Untargeted metabolic footprinting reveals key differences between fermented brown milk and fermented milk metabolomes

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ABSTRACT

Fermented brown milk has gained popularity because of its unique taste and flavor. *Lactobacillus bulgaricus* ND02 is a starter culture that has good milk fermentation characteristics. This study aimed to profile the metabolites produced during Maillard browning and to identify metabolomic differences between fermented brown milk and fermented milk produced by the ND02 strain. This study used liquid chromatography–mass spectrometry to compare the metabolomes of milk, fermented milk, brown milk, and fermented brown milk. Significant differences were observed in the abundances of various groups of metabolites, including peptides, AA, aldehydes, ketones, organic acids, vitamins, and nucleosides. The Maillard browning reaction significantly increased the intensity of a wide spectrum of flavor compounds, including short peptides, organic acids, and compounds of aldehydes, ketones, sulfur, and furan, which might together contribute to the unique flavor of brown milk. However, Maillard browning led to an increase in N ϵ -(carboxymethyl)lysine, which might cause negative health effects such as diabetes, uremia, or Alzheimer's disease. On the other hand, fermenting brown milk with the ND02 strain effectively countered such an effect. Finally, 5 differentially abundant metabolites were identified between fermented brown milk and fermented milk, including L-lysine, methylglyoxal, glyoxal, 2,3-pentanedione, and 3-hydroxybutanoic acid, which might together contribute to the different nutritional qualities of fermented brown milk and fermented milk. This study has provided novel information about the Maillard reaction and compared the metabolomes of the 4 types of dairy products.

Key words: fermented brown milk, Maillard reaction, metabolomics, *Lactobacillus bulgaricus* ND02

INTRODUCTION

For many years, certain foods have been found to be beneficial to health. Fermented milk is a classic example, as it contains a wide range of bioactivities (e.g., modulating digestive and gastrointestinal functions, lowering cholesterol, and the capacity of immunoregulation; Buttriss, 1997). In terms of nutritional composition, fermented milk is an excellent source of high-quality protein, calcium, phosphorus, magnesium, zinc, niacin, vitamin B12, and riboflavin. *Lactobacillus* fermentation decreases the content of milk lactose, subsequently produces lactic acid production, and curdles the milk protein. These chemical changes offer the unique organoleptic characteristics and nutritional value to fermented dairy products. The main group of bacteria responsible for milk fermentation is lactic acid bacteria (**LAB**), and the fermentation process involves the following: (1) the formation of a large amount of lactic acid from lactose reduction; (2) an increase in free biomolecules, such as free AA, small peptides, and fatty acids; and (3) the production of flavor compounds from the metabolisms of carbohydrates and citrate, AA, and fatty acids.

The development of cold chain technology has brought about new varieties of yogurt products. Brown yogurt, also known as char-grilled yogurt, is a new type of yogurt product. Instead of fresh milk, the milk protein and reducing sugar undergo the Maillard reaction at high temperature for a long time before fermentation by the starter culture. Fermented brown milk not only has a unique color and flavor as a result of the Maillard reaction (Han et al., 2019) but is beneficial to human health (Shida et al., 2017) due to the *Lactobacillus* fermentation products. Hence, fermented brown milk has become popular among consumers, particularly in China, Japan, Southeast Asia, Europe, and other regions (Xu et al., 2010). However, the complex Maillard reaction has been reported to produce a variety of potentially harmful compounds (Rannou et al., 2016). Therefore, detailed investigation into fermented brown milk metabolomes would be of great importance for

Received June 9, 2021.

Accepted November 10, 2021.

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assessing the health impact of consuming such products and ensuring product quality.

Metabolomics is a newly developed discipline after genomics and proteomics, and it provides an effective research platform for studying cell metabolites and building metabolic pathways (Rochfort, 2005). Metabolomic studies analyze low-molecular-weight substances (molecular weight <1,000) of a certain organism or subject under certain physiological conditions. These small compounds usually include short peptides, AA, nucleic acids, sugars, organic acids, vitamins, polyphenols, and other compounds such as alkaloids. Metabolomics can be divided into nontargeted metabolomics and targeted metabolomics based on research characteristics (Roberts et al., 2012). Targeted metabolomic analysis aims to quantify specific metabolites in environmental samples by building calibration curves of authentic standards (Roberts et al., 2012). Nontargeted metabolomic studies mainly detect dynamic changes of a large number of metabolites before and after an environmental disturbance or stimulation. Commonly used methods in metabolomics include GC-MS, nuclear magnetic resonance, and liquid chromatography (LC)-MS. Among them, LC-MS combines the advantages of chromatography and MS that exhibits high separation ability for complex samples with a high selectivity and sensitivity, together with a powerful capacity in acquiring relative molecular mass and structural information. Metabolomic analyses are widely applied in many fields (e.g., medical studies, environmental, and food analysis).

