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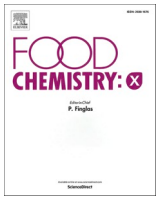

Identification of pasteurized mare milk and powder adulteration with bovine milk using quantitative proteomics and metabolomics approaches

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ABSTRACT

Adulteration in dairy products presents food safety challenges, driven by economic factors. Processing may change specific biomarkers, thus affecting their effectiveness in detection. In this study, proteomics and metabolomics approaches were to investigate the detection of bovine milk (BM) constituents adulteration in pasteurized mare milk (PMM) and mare milk powder (MMP). Several bovine proteins and metabolites were identified, with their abundances in PMM and MMP increasing upon addition of BM. Proteins like osteopontin (OPN) and serotransferrin (TF) detected adulteration down to 1 % in PMM, whereas these proteins in MMP were utilized to identify 10 % adulteration. Biotin and N6-Me-adenosine were effective in detecting adulteration in PMM as low as 10 % and 1 % respectively, while in MMP, their detection limits extend down to 0.1 %. These findings offer insights for authenticating mare milk products and underscore the influence of processing methods on biomarker levels, stressing the need to consider these effects in milk product authentication.

1. Introduction

Mare milk (MM) products are increasingly garnering attention in the dairy sector due to their unique composition and beneficial physiological properties. MM is noted for its similarity to human milk, particularly in terms of lactose, proteins, and minerals (Ji et al., 2024; Pietrzak-Fiećko & Kamelska-Sadowska, 2020). Importantly, MM exhibits lower allergenicity compared to bovine milk (BM) (Duan et al., 2021). The limited production and distinctive nutritional value of MM confer upon it a commercial value substantially exceeding that of BM. The practice of adulterating high-value specialty milk products with lower-cost BM—a phenomenon known as heterogeneous milk adulteration—represents a deceptive commercial tactic employed by some vendors for financial advantage (Azad & Ahmed, 2016). These adulterated milk products pose a threat not only to consumer rights but also to the health of individuals with BM allergies, particularly children (Manuyakorn & Tanpowpong, 2019).

Heat treatment is widely employed in dairy processing to enhance the safety and extend the shelf life of milk and dairy products. Although fermented mare milk is a significant dairy product and widely favored among populations in Central Asia (Di Cagno et al., 2004), mare milk can be heated processing for consumers and milk powder for infant are inevitable topics. However, such processing and storage can destabilize assay targets in milk (Benabdelkamel et al., 2017; Zhu, Kebede, Chen, McComb, & Frew, 2020), thereby compromising the accuracy of adulteration detection. Several methods were used to detect specific milk and dairy products adulteration, of these, the Polymerase Chain Reaction is a well-established molecular technique widely used in dairy product testing, renowned for its high sensitivity, specificity, and cost-effectiveness (Deng et al., 2020; Guo et al., 2018). Its limitations, however, include vulnerability of DNA to degradation during heating processes and a lack of quantitative analysis capabilities. Proteomics and metabolomics methods have emerged as highly sensitive and specific, unaffected by matrix effects, and capable of targeting a broader spectrum of compounds, although they are more costly (Böhme, Calo-Mata, Barros-Velázquez, & Ortea, 2019). Mass spectrometry (MS)-based approaches, particularly matrix-assisted laser desorption ionization time-of-flight-tandem MS (MALDI-TOF-MS) and liquid chromatography-

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tandem MS (LC-MS/MS), have also been applied to species identification in various dairy products (Li et al., 2020; Rau et al., 2020). Notably, species-specific peptides from bovine-derived α-lactalbumin have facilitated the detection of bovine whey adulteration in specific milk at levels as low as 0.5 %, using LC-MS/MS (Camerini et al., 2016). Similarly, trypsin-digested peptides from bovine β-lactoglobulin have been instrumental in detecting adulteration exceeding 10 % whey content in BM- or buffalo milk–derived cheese using LC-MS/MS (de Oliveira et al., 2022). Proteomic characterization through MALDI-TOF-MS of bovine liquid milk samples adulterated with varying percentages of BM powder identified specific peptides from whey and casein as markers, enabling detection of adulteration at levels as low as 1 % (Calvano, Monopoli, Loizzo, Faccia, & Zambonin, 2013). Furthermore, β-lactoglobulin and αS1-casein have been identified as BM biomarkers for detecting the adulteration in goat milk and sheep milk using ion-trap proteomics and liquid chromatography electrospray ionization ion-trap MS following multi-enzyme digestion (Nardiello, Natale, Palermo, Quinto, & Centonze, 2018). In previous studies, several proteins, including β-lactoglobulin and β-casein, were identified as potential biomarkers for detecting adulteration in raw MM with BM using LC-MS/MS (Yang et al., 2017; Ji et al., 2023). These findings underscore the potential of proteomic methods to generate informative fingerprints for verifying milk authenticity. However, the specific proteins indicative of adulteration in MM products remain to be thoroughly investigated.

Metabolomics, especially untargeted metabolomics based on LC-MS/MS, is a powerful tool for species-specific milk identification. This method focuses on the simultaneous measurement of thousands of metabolites in milk, which can be processed by chemometrics (Li et al., 2022; Wu et al., 2021). Utilizing LC-MS/MS-based metabolomics, researchers have analyzed the metabolite composition of human, bovine, goat, and mare milk. Notably, certain metabolites, such as orotic acid, found in significantly higher concentrations in BM compared to other types, have facilitated the differentiation of various milk types (Wu et al., 2021). In a study employing LC-MS/MS metabolomics to investigate the composition of BM metabolites from different dairy animals, key metabolites like choline and succinic acid were identified as effective markers for distinguishing among various milk types (Yang et al., 2016). Adulteration of breast milk with BM has been identified using quantitative metabolomics, specifically chemical isotope labeling LC-MS/MS. This method led to the discovery of five metabolites as biomarkers, capable of detecting BM presence in breast milk at concentrations as low as 5 % (Mung & Li, 2018). These studies demonstrate the efficacy of the milk metabolome as a robust tool for differentiating various milk types. However, research is limited concerning the specific metabolites that contribute to quantifying BM adulteration in MM products.

In this study, untargeted proteomics and metabolomics employing high-resolution Orbitrap Fusion Lumos Tribrid MS were utilized to assess the variations in the protein and metabolite compositions between control and adulterated MM products, including pasteurized mare milk (PMM) and mare milk powder (MMP). These findings of this study have the potential to offer fingerprint protein and metabolite biomarkers for the identification of MM products adulterated with BM and may serve as a valuable reference for evaluating adulteration in MM.

2. Materials and methods

2.1. Sample collection

Raw MM samples were obtained from farms in the vicinity of Urumchi, China, and Holstein milk samples were collected from farms near Qingdao, China. The collected milk samples were placed in sterile sampling bottles, initially stored at −20 °C, transferred with dry ice, and subsequently preserved at −80 °C in the laboratory prior to analysis. The protein, lactose and fat levels of studied milk samples are listed in Table S1. Given that the dry matter content of BM is approximately 12.7 % and that of MM is 11.2 %, the adulteration detection procedures were conducted as follows. For pasteurized milk, samples were thawed at 4 °C, Binary mixtures were then prepared by blending BM with MM in proportions of 0 %, 0.1 %, 1 %, 10 %, 20 %, 50 %, and 100 % by volume, followed by pasteurization at 63 °C for 30 minutes. For the milk powder group, raw milk was converted into powder through freeze-drying. BM powder was mixed with MMP in ratios of 0 %, 0.1 %, 1 %, 10 %, 20 %, 50 %, and 100 % by weight. These binary mixtures were then reconstituted using ultrapure water at a ratio of 1:8 (80 mL ultrapure water per 10 g milk powder).

