biotin can effectively identify OJM samples adulterated with 20% NOJM, whereas peptide of Thr-Ala-Val was determined to be markers of OJM; and the trimethylamine *N*-oxide can effectively identify OYM samples adulterated with 30% NOYM. However, generally speaking, the adulteration rate is generally $>$ 30%, even substituting non-organic milk as organic milk to obtain illegal profits in the market circulation because adulteration at lower levels of $<$ 30% is associated with a smaller economic pay-off and is less often expected (Kang et al., 2022b; Wang et al., 2022). Hence, the use of these three markers were effective to determine the authenticity of Jersey and Yak organic milk.

4. Conclusion

This study used untargeted and targeted metabolomics analysis to identify metabolite biomarkers for organic milk both Yak and Jersey. According to the differential analysis between metabolic profiles of organic and non-organic milk, 85 and 34 differential metabolites were identified as candidate molecular biomarkers in OYM and OJM, respectively. Finally, based on targeted metabolomics, peptide of Thr-Ala-Val and D-biotin were further confirmed as metabolite biomarkers for distinguishing OJM from NOJM, and trimethylamine *N*-oxide was confirmed as metabolite biomarkers for distinguishing OYM from NOYM. In the validation experiments, it was found that D-biotin can be used to detect adulteration of OJM with NOJM at levels as low as 20% and trimethylamine *N*-oxide has the ability to identify adulteration of OYM with NOYM at levels as low as 30%. In brief, this study demonstrated that the metabolomics analysis method was effective in distinguishing organic and non-organic milk. In summary, our findings provide new molecular markers to assess the authenticity of organic milk for both Jersey and Yak, and will facilitate the research on new detection methods.

CRediT authorship contribution statement

Min Kang: Investigation, Data curation, Methodology, Writing – original draft. **Hongxia Wang:** Investigation, Methodology. **Chuxin Chen:** Investigation, Methodology. **Ran Suo:** Conceptualization, Methodology, Data curation, Supervision, Writing – review & editing. **Jianfeng Sun:** Conceptualization, Methodology, Data curation, Supervision. **Quanhong Yue:** Methodology. **Yaqiong Liu:** Validation.

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.2023.100786.

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**The research content of the literature is similar to this study, and is the key reference object in the manuscript*

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