Lactobacillus delbrueckii ssp. *bulgaricus* is the most common species used as an industrial dairy starter. The *L. bulgaricus* species has strong proteolytic ability, as its genome contains a cell wall proteolytic enzyme that can degrade CN. The bacteria hydrolyze CN to produce all AA except glutamic acid and cysteine. In particular, *L. bulgaricus* can produce large amounts of proline, lysine, and valine (Do Carmo et al., 2011). In addition, *L. bulgaricus* has a complex lactose-degrading enzyme system that can efficiently convert lactose to lactic acid (Burgos-Rubio et al., 2000). The *L. bulgaricus* strain ND02 was isolated from traditional fermented yak milk in Qinghai, China (Sun et al., 2010). This strain possesses excellent fermentation properties in terms of time, flavor, and water-holding capacity of the produced fermented milk (Sun et al., 2010). The genome of ND02 contains 431 unique genes, including acetyltransferase, glutamate decarboxylase, polysaccharide synthesis protein, multiple glycosyltransferases, and extracellular polysaccharide synthesis cluster. The possession of these genes gives ND02 excellent curd characteristics and ability to produce fermented prod-

ucts of good nutritional value. To gain a comprehensive understanding of the fermentation properties of ND02, it is necessary to characterize the metabolome of the resultant fermented milk products.

To the best of our knowledge, only a few studies have analyzed the metabolomic dynamics of brown milk fermentation, and no study has yet directly compared the metabolomes of fermented brown milk and fermented milk. Therefore, detailed investigation into the metabolites in fermented brown milk, especially Maillard reaction products, is essential for understanding the nutritional value of these products and to further improve the product quality. The aims of this work were to (1) identify and explore the role of the metabolites produced during Maillard browning of milk; (2) identify the metabolomic differences between fermented brown milk and fermented milk; and (3) to gain an in-depth understanding of the nutritional values of the 2 products.

MATERIALS AND METHODS

Materials and Equipment

The *L. bulgaricus* ND02 strain used in the experiment was obtained from the LAB Culture Collection, Inner Mongolia Agricultural University, China. Skim milk powder (protein 30%) was purchased from Fonterra Co. Ltd. (NZMP Ltd.). The Acquity UPLC-Xevo-G2-QTOF-MS system, MassLynx 4.1 Workstation, Progenesis Q1, and the leucine-enkephalin standard were purchased from Waters Corp. Chromatographic grade acetonitrile, ammonium hydroxide solution, and formic acid were purchased from Sigma Aldrich.

Sample Preparation

Preparation of Milk and Fermented Milk. The reconstituted milk was prepared by suspending 10% (wt/wt) skim milk powder in water and then homogenizing it (65°C, 90 MPa) using a high-pressure homogenizer (Hirayamaha-300M). The mixture was sterilized at 95°C and maintained for 10 min, followed by cooling to 42°C. Then, *L. bulgaricus* ND02 (5×10^6 cfu/mL) was inoculated into the sterilized milk, and the mixture was fermented at 42°C until the pH reached 4.5. The whole process used anaerobic fermentation. Milk samples were collected after reaching the fermentation end point, and each sample was processed in sextuplicate. All samples were stored at -80°C before analysis.

Preparation of Brown Milk and Fermented Brown Milk. Brown milk and fermented brown milk were prepared and sampled in the same way as the milk

and fermented milk except that they were incubated in a thermostatically controlled water bath for 3 h for browning to occur after heating to 95°C instead of directly cooled to 42°C.

Acidity and Viable Counts

The fermentation process was monitored by measuring the acidity of the fermented milk and the fermented brown milk. The pH value was determined by a pH meter (Leici). Titratable acidity (TA) was measured as described by Zha et al. (2021). Viable cell counts of *L. bulgaricus* ND02 were evaluated using the standard dilution method in de Man, Rogosa, and Sharpe (MRS) agar medium (Dan et al., 2019).