2.2. Reagents used

2-Chlorophenylalanine, HPLC-grade acetonitrile, acetic acid, ethanol, methanol, acetone, and formic acid (FA); Tris, sodium dodecyl sulfate, hydrochloric acid, dithiothreitol (DTT), iodoacetamide (IAA), and ammonium bicarbonate were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China. Sodium tricitrate, DL-dithiotriol, urea, sodium dihydrogen phosphate, hydroxypropyl methylcellulose were purchased from Sigma-Aldrich company, USA. Bicinchoninic acid (BCA) assay were purchased from Beyotime Biotechnology, Shanghai, China. Trypsin (TPCK-treated) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Pure water for the experiment was prepared with ultrapure water (18.2 MΩ·cm, 25 °C).

2.3. Sample preparation for proteomics

Three replicates each of both MM and BM products, along with their mixtures, were subjected to centrifugation at 4,000 × *g* and 4 °C for 30 minutes. The upper layer of milk fat was discarded, and the middle layer, comprising skim milk rich in milk proteins, was collected. Protein concentrations in these gradient-diluted samples were quantified using the BCA assay, with bovine serum albumin as the standard. The procedure for peptide preparation was as described in our previous study (Ji et al., 2023). Briefly, 30 μg milk protein was denatured through heated treatment, reduced with dithiothreitol and alkylated with iodoacetamide. The protein samples were then digested with 50 μL ammonium bicarbonate solution containing 1 μg of trypsin, and incubated at 37 °C for 16–18 hours. Finally, formic acid was added to terminate the enzymatic reaction, and the peptides were purified using a SPE column (Thermo Fisher Scientific, Milford, MA, USA), dried, and resuspended in 300 μL of 0.1 % formic acid solution.

2.4. Data-dependent acquisition analysis by nLC-MS/MS

The reconstituted peptide mixtures were subjected to analysis using an Easy nLC 1000 system coupled with an Orbitrap Fusion Lumos instrument (Thermo Fisher Scientific, Milford, MA, USA), as referenced in our previous study (Ji et al., 2023). Peptide mixtures were automatedly loaded onto a C18 trap column (Thermo Fisher Scientific, Milford, MA, USA; PepMap 100 μm × 20 mm, 5 μm) and then passed through a C18 analytical column (Thermo Fisher Scientific, Milford, MA, USA; PepMap 75 μm × 150 mm, 3 μm) for gradient elution. Briefly, peptide samples were loaded and separated using mobile phase A [0.1 % formic acid] and mobile phase B [0.1 % (v/v) formic acid in 80 % acetonitrile] at a flow rate of 300 nL/minutes as follows: 0–5 minutes (4 %–10 % B), 5–63 minutes (10 %–30 % B), 63–72 minutes (30 %–40 % B), 72–80 minutes (40 %–100 % B), and finally maintained at 100 % for 10 minutes. MS was performed in the positive ion mode with a resolution power of 60,000 at 300–1,800 *m/z*. The top 20 precursor ions with multiple charged ions were analyzed by MS/MS using high-energy dissociation (normalized collision energy: 27).

2.5. Protein identification and quantification

The raw data were captured using Xcalibur software (Thermo Fisher

Scientific, Milford, MA, USA) and subsequently imported into MaxQuant software (version 2.0.3.0, Max-Planck-Gesellschaft, Berlin, Germany) for searching against a housed database (*Bos taurus* and *Equus caballus*, 2020.12) obtained from UniProt (https://www.uniprot.org/). The search parameters included: trypsin specificity, allowance of up to two missed cleavages, MS/MS tolerance set at 0.05 Da, carbamidomethylation of cysteine defined as a fixed modification, protein *N*-terminal acetylation, and oxidation of methionine defined as a variable modification. Protein relative quantification was performed using razor and unique peptides according to the label-free quantification (LFQ) workflow. Acceptance criteria for peptides and proteins included a false discovery rate of no more than 0.01 %. Identified proteins were further filtered by requiring a minimum of two identified peptides.

2.6. Quantitative protein analysis by capillary electrophoresis

To evaluate potential biomarkers, samples of MM, BM, and their mixtures underwent analysis using capillary electrophoresis (CE) (Beckman Instruments, California, USA). Milk protein samples were diluted to 1 mg/mL in a sample reduction buffer containing 5 mmol/L sodium tricitrate, 5 mmol/L DL-dithiotriol, and 8 mol/L urea at pH 8.0. After dilution, the samples were vortexed and incubated for 1 hour at room temperature. Prior to CE analysis, the protein samples were filtered through a 0.22 μm filter.

CE analysis was conducted using a Beckman PA800 plus CE system, operated with 32Karat^{TM} software (Beckman Instruments, California, USA). The coated capillary column had dimensions of 580 mm × 50 μm × 375 μm. The distance from the detection window to the outlet was 80 mm. The separation process utilized a voltage of 20 kV, resulting in a final current of approximately 30 μA. Samples were injected into the system at a pressure of 20 psi (1 psi = 6894.76 Pa) for 10 seconds. Detection of the analytes was performed at a wavelength of 214 nm. Before each sample injection, the capillary was sequentially flushed with purified water for 2 minutes and then with a running buffer (composed of 11.5 mmol/L sodium dihydrogen phosphate, 8.5 mmol/L hydroxypropyl methylcellulose, and 8 mmol/L urea at pH 2.50) for 5 minutes at 30 psi.

Bovine β-casein was isolated following the method described by previous research (Post, Arnold, Weiss, & Hinrichs, 2012). To quantify β-casein in samples, β-Casein was electrophoresed at five different concentrations: 20, 50, 100, 200, and 500 μg/mL.

2.7. Statistical analysis of proteomics

Protein data derived from three parallel samples for each treatment was imported into Perseus (https://www.perseus-framework.org) for statistical analysis. This analysis included principal component analysis (PCA), loading plots, and hierarchical cluster analysis. Proteins exhibiting *P*-value < 0.05 were considered significantly different. On this basis, if a protein in adulterated samples shows a FC value ≥ 2 relative to unadulterated samples, this protein is considered to have potential as a biomarker for the detection of adulteration. Volcano plots were created using GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, California, USA). For the CE data, the quantification of bovine β-casein in adulterated samples was determined by correlating the concentration and peak area of isolated β-casein, using Microsoft Office Excel 2010.

2.8. Sample preparation for metabolomics

Eight replicates each of MM and BM products, as well as their mixtures, were prepared. These samples were centrifuged at 4,000 × *g* at 4 °C for 30 minutes to produce skimmed milk samples. For the quality control (QC), equal aliquots from each sample were pooled. Metabolites were extracted by mixing 1 mL of the skimmed milk sample with 10 μL of 3 mg/mL 2-chlorophenylalanine as an internal standard and 30 μL of 33 % acetic acid to adjust the pH for protein precipitation. This mixture was then centrifuged at 10,000 × *g* for 15–20 minutes. The resulting supernatant was transferred to a 2 mL centrifuge tube and lyophilized. Post-lyophilization, 2 mL of 90 % ethanol was added to the dried sample, shaken at 750–1000 rpm for 5 hours, followed by centrifugation at 4 °C 10,000 × *g* for 15 minutes. The final supernatants were lyophilized again, reconstituted in 100 μL of ultrapure water and methanol (1:1), and centrifuged at 10,000 × *g* for 15 minutes. The clear supernatant was then prepared for further analysis.

2.9. Metabolite analysis by LC-MS/MS

Metabolites were analyzed using a Dionex UltiMate 3000 UHPLC system coupled with an Orbitrap Fusion Lumos system (Thermo Fisher Scientific, Milford, MA, USA). Each sample was run twice with technical repetitions. Metabolite samples were injected into an Agilent Poroshell 120 SB-C18 column (2.1 × 100 mm, 2.7 μm) using an autosampler. Chromatographic separation was achieved with two mobile phases: mobile phase A [5 % (v/v) acetonitrile and 0.1 % (v/v) formic acid] and mobile phase B [0.1 % (v/v) formic acid in acetonitrile]. The flow rate was set at 0.2 mL/minutes. The elution program was structured as follows: 0–2 mL/minutes with 100 % mobile phase A, followed by a gradient from 100 % to 0 % A over the next 10 minutes, and then a 4 mL/minutes maintenance period.

The mass spectrometer parameters for this experiment were as follows: electrospray voltage in positive mode was 3.5 kV; the temperature of the electrospray ionization source was 350 °C; the resolution of the initial full scan was 70,000; the scan range extended from 100 to 1,000 *m/z*; the resolution of the secondary data dependency scan was 35,000; and the automated gain control target was set to 1e6. To ensure the stability of the analysis, the QC was conducted by injecting a sample once every 14 samples throughout the analysis to monitor the consistency of sample preparation and instrument performance.

2.10. Metabolite data acquisition

Raw metabolomics data were obtained using Xcalibur and uploaded into Compound Discoverer 3.13 (Thermo Fisher Scientific, Milford, MA, USA). This software facilitated the extraction of mass spectral peak lists, mass annotation, and deconvolution, incorporating the analysis of relevant fragment ion and isotope distribution. The retention time tolerance window was set to 20 seconds for definitive identification searches, and a mass tolerance of 0.005 Da was applied for the putative searches. Retention time alignment was performed using a mass tolerance of 5 ppm for both precursor and fragment ions, with a maximum time shift of 30 seconds. Compound detection parameters involved a mass tolerance of 10 ppm, an intensity tolerance of 30 %, and a signal-to-noise ratio threshold of 3. Information on protonated molecular ion $[M+H]^+$, $[M+K]^+$, $[M+Na]^+$, and $[M+2H]^+$, acquired from MS, was utilized for metabolic profile analysis and characterization. A fragmentation score ranked the potential identification of high-energy accurate mass fragment ions against the theoretical dissociation of the molecule. The outcomes of the quantitative and statistical analyses, encompassing metabolites and peak area, were subsequently obtained.

2.11. Statistical analysis of metabolites

The metabolites identified through database searches and substances retrieved via MS2 were retained for accuracy assurance. The data were then imported into Perseus (https://www.perseus-framework.org) for comprehensive multivariate statistical analysis included PCA. This process strictly followed the established methodologies outlined in the Perseus instructions, encompassing several key steps: 1) Importation and classification of data into respective groups within the Perseus software. 2) Removal of low-quality data. 3) Logarithmic transformation of the data, with missing values imputed using interpolation methods

align with the assumptions of normal or uniform distribution assumptions. 4) Execution of statistical analyses including one-way ANOVA, PCA, and clustering analysis were performed, followed by their visual representation. Additionally, SIMCA 14 (Umetrics, Umeå, Sweden) was utilized for orthogonal partial least-squares discriminant analysis (OPLS-DA) and the generation of loading plots based on OPLS-DA. Its analytical procedures mirrored those of Perseus. PCA and OPLS-DA were employed to elucidate potential clustering and trends among the samples, and loading plots were utilized to highlight metabolite differences between samples. Metabolites were considered significantly different if they exhibited a *P*-value < 0.05 and a variable importance for projection (VIP; as calculated in the OPLS-DA model) ≥ 1. On this basis, if a metabolite in adulterated samples shows a FC value ≥ 1 relative to unadulterated samples, this metabolite is considered to have potential as a biomarker for the detection of adulteration. The univariate receiver operating characteristic (ROC) curve analysis was employed to assess the performance of metabolites as biomarkers, using MedCalc version 9.5.2.0 statistical software (MedCalc Software) for the ROC analysis. The area under the curve (AUC) values were interpreted as follows: an AUC of 1.0 indicated a perfect test; 0.9 to 0.99, an excellent test; 0.8 to 0.89, a good test; 0.7 to 0.79, a fair test; and < 0.7, a test not considered useful.

3. Results

3.1. Proteomics profiles of MM products with different levels of adulteration

In this study, 2,004 and 1,931 tryptic peptides were identified in the PMM and MMP groups, respectively, and are detailed in Tables S2 and S3. Among these, several peptides were found to originate from bovine-derived proteins. Specifically, 17 peptides from bovine-derived αS1-casein were identified in both PMM and MMP groups, 20 peptides from bovine αS2-casein in the PMM group and 21 peptides in the MMP group, four peptides from bovine β-2-microglobulin (B2M) in both groups, and seven peptides from bovine osteopontin (OPN) in the PMM group as opposed to six in the MMP group. Statistical analysis revealed the identification of 740 differentially abundant peptides from bovine-derived proteins in the PMM group and 634 in the MMP group, as listed in Tables S4 and S5. The relative abundances of these proteins was quantified using the LFQ workflow, focusing on bovine-specific peptides that elucidate the protein differences between MM products and BM.

A total of 254 proteins, each identified by at least two peptides, were detected in the PMM group, as detailed in Table S6. The clustering analysis of these quantitatively assessed proteins in the PMM group is depicted in Fig. S1a, Notably, pure PMM and adulterated PMM samples at 0.1 % and 1 % formed a subcluster, while the 10 %, 20 %, and 50 % adulterated PMM samples clustered together with pasteurized bovine