Ultra Performance Liquid Chromatography-Quadrupole Time-of-Flight MS with MS^E Data Acquisition

Sample Pretreatment and Application. One milliliter of the sample was pipetted and mixed with 1 mL of acetonitrile solution in a 5-mL Eppendorf tube for 1 min. Afterward, the samples were centrifuged at $10,000 \times g$ at 4°C for 10 min to remove proteins. The supernatant (1 mL) was added into a new 5-mL Eppendorf tube which contained 3 mL of acetonitrile solution, and the solution was allowed to stand at 4°C for 2 h. The mixture was concentrated for 9 h using a vacuum rotary evaporation and then reconstituted by 40% acetonitrile solution. Reconstituted samples were filtered through a 0.22- μ m microporous membrane. A quality control (QC) sample was prepared by combining equal portion of all samples. Acetonitrile was used as a blank. Blank and QC samples were injected to the chromatographic system initially to ensure the stability of system and conditions. A total of 24 samples (6 each of milk, fermented milk, brown milk, and fermented brown milk) were injected randomly to minimize the effect of instrumental drift. A QC sample was injected every 3 samples and a standard QC to evaluate the mass accuracy.

Liquid Chromatography Conditions. Chromatographic analysis was performed using Waters Acquity ultra performance liquid chromatography (UPLC) system. The chromatographic column, HSS T3 (1.8 μ m, 2.1 mm $\times$ 100 mm), was used for the separation. The column was maintained at 45°C during analysis. The injection volume was 5 μ L, and the flow rate was 0.45 mL/min. The positive ion mode (electrospray ionization-positive, ESI⁺) mobile phase was made of 0.1% formic acid aqueous solution (A1) and 0.1% formic acid acetonitrile solution (B1). The negative

ion mode (ESI⁻) mobile phase was made of 0.1% ammonium hydroxide (A2) and acetonitrile solution (B2). The gradient elution program used in both the ESI⁺ and ESI⁻ modes was as follows: 0.25 min, 98% A, 2% B; 12 min, 2% A, 98% B; 14 min, 2% A, 98% B; 14.10 min, 98% A, 2% B; 17 min, 98% A, 2% B. The total run time was 17 min.

Mass Spectrometry Conditions. Metabolites analysis of fermented milk and fermented brown milk was performed using UPLC-quadrupole-time-of-flight (UPLC-Q-TOF MS^E). Mass spectrometry was performed using ESI⁺ and ESI⁻ (MS^E). Highly purified nitrogen and helium were used as the desolvation gas and collision gas, respectively. The data acquisition range was 50 to 1,200 m/z . The collision energy of 20 eV to 40 eV was applied to crush the parent ions. For both ESI⁺ and ESI⁻ modes, the source parameters were set as follows: the capillary voltage was 3.0 KV; the sample cone voltage was 40 V; the source temperature was 120°C; the desolvation temperature was 500°C; the desolvation gas flow was 800 L/h; the cone gas flow was 50 L/h; and the scan time was 0.2 s. A lock mass of leucine-enkephalin (concentration: 200 pmol) was used as the calibration solution (ESI⁺: m/z 556.2771; ESI⁻: m/z 554.2615) and was introduced by Lockspray at 10 μ L/min. The scan time was 0.2 s, and the acquisition interval was 20 s for real-time data calibration.

Data Treatment and Metabolite Identification. The MS^E raw data, including mass-to-charge ratio (m/z), retention time, and ionic strength, were collected by MassLynx 4.1 workstation. The MS^E data were imported to the Progenesis QI software without any conversion to complete peak extraction, peak alignment, deconvolution, and normalization. According to standard ionization behavior, the adduct ions selected for data collection in the ESI⁺ mode included [M+H]⁺, [M+Na]⁺, [M+NH₄]⁺, [M+K]⁺, and [2M+H]⁺; and the adduct ions selected for data collection in the ESI⁻ mode included [M-H]⁻, [M+Na-2H]⁻, [M-2H]²⁻, [M+HCOO]⁻, and [M+Cl]⁻. Peak alignment was carried out using all runs in the experiment as reference. Peak picking threshold of sensitivity was set at 3. All data were normalized to the summed total ion intensity per chromatogram.

The obtained data matrices were imported into MetaboAnalyst 4.0 (<https://www.metaboanalyst.ca>), a web server designed to facilitate analysis of metabolites and data pre-processing, including missing value estimation, median centering, log-transformation, and Pareto scaling. Features existed in at least 50% of samples, and features with coefficient of variation less than 25% in the QC samples were further analyzed (Liu et al., 2020). Nonparametric tests (Wilcoxon rank-sum tests)

Table 1. Acidity and viable counts of milk and brown milk fermented by *Lactobacillus bulgaricus* ND02 ($\pm$ SD)

Sample	Fermentation time (h)	pH	Titrateable acidity ($^{\circ}$ T)	Viable counts ($\times 10^8$ cfu/mL)
Fermented milk	7.00 $\pm$ 0.58	4.49 $\pm$ 0.02	86.24 $\pm$ 0.80	5.15 $\pm$ 0.04
Fermented brown milk	7.20 $\pm$ 0.20	4.50 $\pm$ 0.00	83.12 $\pm$ 0.41	8.54 $\pm$ 0.10

were used to evaluate the significance of variables. The adjusted P -value (false discovery rate) cutoff was set as 0.05 before further pattern recognition analysis (principal component analysis, **PCA**; orthogonal partial least square discriminant analysis, **OPLS-DA**; Liu et al., 2020). To visualize group-based clustering of metabolites, OPLS-DA was used. Differential metabolites were defined as follows: variable important in projection (**VIP**) value >1 in OPLS-DA analysis; $P < 0.05$ in single-dimensional statistical analysis; and fold-change between 2 groups >2 . Furthermore, a permutation test was also performed to assess the quality of the OPLS-DA models. The goodness-of-fit was represented by R^2Y , and the predictive ability was indicated by Q^2 .

Metabolite Annotation and Pathway Analysis.

Pathway enrichment analysis and metabolite annotation were performed using Mummichog 5 (Li et al., 2013a). The “.csv” files were input as 3 columns, corresponding to m/z features, P -values, and statistical scores of 2 sample groups in both ESI^+ and ESI^- modes, into Mummichog to conduct pathway and module analysis. The Kyoto Encyclopedia of Genes and Genomes *Bacillus subtilis* pathway library was selected, and the mass accuracy of instrument was 5 ppm. The P -value cutoff was set to 0.05 to identify significantly enriched m/z features. Default settings were applied to other parameters. The results of the metabolite annotation and Mummichog pathway analysis were presented by network visualization analysis. Identified differential metabolites were subjected to MetaboAnalyst 4.0 for exploratory receiver operating characteristic (**ROC**) analysis. Random forest algorithms were used for ROC curve construction (Liu et al., 2020).

RESULTS

Acidity and Viable Counts of Fermented Milk and Fermented Brown Milk

The acidity and viable counts of milk and brown milk fermentation by *L. bulgaricus* ND02 are shown in Table 1. The pH decreased within the process of fermentation of milk and brown milk by *L. bulgaricus* ND02, and the TA gradually increased; the acidification continued until reaching the fermentation endpoint of pH

4.5 after around 7 h. The TA ($^{\circ}$ T) of fermented milk and fermented brown milk was 86.24 $^{\circ}$ T and 83.12 $^{\circ}$ T, respectively; both were between 70 and 110 $^{\circ}$ T, indicating that *L. bulgaricus* ND02 had the ability to produce acid and the acidity was within an acceptable range for consumers (Sudun et al., 2013). The viable count of the LAB directly affected the quality and function of fermented dairy products, and the viable counts of *L. bulgaricus* ND02 in both the fermented milk and brown fermented milk were above 10^8 cfu/mL, indicating that *L. bulgaricus* ND02 had good fermentation performance.

Analysis of UPLC-Q-TOF MS^E Chromatograms

Typical base peak intensity chromatograms of the 4 types of milk samples, including milk, brown milk, fermented milk, and fermented brown milk, are shown in Figure 1. The chromatograms depict the changes in intensity of detected ion signals during each run, and they reflect the overall information of the sample. The chromatographic profiles of the fermented milk and fermented brown milk were highly similar, and those of milk and brown milk were also similar to each other. However, significant differences were seen in the chromatographic profiles between nonfermented and fermented milks.

Metabolomics Analysis

Quality Control. A QC standard was prepared by pooling equal aliquots of all samples. The injections showed a stable run condition with small variation (less than ± 2 SD; Figure 2A). The close clustering of the QC samples on the PCA score plot confirmed that the chromatographic runs were stable and reproducible (Figure 2B; Figure 3). The 4 sample groups clustered distinctly on the PCA score plot on ESI^+ , and most sample groups except fermented milk clustered closely on the PCA score plot on ESI^- (Figure 3).