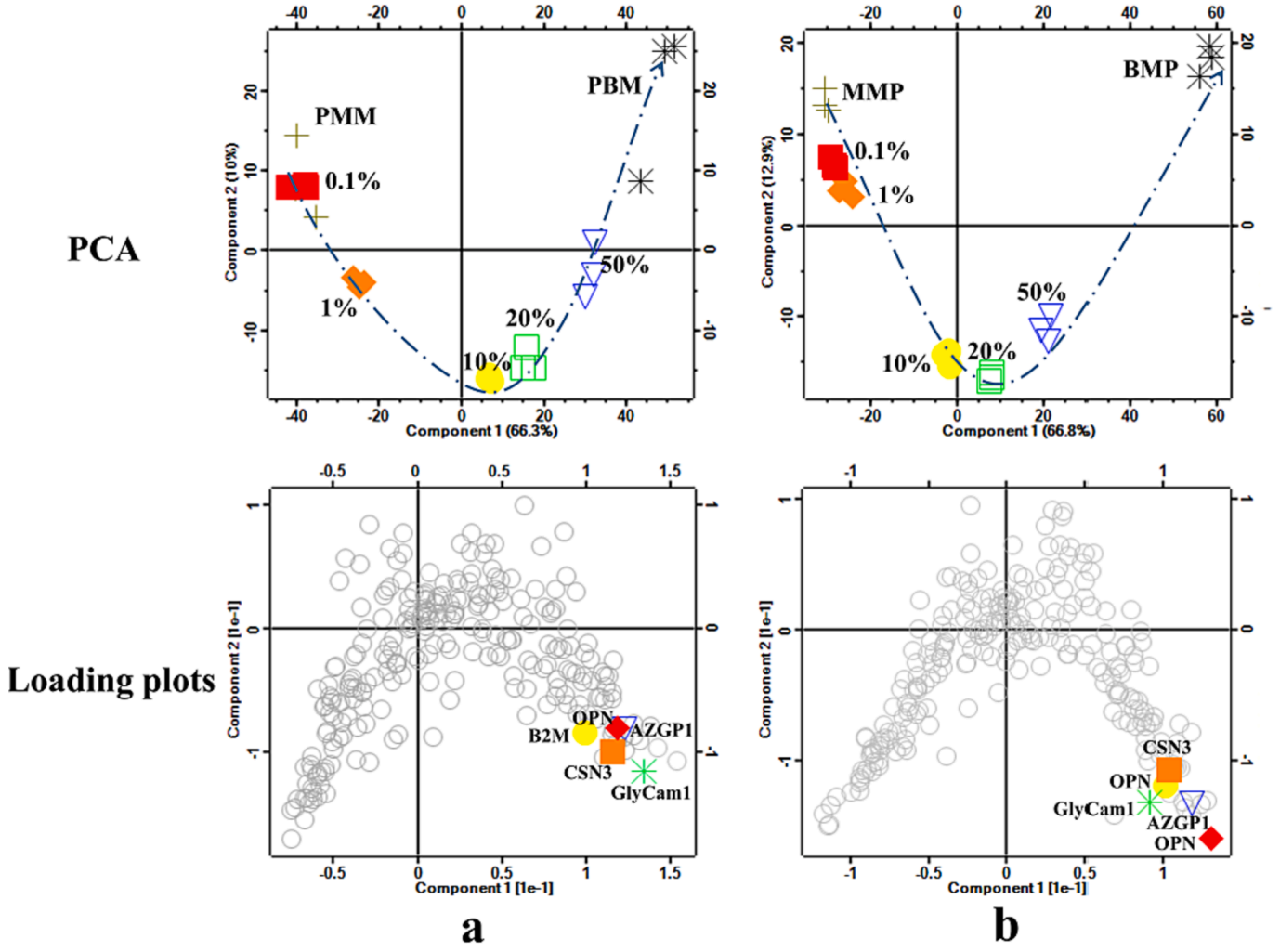

Fig. 1. Principal component analysis (PCA) score plots and PCA-based loading plots of all milk proteins using proteomics methods. PMM, PBM, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BM in PMM (a). MMP, BMP, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BMP in MMP (b).

milk (PBM) in a larger, separate grouping. The PCA score plots, as depicted in Fig. 1a, revealed a distinct separation in the protein profiles correlated with the levels of adulteration. Notably, samples at different adulteration levels were distinctly isolated from each other, with the exception that PMM and the 0.1 % and 1 % adulterated mixtures were not clearly differentiated. In terms of the loading plots corresponding to these score plots, specific bovine proteins such as B2M, κ-casein, and glycosylation-dependent cell adhesion molecule 1 (GlyCam1) were identified as significant proteins to the differentiation of adulterated samples.

In the MMP group, a total of 241 proteins, each identified by at least two peptides, were detected (Table S7). The clustering analysis of these proteins, presented in Fig. S1b, revealed that the pure MMP and adulterated MMP samples at 0.1 % and 1 % formed a subcluster, while 10 %, 20 %, and 50 % adulterated MMP samples constituted another. Bovine milk powder (BMP) alone clustered separately, and joined these two clusters. The PCA score plots for the MMP group, as shown in Fig. 1b, indicated that the protein profile's apparent separation was linked to the proportion of BMP ingredients added, displaying similarities to the PMM group. In alignment with these loading plots, several bovine proteins including B2M, κ-casein, OPN, and GlyCam1, were identified on the loading plots as key proteins to the differentiation of the adulterated samples in MMP.

3.2. Statistical analysis of differentially abundant proteins

Statistical analysis of the identified proteins identified in the PMM group revealed 193 proteins to be significantly, as listed in Table S8. Notably, bovine-derived β-casein, κ-casein, αS1-casein, and αS2-casein were observed to increase in adulterated PMM samples with BM content (ranging from 0.1 to 100 %), exhibiting a FC $\geq$ 2 and *P*-value $<$ 0.05. The levels of bovine-derived proteins such as α-lactalbumin, B2M, serotransferrin (TF), zinc-α-2-glycoprotein (AZGP1), and OPN in adulterated PMM significantly increased in adulterated PMM samples with 1 %–100 % BM content. Additionally, β-lactoglobulin levels significantly increased in adulterated PMM samples adulterated with 10 %–100 % BM content. Volcano plots were employed to illustrate the significantly different proteins between the PMM and other studied groups. Several different proteins including α-lactalbumin, OPN, and caseins were labeled, as depicted in Fig. 2a. Corresponding to the varying adulteration levels, the relative abundance changes of caseins, TF and OPN are detailed in Fig. S2a.

Statistical analysis of the proteins identified in the MMP group revealed that 193 proteins were significantly different, as documented in Table S9. Within this group, bovine-derived proteins such as lactoferrin, αS1-casein, and αS2-casein, were detected in MMP samples adulterated with 0.1 %–100 % BMP, exhibiting a FC of $\geq$ 2 and *P*-value $<$ 0.05. Significantly different bvine-derived proteins including β-Casein, κ-casein, and B2M were identified in MMP adulterated with 1 %–100 % BMP. Additionally, the levels of TF, α-lactalbumin, AZGP1, OPN, β-lactoglobulin, and GlyCam1 significantly increased in MMP adulterated with 10 %–100 % BMP. Volcano plots, as illustrated in Fig. 2b, highlighted the differential proteins between MMP and the other studied groups, specifically annotating variations in β-lactoglobulin and caseins. Corresponding to the varying adulteration levels, the relative abundances changes of caseins, AZGP1, and OPN are detailed in Fig. S2b.

To further elucidate the findings, the mean relative abundances of some proteins or metabolites identified as potential markers in various adulterated samples were detailed, alongside their respective ANOVA *P*-value and Post hoc Tukey's HSD test results, in Table S10 and Table S11. Additionally, clustering analysis was employed to directly present the differential proteins in the PMM and MMP groups, as depicted in Fig. 3. It was observed that the variations in protein levels identified in PMM were similar to those in MMP, with specific variations in proteins like β-lactoglobulin and α-lactalbumin, likely attributable to variations in milk processing methods. This point previously addressed in this study.

The presence of bovine β-casein in adulterated PMM and MMP samples was conclusively verified using CE. Chromatograms representing PMM and MMP at different adulteration levels (0 %, 0.1 %, 1 %, 10 %, 20 %, 50 %, and 100 %), as depicted in Fig. S3. Quantification of β-casein in PMM and MMP adulterated with BM, as depicted in Fig. S4. In PMM adulterated with 0.1 %–100 % BM, β-casein levels increased from 32.94 $\pm$ 1.23 μg to 181.63 $\pm$ 10.99 μg, and in MMP adulterated with 0.1 %–100 % BMP, the increase was from 29.06 $\pm$ 0.73 μg to 144.26 $\pm$ 9.04 μg. These changes in β-casein levels in adulterated PMM and MMP showed a gradual increase commensurate with the increasing BM components, similar to our proteomics results.

3.3. Metabolomics profiles of MM products with different levels of adulteration

The raw data from the PMM and MMP groups were processed using Compound Discoverer 3.0, leading to the identification of 1,270 and 1,105 metabolites, respectively, as listed in Tables S12 and S13. To illustrate the changes in the metabolome profile, PCA of the quantitative metabolites from both the PMM and MMP groups was conducted, as shown in Fig. 4. In the PMM group, different adulteration levels of PMM were distinctly separated, with each level forming a tight cluster. The metabolite profile of adulterated PMM changed in accordance with the increase in BM supplementation, and the unsupervised PCA model effectively distinguished these variations. In addition, in the MMP group, pure MMP, 0.1 % and 1 % adulterated MMP showed slight overlap, the other groups were tightly clustered and distinctly separated from each other. The metabolite profiles of the MMP group demonstrated a pattern of regularity akin to that observed in the PMM samples.