Changes in the Milk Metabolome After the Maillard Reaction. A total of 2,523 spectral features were yielded in the milk and brown milk metabolomes generated in ESI^+ and ESI^- modes after data filtering and missing value removal. The OPLS-DA of the milk

and brown milk metabolomes was performed (Figure 4A), which was a supervised modeling method. The milk samples were located distantly from the brown milk samples, indicating that marked differences existed between the metabolomes of the 2 sample groups. In ESI⁺ mode, the cumulative R²X, R²Y, and Q² of the OPLS-DA between the milk and brown milk groups were 0.710, 1, and 0.958, respectively (Table 2). In ESI⁻ mode, the cumulative R²X, R²Y, and Q² of the OPLS-DA between the milk and brown milk groups were 0.790, 0.995, and 0.754, respectively (Table 2). Both OPLS-DA models showed goodness-of-fit and high prediction ability. Permutation tests were carried out to confirm the stability and robustness of the OPLS-DA models presented in this study (Figure 4B). The permutations for 200 times was applied to assess the risk that the current OPLS-DA model was spurious, and

the results indicated no overfitting (Worley and Powers, 2016). To identify significant metabolites that contributed to the discrimination, an S-plot (Figure 4C) was generated from the OPLS-DA regression. In the S-plot (Figure 4C), red squares indicated substances with VIP value >1. The S-plot was used to identify differential compounds based on covariance and loading profiling correlation of each metabolite. Each dot in the S-plot represented a single metabolite. Metabolites with large contribution and significant differences would be located away from the origin on the S-plot, which would be considered as potential biomarker candidates.

Significant biomarker candidates fulfilled the following criteria: VIP value > 1, *P* < 0.05, and fold-change > 2. The significant differential retention time exact mass pairs obtained from Mummichog and OPLS-DA predictions were selected and imported back into

Figure 1. Representative based peak intensity chromatograms of different milk samples. (A) Milk; (B) brown milk; (C) fermented milk; (D) fermented brown milk.

Figure 2. Assessment of quality control (QC) samples. (A) Trend plot showing the variation of $t[1]$ against all QC runs. The x-axis represents the QC sample number, and the y-axis, $t[1]$, represents the fluctuation of signal of QC samples in arbitrary units. The upper and lower ranges up to 3 SD are shown. (B) Principal component analysis (PCA) score plot of the milk samples and QC samples. $t[1]$ and $t[2]$ are the 2 most important new variables in summarizing the dataset.

Progenesis QI software for compound identification. Then, 3 individual searches were applied as follows: (1) peak picking threshold of sensitivity was 3; (2) the elemental composition should fit precursor tolerance within 1 ppm difference of the candidate compounds searched by online available databases such as Chemspider (<http://www.chemspider.com/>) and Kyoto Encyclopedia of Genes and Genomes (<https://www.kegg.jp/>); and (3) fragment m/z should be selected in the high-energy function (MS^E). The 3 individual searches methods resulted in 56 signals from the 2,523 identified spectral features, and they were considered remarkably different between the milk and brown milk groups. A total of 20 robust known chemical markers

(Table 3) were identified. The diagnostic accuracy of the differential metabolites identified between milk and brown milk samples was also evaluated, and 17 showed a good diagnostic value by area under the curve (AUC) >0.8 (Table 3). To visualize chemical changes after the Maillard reaction, a heatmap was generated to show the relative abundances of identified differential components in individual milk and brown milk samples (Figure 4E).

Multivariate Analysis of the Fermented Brown Milk and Brown Milk Metabolomes. Similar methods described above were used to generate OPLS-DA models and identify differential metabolites between fermented brown milk and brown milk (Figure 5A). A

Figure 3. Principal component analysis (PCA) score plots of 4 groups of samples in (A) positive [electrospray ionization (ESI^+)] and (B) negative (ESI^-) ion modes. Sample groups in this analysis: brown milk (BM), fermented brown milk (FBM), fermented milk (FM), and milk (M). QC samples run in ESI^+ mode and ESI^- are represented by POS-QC and NEG-QC, respectively. Six samples were analyzed from each group. Each color symbol represents the metabolome of one sample. The x-axis represents the correlation coefficients of principal components and metabolites, and the y-axis represents the correlation coefficient between principal components and metabolites.

Figure 4. Metabolomic analysis of brown milk (BM) and milk (M). (A) Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA), (B) permutation test (200 random permutations), (C) receiver operating characteristic (ROC) curve, and (D) S-plot of orthogonal partial least squares discriminant analysis (OPLS-DA) of BM and milk M metabolomes. Red squares indicate substances with variable importance in projection (VIP) value > 1 . (E) Heatmap showing the relative abundance of differential metabolites between BM and M. In panel B, the y-axis represents the values of R and Q, and the x-axis is the similarity to the original model. M2.DA = drawing number in the software. TPR = true positive rate. POS = receiver operating characteristic (ROC) curve in positive [electrospray ionization (ESI)⁺] ion modes. The color scale represents the relative abundance of significant differential abundant metabolites: blue indicates the lowest abundance and red the highest. The relative abundance is preprocessed by median centering, Log2 transformation, and Pareto scaling.

total of 2,809 spectral features were retained after data filtering. The OPLS-DA model achieved significant separation between groups and showed goodness-of-fit

and high prediction ability (ESI⁺: $R^2X = 0.772$, $R^2Y = 1$, $Q^2 = 0.987$; ESI⁻: $R^2X = 0.726$, $R^2Y = 0.956$, $Q^2 = 0.892$; Table 2). The permutation test showed that

Table 2. Pairwise comparison of data sets generated in positive ion mode and negative ion mode using principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) models

Mode and sample pair	Statistical model	R ² X (cumulative)	R ² Y (cumulative)	Q ² (cumulative)
Positive ion mode				
Brown milk and milk	PCA	0.539	—	0.219
	OPLS-DA	0.710	1.000	0.958
Fermented brown milk and brown milk	PCA	0.645	—	0.393
	OPLS-DA	0.772	1.000	0.987
Fermented milk and milk	PCA	0.966	—	0.943
	OPLS-DA	0.990	0.995	0.995
Fermented brown milk and fermented milk	PCA	0.623	—	0.386
	OPLS-DA	0.753	1.000	0.965
Negative ion mode				
Brown milk and milk	PCA	0.621	—	0.091
	OPLS-DA	0.790	0.995	0.754
Fermented brown milk and brown milk	PCA	0.513	—	0.122
	OPLS-DA	0.726	0.956	0.892
Fermented milk and milk	PCA	0.873	—	0.739
	OPLS-DA	0.965	0.960	0.820
Fermented brown milk and fermented milk	PCA	0.621	—	0.208
	OPLS-DA	0.776	1.000	0.964

the supervised models had good stability and robustness (Figure 5B). In the S-plots, each scattered point represented a variable, and the further away from the origin, the greater the contribution of this variable to the difference between groups. In ESI⁺ mode, 246 metabolites showed significant deviation away from the origin (VIP value >1), which were potential differential metabolites between these 2 sample groups (Figure 5D). An individual search for metabolites of VIP value > 1 identified a total of 493 signals from the 2,809 spectral

features that showed marked difference in abundance between the fermented brown milk and brown milk. A total of 21 significant differential metabolites were further selected based on the criteria: VIP value >1, $P < 0.05$, and fold-change >2 (Table 4). Sixteen of the 21 differential metabolites showed a good diagnostic value of AUC >0.8, including L-lysine, Glu-Glu, and 2-methylquinolin-4-ol (Table 4). A heatmap was generated to visualize differences in the relative abundances of these differential abundant metabolites (Figure 5E).

Table 3. Significant differential metabolites between brown milk and milk¹

Metabolite no.	Compound	Formula	Actual mass	RT (min)	VIP value	P-value	log ₂ (FC) (BM/M)	AUC
1	L-Lysine	C ₆ H ₁₄ N ₂ O ₂	146.1055	1.51	1.63696	1.97E-06	-6.7087	1
2	L-Phenylalanine	C ₉ H ₁₁ NO ₂	165.1900	1.61	1.29904	4.13E-06	-6.6437	1
3	L-Asparagine	C ₄ H ₈ N ₂ O ₃	132.0535	3.38	1.46968	7.29E-04	-6.6991	1
4	L-Leucine	C ₆ H ₁₃ NO ₂	131.0946	3.84	1.42895	4.53E-04	-7.1923	1
5	L-Glycine	C ₂ H ₅ NO ₂	131.1000	2.69	1.23535	6.59E-03	-3.0096	0.9444
6	L-Alanine	C ₃ H ₇ NO ₂	89.0477	0.33	1.31996	2.36E-03	-7.0079	1
7	L-Serine	C ₃ H ₇ NO ₃	105.0426	5.68	2.27095	9.52E-04	-6.6437	1
8	L-Proline	C ₅ H ₉ NO ₂	165.1900	1.60	1.20063	3.07E-02	-2.2153	1
9	Acrylamide	C ₃ H ₅ NO	71.0371	1.45	1.54318	2.69E-05	4.0223	1
10	Carboxy methyl lysine	C ₈ H ₁₆ N ₂ O ₄	204.2236	1.28	1.67833	1.67E-14	8.2360	1
11	4-Hydroxypropylleucine	C ₅ H ₉ NO ₃	131.1300	0.90	1.45412	2.69E-04	6.8442	—
12	Glutaminylcysteine	C ₂₆ H ₃₁ N ₄ O ₆	496.5700	1.60	1.66272	7.40E-10	8.2287	—
13	6-Aminotryptophan	C ₁₁ H ₁₃ N ₃ O ₂	219.1088	0.61	2.31326	5.30E-04	7.2703	—
14	1-(4-Propoxy-phenyl)-pyrrole-2,5-dione	C ₁₃ H ₁₃ NO ₃	231.5867	2.45	1.41189	5.99E-04	6.3655	1
15	1-(3-Pyridyl)-1-butanone	C ₉ H ₁₁ NO	149.0940	3.09	1.67665	2.58E-13	1.2874	0.88889
16	5-Ethyl-4-hydroxy-3(2H)-furanone	C ₆ H ₈ O ₃	442.4800	5.91	1.54674	2.20E-05	7.3361	1
17	2-Heptyl-1,3-dithiane	C ₁₁ H ₂₂ S ₂	220.1239	3.09	1.34043	1.86E-03	4.0921	1
18	2,2,4-Trimethyl-1,3-dithiane	C ₇ H ₁₄ S ₂	130.1760	0.64	1.6213	3.83E-07	6.9986	1
19	Bipyrazine	C ₈ H ₆ N ₄	158.0661	0.95	1.20063	9.13E-03	5.0440	—
20	2-(2-Furyl)pyridine	C ₉ H ₇ NO	146.0607	1.33	1.67933	2.42E-16	8.4849	1