3.4. Identification of differentially abundant metabolites

To identify differentially abundant metabolites, all metabolites from the studied groups underwent multivariate statistical analysis, employing a pattern recognition method based on supervised OPLS-DA. The OPLS-DA model of the PMM group is depicted in Fig. 5a. Utilizing the first six principal components, the OPLS-DA model yielded parameters R2(X), R2(Y), and Q2 of 0.856, 0.963, and 0.855, respectively, indicating that the models had good adaptability and predictive ability. The loading plots, illustrating the correlation between X and variables and Y-variables, highlight the metabolite differences between pure PMM and the adulterated samples based on OPLS-DA. Important variables contributing to the differentiation between authentic and adulterated samples were discerned using VIP values. In Fig. 5a, metabolites with a VIP $\geq$ 1 were highlighted in red, with 2-aminonicotinic acid and 7-methylguanine were specifically labeled. Based on the criteria of VIP of $\geq$ 1 and *P*-value $<$ 0.05, a total of 125 metabolites were found to be significantly different in the PMM group, as documented in Table S14. Among these, with a FC $\geq$ 1, the levels of N6-Me-adenosine, prostaglandin A2, and two unidentified metabolites with molecular weights (MW) of 226.0757 and 317.1757 increased significantly in proportion to the BM content, ranging from 0.1 % to 100 %. Additionally, the abundances of 7-Methylguanine, 2-aminonicotinic acid, and 2,4-quinolinediol showed a significant increase with BM additions of 1 % to 100 % in PMM. The metabolites choline, 7-methylguanine, 2,4-quinolinediol, N6-Me-adenosine and 2-aminonicotinic acid were observed to increase in abundance with rising levels of BM adulteration in PMM, as demonstrated in Fig. S5a. Furthermore, ROC curves for N6-Me-adenosine, 2-aminonicotinic acid, 2,4-quinolinediol, and 7-methylguanine, employed to discriminate between pure PMM and PMM adulterated with 0.1 %, 1 %, and 10 % BM, are listed in Fig. S6a. Analysis revealed that the abundance of these metabolites enhanced in tandem with the increase in BM content in PMM. In the PMM group adulterated with at least 1 %, the AUC for these metabolites was at least 0.875, as illustrated in Fig. S6a.

The OPLS-DA model for the MMP group is illustrated in Fig. 5b. This

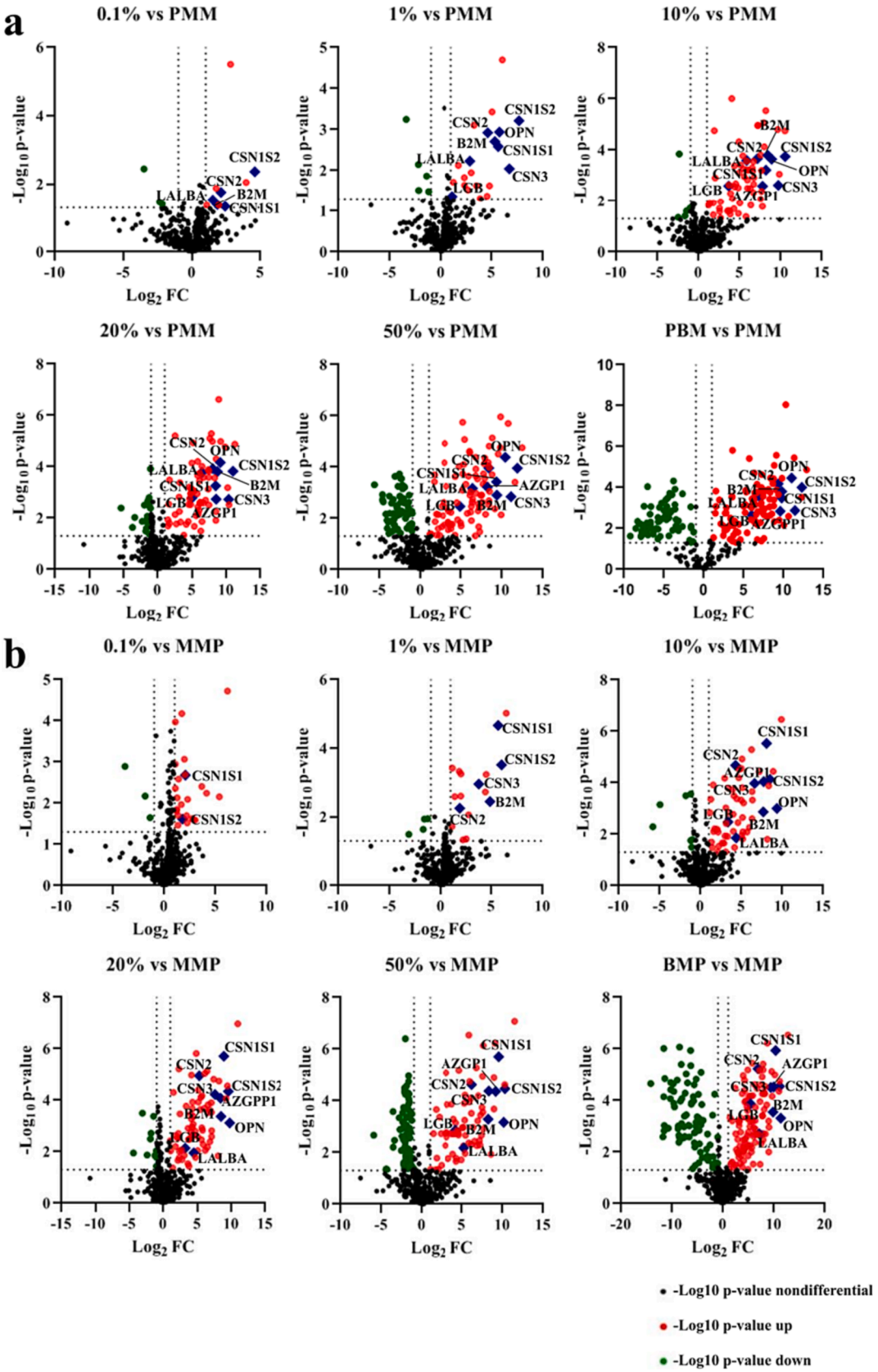

Fig. 2. Volcano plots of differential abundant proteins from all milk samples. PMM, PBM, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BM in PMM (a). MMP, BMP, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BMP in MMP (b).

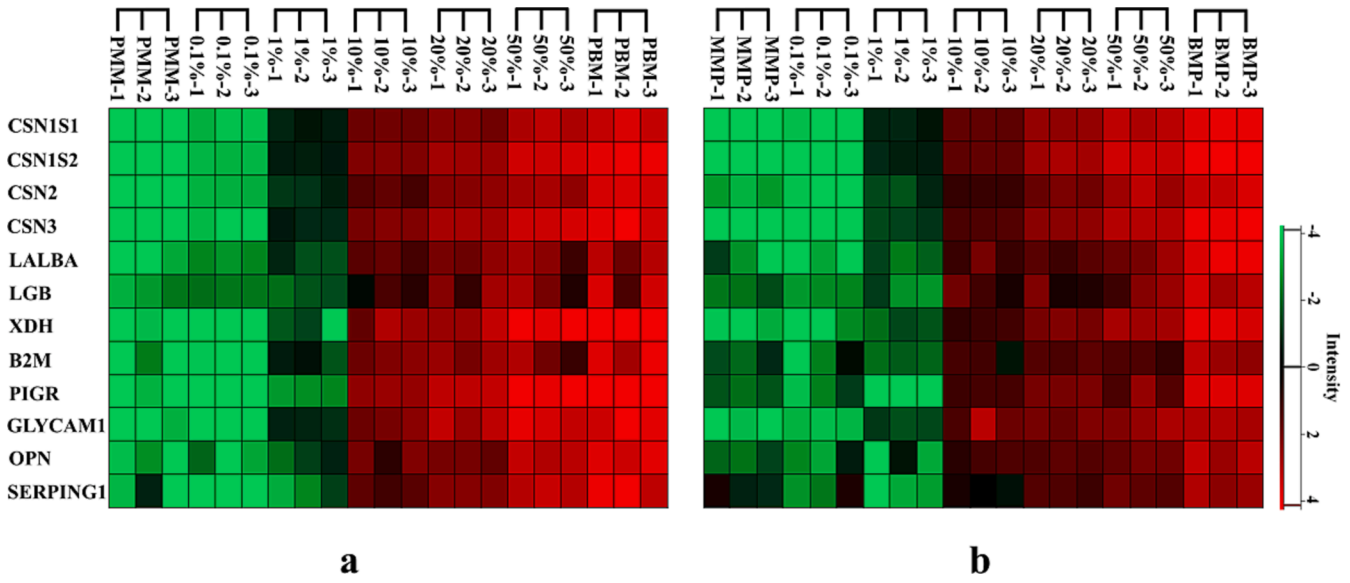

Fig. 3. Cluster analysis of some differential abundant proteins from all milk samples. PMM, PBM, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BM in PMM (a). MMP, BMP, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BMP in MMP (b).

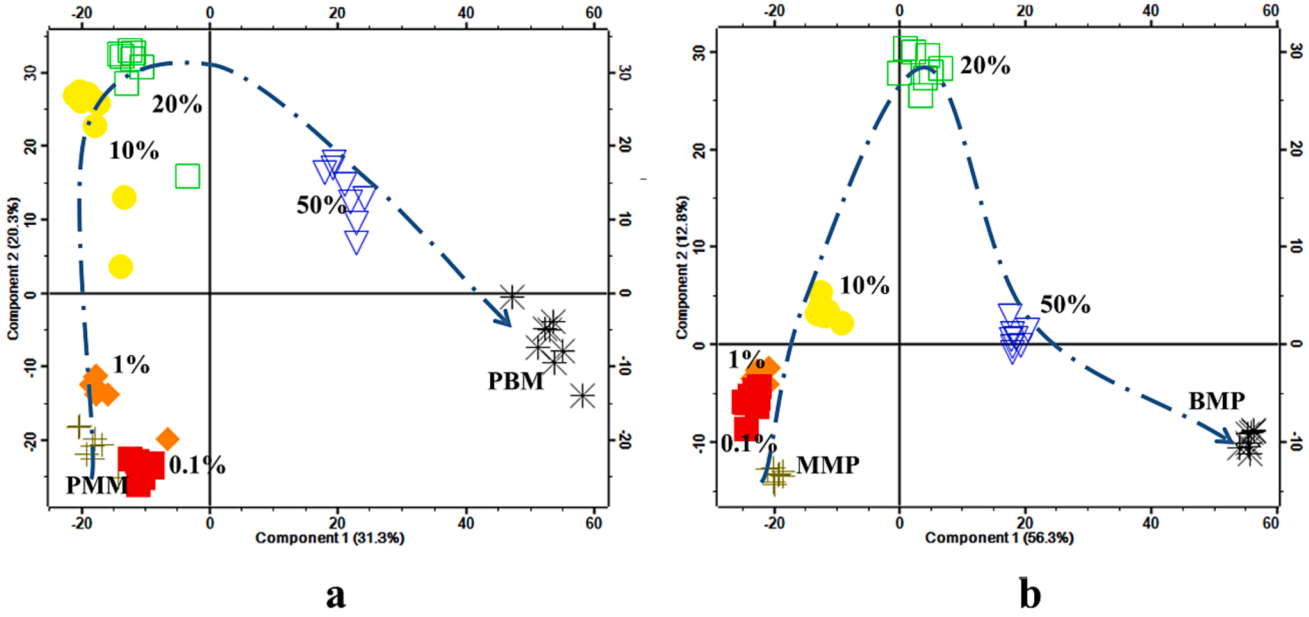

Fig. 4. Principal component analysis (PCA) of metabolite profile from all milk samples. PMM, PBM, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BM in PMM (a). MMP, BMP, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BMP in MMP (b).

model, based on the first six principal components, demonstrated R2(X), R2(Y), and Q2 parameters of 0.699, 0.787, and 0.656, respectively, signifying effective predictive reliability. Metabolites with VIP $\geq$ 1, such as biotin and 2-aminonicotinic acid were labeled on the loading plots of OPLS-DA (Fig. 5b). From the analysis of metabolites with a VIP $\geq$ 1 and *P*-value $<$ 0.05, a total of 147 metabolites were identified as significantly different in the MMP group, as detailed in Table S15. Among the identified metabolites with a FC of $\geq$ 1, biotin, 2-aminonicotinic acid, N6-Me-adenosine, and an unknown metabolites (MW = 254.0784) significantly increased with BMP ranging from 0.1 % to 100 %. Additionally, 7-Methylguanine, 6-methylflavone, and another unknown metabolite (MW = 326.1729) significantly increased in MMP adulterated with 1 % to 100 % BMP. Meanwhile, the abundances of biotin, 6-methylflavone, 2-aminonicotinic acid, N6-Me-adenosine and 7-Methylguanine increased with the rising levels of BMP adulteration, as depicted in Fig. S5b. Furthermore, ROC curves for N6-Me-adenosine, 2-aminonicotinic acid, biotin, and 7-methylguanine, aimed at distinguishing between MMP and MMP adulterated with 0.1 %, 1 % and 10 % BMP, are presented in Fig. S6b. In the MMP group adulterated with at least 1 % BMP, the AUC for these metabolites was at least 0.922, as shown in Fig. S6b. Additionally, it was observed that most of the differentially abundant metabolites identified in the PMM group were similar to those detected in the MMP group.

4. Discussion

In this study, PMM and MMP samples adulterated with varying amounts of BM components were analyzed using proteomic and metabolomic approaches. Multivariate statistical analysis revealed that the abundances of several proteins, including B2M, OPN, and