¹RT = retention time; VIP = variable importance in projection; FC = fold-change; BM = brown milk; M = milk; AUC = area under the curve (only metabolites with AUC value >0.8 are shown).

Multivariate Analysis of the Fermented Milk and Milk Metabolomes. Multivariate analysis was performed between the fermented milk and milk me-

tabolomes (Figure 6). In total, 2,989 spectral features were retained after data filtering. The OPLS-DA model achieved significant separation between groups. For the

Figure 5. Metabolomic analysis of fermented brown milk (FBM) and brown milk (BM). (A) Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA), (B) permutation test (200 random permutations), (C) receiver operating characteristic (ROC) curve, and (D) S-plot of orthogonal partial least squares discriminant analysis (OPLS-DA) of FBM and BM metabolomes. Red squares indicate substances with variable importance in projection (VIP) value >1. (E) Heatmap showing the relative abundance of differential metabolites between fermented brown milk and brown milk. In panel B, the y-axis represents the values of R and Q, and the x-axis is the similarity to the original model. TPR = true positive rate. POS = receiver operating characteristic (ROC) curve in positive [electrospray ionization (ESI)⁺] ion modes. The color scale represents the relative abundance of significant differential abundant metabolites: blue indicates the lowest abundance and red the highest. The relative abundance is preprocessed by median centering, Log₂ transformation, and Pareto scaling.

Table 4. Significant differential metabolites between fermented brown milk and brown milk¹