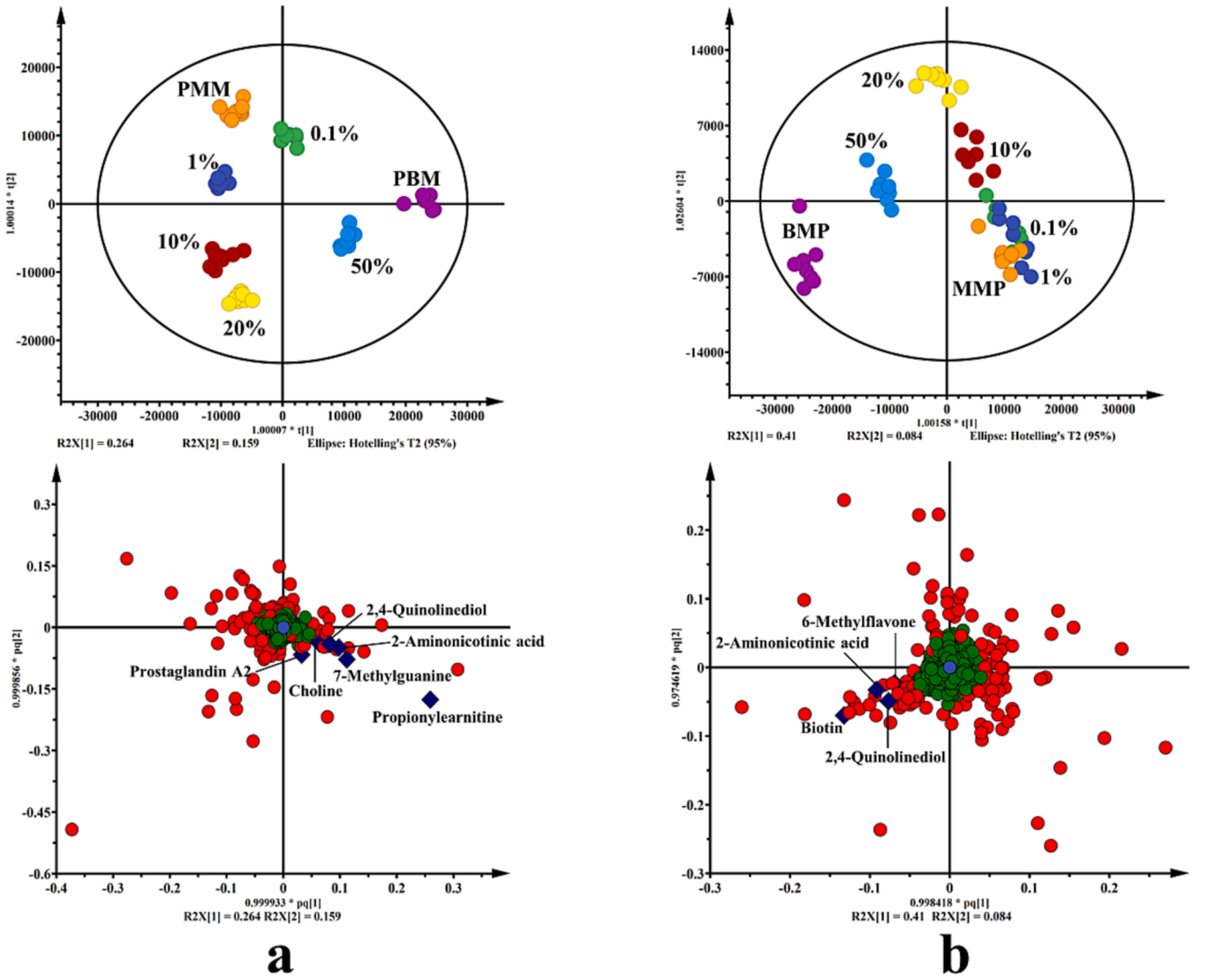

Fig. 5. Orthogonal partial least-squares discriminant analysis (OPLS-DA) and OPLS-DA -based loading plots of metabolites from all milk samples using metabolomics approach, metabolites with VIP ≥ 1 were marked in red. PMM, PBM, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BM in PMM (a). MMP, BMP, and 0.1 %, 1 %, 10 %, 20 %, and 50 % BMP in MMP (b).

metabolites such as 2-aminonicotinic acid and N6-Me-adenosine, increased with the level of BM adulteration in both PMM and MMP. These specific proteins and metabolites were identified as biomarkers and employed to detect adulteration of dairy products with BM, even at levels as low as 0.1 %. The differential expression patterns of these proteins and metabolites, potentially originating from mare and bovine mammary glands, provide valuable insights into the adulteration of MM products with BM.

4.1. *Proteomics analysis of markers*

The caseins αS1-casein, αS2-casein, β-casein, and κ-casein were identified as key markers for distinguishing PMM and MMP adulterated with BM components in this study. In prior research, α-casein was effective in detecting the addition of ≥ 5 % BM to goat milk using the differing retention times of α-casein from the two species. However, when employing reversed-phase high performance liquid chromatography, caseins were not capable of identifying the addition of BM to sheep milk (Veloso, Teixeira, & Ferreira, 2002). αS1-Casein has been utilized for the detection and quantitation of BM in buffalo mozzarella cheese via multiple reaction monitoring MS (Gunning, Fong, Watson, Philo, & Kemsley, 2019). Bovine-specific peptides derived from αS1-casein and αS2-casein have been employed as biomarkers to detect a 5 % BM presence in goat and sheep milk based on different tryptic peptides between bovine and goat or sheep using MALDI-TOF-MS (Calvano, Ceglie, Monopoli, & Zambonin, 2012). κ-Casein has been identified as a biomarker for detecting as low as 2 % BM components in goat milk. This detection is based on the differences in MW and isoelectric point as observed on 2D-gels (Jamnik, Volk, Ogrinc, & Jerek, 2019). As previously mentioned, caseins, due to differences in amino acid sequences and physicochemical properties, can serve as biomarkers for distinguishing milk from different species. Utilizing the the Uniprot database, we conducted a comparative analysis of the amino acid sequences of αS1-casein, αS2-casein, β-casein, and κ-casein between mare and bovine, revealing sequence similarities of 30.1 %, 66.7 %, 67.5 %, and 51.3 %, respectively. Through MS, we identified several tryptic peptides specific to bovine caseins and observed that the abundances of bovine caseins in both PMM and MMP increased with the addition of increasing amounts of BM. Consequently, caseins can be considered as fingerprint proteins for identifying PMM and MMP that containing BM. Our study found that the levels of BM κ-casein and β-casein levels in PMM significantly increased with BM adulteration ranging from 0.1 % to 100 %,

and in MMP with BMP adulteration from 1 % to 100 %. The difference in detection limits of κ-casein and β-casein between PMM and MMP groups may be attributed to the adhesion of casein and whey protein to the surface of fat globules, a phenomenon potentially caused by shear forces during milk powder reconstitution (Holzmüller & Kulozik, 2016; Zheng et al., 2020). Then, β-casein in PMM and MMP adulterated with BM components was quantified using CE. It was observed that the peak area of β-casein increased with the quantity of BM. This observation confirms the reliability of bovine β-casein as a biomarker for identifying adulteration in PMM and MMP.

Numerous proteins, including B2M, α-lactalbumin, β-lactoglobulin, GlyCam1, TF, AZGP1, and OPN, have been employed to detect adulteration in PMM and MMP. TF, a protein originating from blood and identified in milk, has been observed to be significantly higher in BM whey compared to that from goat and camel (Yang et al., 2013). B2M, an integral component of the major histocompatibility complex class 1, is a low-molecular-weight protein of approximately 12 kDa. The expression of the B2M gene was found to be elevated in somatic cell samples from mastitic milk compared to healthy samples, as identified through RNA-sequencing technology (Asselstine et al., 2019), whereas the levels of this protein in serum and milk proteins during mastitis, according to TMT proteomics. (Turk et al., 2021). B2M has been widely indicated to be involved in immune modulation and is critical for protection against bacterial infections (Argov-Argaman et al., 2010). Interestingly, a comparative analysis of milk whey between Kashmiri and Jersey cattle revealed B2M as a milk protein in Jersey cattle (Bhat et al., 2020). In addition, in our previous study, an increase in B2M in raw MM adulterated with BM was detected using a data-independent acquisition proteomics approach (Ji et al., 2023). In the current study, several peptides such as f53-69 and f43-52 derived from bovine B2M, which increased significantly in PMM and BMP adulterated with 1 %–100 % BM, were identified, thus serving as useful fingerprint proteins to detect PMM and MMP adulterated with BM.

α-Lactalbumin and β-lactoglobulin are primary constituents of bovine whey proteins. Various studies have indicated the efficacy of β-lactoglobulin as a marker for identifying BM in adulterated milk. For instance, a specific β-lactoglobulin variant was utilized to detect adulteration in buffalo milk and mozzarella cheese with as little as 1 % BM, based on retention time and peak areas using high performance liquid chromatography (Enne et al., 2005). In their study, the peak areas and heights of β-lactoglobulin were significantly lower in the cheese matrix compared to the milk. This reduction was attributed to protein degradation during the cheesemaking process. In a recent study, β-lactoglobulin was employed to detect adulteration of camel milk powder with BM at levels as low as 5 %, using ultra-high performance liquid chromatography (Li et al., 2021). As discussed previously, the differences in amino acid sequences and physicochemical properties of β-lactoglobulin contribute to its use as a fingerprint protein for detecting milk adulteration. In our study, bovine β-lactoglobulin was identified as a specific marker for BM in PMM, with a detection limit of 10 %, based on the distinct tryptic peptides of β-lactoglobulin between mare and bovine. α-Lactalbumin has been used in detecting adulteration in goat, camel, yak, and buffalo milk with BM, as evidenced by its spots on 2D-gel maps (Yang et al., 2014). More recently, α-lactalbumin was identified as a marker for detecting BM in buffalo milk at a 1 % level using CE (Trimboli et al., 2019). In our study, bovine α-lactalbumin was effectively identified as a biomarker capable of detecting as little as 1 % BM in PMM. Interestingly, bovine β-lactoglobulin and α-lactalbumin were identified as markers for BM in MMP, with a detection limit of 10 %. This could be attributed to the partial adhesion of bovine whey protein to fat globules. Thus, it is evident that the quantification and detection of biomarkers in MM and its products can be influenced by the milk processing procedures.

GlyCam1, a hormone-regulated secreted glycoprotein, is the most abundant host defense protein found in whey. It has been identified in whey and milk fat globule membrane from various types of milk (Han et al., 2022; Ma, Zhang, Wu, & Zhou, 2019). Recently, bovine-derived GlyCam1 peptides have been utilized as markers for assessing BM constituents in fresh bovine, buffalo, and goat milk (Sassi, Arena, & Scaloni, 2015). Interestingly, we observed a significant increase in the abundance of GlyCam1 in PMM and MMP when mixed with various amounts of BM. Thus, we propose that GlyCam1 can serve as a biomarker to detect adulteration in PMM and MMP with BM ingredients, leveraging the differences in GlyCam1 peptides between mare and bovine.

Transferrin has also been selected as a biomarker for plasma powder to enhance the identification of processed animal proteins in feed or feed materials (Lecrenier et al., 2016). In our study, we observed that TF levels significantly increased in PMM adulterated with 1 %–100 % BM and in MMP adulterated with 10 %–100 % BMP. Our findings underscore the potential of TF as a biomarker for detecting the authenticity of milk. OPN, an acidic and highly phosphorylated glycoprotein, is synthesized by a wide range of cells and tissues, including the mammary gland, blood, and immune organs. This protein has been extensively identified in the milk of human and various dairy animals (Jin et al., 2021; Yang et al., 2013). OPN is known for multiple beneficial functions, such as facilitating cellular migration and modulating immune responses (Christensen & Sørensen, 2016). In BM, OPN exists as a full-length 60-kDa protein and a truncated 40-kDa isoform, both characterized by MS as being highly phosphorylated and glycosylated (Bissonnette, Dudemaine, Thibault, & Robitaille, 2012). Recently, OPN has been employed as a target protein for detecting and quantifying ruminant proteins in animal feed, effective even at an adulteration level of 1 % (w/w) (Lecrenier et al., 2021; Steinhilber et al., 2019). In our study, we observed an increase in OPN levels corresponding to BM constituents adulteration ranging from 1 % to 100 % in PMM and 10 % to 100 % in MMP. This increase could be linked to peptides such as f153-161, f31-51, and f36-51 derived from bovine OPN.

AZGP1, a 41-kDa multifunctional glycoprotein, has been detected in serum, saliva, and milk (Yang et al., 2013). This protein, functioning as a lipid-mobilizing factor, stimulates lipid degradation in adipocytes and binds to polyunsaturated fatty acids (Zahid et al., 2021). While a previous study using a labeled proteomic approach did not report a significant difference in AZGP1 abundance between Holstein and goat or camel milk (Yang et al., 2013), our study identified several peptides, such as f287-295, f283-295, and f231-238, derived from bovine AZGP1. These peptides showed increased levels in PMM adulterated with BM at 1 %–100 % BM and in MMP adulterated with 10 %–100 % BMP. Additionally, the observed differences in the detection limits of the protein between PMM and MMP might be attributable to the adhesion of some of these proteins to fat globules, as previously discussed. Collectively, the current study demonstrates that variations in several bovine proteins increase in tandem with the amount of BM adulteration in MM products, and the phenomenon potentially influenced by the milk processing procedures. Additional studies are essential to evaluate the suitability of these proteins as potential markers for addressing wider aspects of milk authentication.

4.2. Metabolomics analysis of markers

In this study, we observed a significant increase in the levels of several metabolites, including N6-Me-adenosine, 2-aminonicotinic acid, and biotin, consequent to the addition of BM constituents to PMM and MMP. To date, the characteristics of N6-Me-adenosine and 2-aminonicotinic acid in milk remain largely unexplored. We found that the abundances of N6-Me-adenosine and 2-aminonicotinic acid significantly increased with the BM constituents added to PMM and MMP. Thus, N6-Me-adenosine and 2-aminonicotinic acid might serve as novel markers for detecting BM adulteration in MM products, even at levels as low as 1 %. A previous study delved into the origin of 2,4-quinolinediol in BM, uncovering that it is exclusively present in the gastrointestinal tract of bovine and notably absent in forage, including hay, grass silage, and maize silage, as well as in rumen juice (Rouge et al., 2013). These

findings might indicate that 2,4-quinolinediol in milk is a product of gut metabolism in bovine. Considering the clear differences between bovine and mare milk, this specific metabolite emerges as a promising candidate for detecting the adulteration of MM products with BM constituents. In our research, we observed an increase in the abundance of 2,4-quinolinediol correlating with the rising levels of BM in PMM. Therefore, we posit that this metabolite could serve as an effective fingerprinting tool to detect the presence of BM in PMM, even at levels as low as 1 %.

Biotin is a water-soluble B vitamin, acts as a cofactor for enzymes like pyruvate carboxylase and propionyl-coenzyme A carboxylase, playing a role in various metabolic reactions (Lombard & Moreira, 2011). In our study, we found a significant increase in biotin levels corresponding to the addition of 1 %–100 % BMP to MMP. This suggests its potential as a specific marker for detecting BMP adulteration in MMP. Water-soluble vitamins, including biotin, are known to be more sensitive to heat treatment than fat-soluble vitamins (Bendicho, Espachs, Arantegui, & Martín, 2002). Previous research has shown that pasteurization processes reduce the concentrations of various vitamins, including vitamin B12, vitamin E, vitamin C, and folic acid (Zhu et al., 2021). Therefore, the biotin content in PMM may be partially diminished compared to MMP. In addition, biotin did not show a significant increase in PMM adulterated with BM. Furthermore, 7-methylguanine and 6-methylflavones showed variability in expression between BMP and MMP. These metabolites increased significantly with the addition of BMP to MMP, aiding in the detection of MMP adulteration with BMP at levels as low as 1 %. However, further research is needed to fully understand the characteristics of these metabolites. Choline, an essential nutrient for normal fetal development, has also been studied in the context of milk adulteration. A mathematical model utilizing nuclear magnetic resonance and 10 different metabolites, including choline, was applied to detect goat milk mixed with 5 % BM (Li et al., 2016). In our study, we noted that the abundance of choline in PMM and MMP increased in tandem with the addition of BM, particularly at levels ranging from 10 % to 100 %. Consequently, choline could potentially serve as a biomarker for assessing BM adulteration in both PMM and MMP.

In our study, we noted a significant increase in the levels of several unknown metabolites with MW of 226.0757 and 317.1757 in PMM adulterated with BM. Additionally, other unknown metabolites with MW of 254.0784 and 326.1729 showed increased levels in MMP adulterated with BMP. Collectively, these metabolites have been instrumental in identifying MM products adulterated with BM at levels as low as 0.1 %. However, the specific characteristics of these metabolites, especially in relation to their fragment ions in milk, warrant further exploration in future studies.

5. Conclusions

In this study, the changes in protein and metabolite profiles in PMM and MMP when adulterated with various proportions of BM components were characterized by proteomics and metabolomics approaches. Crucially, our research underscores the transformative effects of dairy processing on these profiles. Proteins including AZGP1, OPN, and TF were effective in detecting adulteration in PMM as low as 1 %, while in MMP group, the detectable limit of adulteration was 10 %. For metabolites, N6-Methyladenosine exhibited a detectable adulteration limit of 0.1 % in the MMP group, compared to 1 % in the PMM group. Furthermore, to obtain robust results, multiple commercial samples could be required for assessing the MM products adulteration, although we have provided an internal validation strategy.

CRediT authorship contribution statement

Zhongyuan Ji: Writing – original draft, Investigation, Data curation. **Junyu Zhang:** Resources, Investigation. **Chunxia Deng:** Visualization. **Tongjun Guo:** Supervision, Resources. **Rongwei Han:** Funding acquisition. **Yongxin Yang:** Writing – review & editing, Validation, Methodology. **Changjiang Zang:** Writing – review & editing, Formal analysis, Conceptualization. **Yong Chen:** Project administration.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101265.

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