Metabolite no.	Compound	Formula	Actual mass	RT (min)	VIP value	P-value	log2(FC) (FBM/BM)	AUC
1	L-Lysine	C ₆ H ₁₄ N ₂ O ₂	146.1055	2.14	1.29619	1.47E-03	9.0539	1
2	L-Proline	C ₅ H ₉ NO ₂	115.0633	3.79	1.64813	3.53E-13	9.6729	1
3	N,N,N-Trimethylglycine	C ₅ H ₁₁ NO ₂	117.0790	3.34	1.5317	1.42E-05	9.0539	1
4	L-Glutamic acid	C ₅ H ₉ NO ₄	147.0532	0.70	1.3816	9.20E-04	8.0323	1
5	N-Acetyl-L-glutamic acid	C ₇ H ₁₁ NO ₅	189.0637	7.07	1.3816	6.56E-04	9.5629	0.91667
6	Gly-Gly-Pro	C ₉ H ₁₅ N ₃ O ₄	457.2000	0.33	1.25585	4.49E-03	3.3611	0.94444
7	Gly-Gln-Ala-Arg	C ₁₆ H ₃₀ N ₆ O ₆	428.2300	5.68	1.22857	1.07E-02	9.3372	1
8	Asp-Trp-Asp-Ser	C ₂₂ H ₂₇ N ₅ O ₁₀	520.1700	1.60	1.2252	1.03E-02	9.3726	—
9	Glu-Glu	C ₁₂ H ₁₆ N ₄ O ₅	335.0700	1.45	1.39395	2.68E-04	3.1861	1
10	2-Methylquinolin-4-ol	C ₁₀ H ₉ NO	159.0684	3.79	1.64057	8.87E-11	9.5570	1
11	(S)-2-Aceto-2-hydroxybutanoate	C ₆ H ₁₀ O ₄	146.0579	7.69	1.33338	1.47E-03	5.7179	1
12	Galactosylglycerol	C ₉ H ₁₈ O ₈	254.1002	0.58	1.39176	5.66E-04	9.5389	1
13	N-Succinyl-L-LL-2,6-diaminoheptanedioate	C ₁₁ H ₁₈ N ₂ O ₇	290.1114	5.69	1.60635	2.68E-07	9.4835	1
14	L-Rhamnulose 1-phosphate	C ₆ H ₁₃ O ₈ P	244.0348	3.56	1.64861	6.94E-14	9.6039	1
15	Pyridoxal	C ₈ H ₉ NO ₃	167.0582	3.79	1.64495	5.12E-12	9.6211	1
16	4-Hydroxy-3-methoxybenzoic acid	C ₈ H ₈ O ₄	168.0423	2.35	1.18448	1.03E-02	9.3726	0.83333
17	Guanine	C ₅ H ₅ N ₅ O	151.0494	0.68	1.49706	4.86E-05	−9.468	—
18	Hypoxanthine	C ₅ H ₄ N ₄ O	136.0385	0.56	1.64688	3.83E-13	−9.461	1
19	Cytosine	C ₄ H ₅ N ₃ O	111.0433	6.80	1.65069	3.63E-19	−9.4259	—
20	Uridine	C ₉ H ₁₂ N ₂ O ₆	244.0695	0.52	1.59743	2.63E-09	9.7548	—
21	Vitamin A	C ₂₀ H ₃₀ O	286.4500	3.78	1.51778	2.54E-05	7.8521	—

¹RT = retention time; VIP = variable important in the projection; FC = fold-change; FBM = fermented brown milk; BM = brown milk; AUC = area under the curve (only metabolites with AUC value > 0.8 are shown).

ESI⁺ mode, the cumulative R²X, R²Y, and Q² of the OPLS-DA model between the fermented milk and milk group were 0.990, 0.995, and 0.995, respectively (Table 2). For the ESI[−], the cumulative R²X, R²Y, and Q² of the OPLS-DA model between these 2 sample groups were 0.965, 0.960, and 0.820, respectively (Table 2). Both OPLS-DA models showed goodness-of-fit and high prediction ability (Figure 6B). The S-plot showed a plurality of ions away from the origin, indicating the presence of differential metabolites between the fermented milk and milk samples (Figure 6D).

The 3 individual searches found 68 signals out of the 2,809 spectral features that showed marked differential abundance between these 2 milk groups. A total of 23 robust chemical markers were identified (Table 5). Seventeen of these differential metabolites showed good diagnostic value with AUC > 0.8 (Table 5). A heatmap was generated to visualize differences in the relative abundances of these differential abundant metabolites (Figure 6E).

Multivariate Analysis of Fermented Brown Milk and Fermented Milk Metabolomes. Multivariate analysis was performed between the fermented brown milk and fermented milk metabolomes (Figure 7). A total of 1,621 spectral features were retained after data filtering. The OPLS-DA models of the fermented milk and the milk groups achieved significant separation in both ESI⁺ mode (cumulative R²X, R²Y, and Q² were 0.753, 1, and 0.965, respectively; Table 2) and

ESI[−] mode (cumulative R²X, R²Y, and Q² were 0.776, 1, and 0.964, respectively; Table 2). Both OPLS-DA models showed goodness-of-fit and high prediction ability. The permutation test showed that the supervised models had good stability and robustness (Figure 7B). The S-plot found a plurality of ions away from the origin, suggesting the presence of differential metabolites between these 2 sample groups (Figure 7D). By performing the 3 metabolite searches described above, a total of 16 significant differential metabolites were identified between these 2 sample groups (Table 6), and 6 of these showed a high AUC value > 0.8 (Table 6). A heatmap was generated to visualize differences in the relative abundances of these differential abundant metabolites (Figure 7E). Differences in the abundance of these 6 metabolites between the 2 fermented milk types are shown in Figure 8A, and the ROC curves of these differential metabolites are shown in Figure 8B.

Prediction of Mummichog Pathway Activity Between Sample Pairs. The Mummichog pathway activity was predicted between different sample pairs (Figure 9). Pathways related to pyrimidine metabolism, lysine biosynthesis, and pantothenate and CoA biosynthesis were enriched in the metabolomes of the fermented brown milk compared with brown milk ($P < 0.05$; Figure 9A). Pathways related to valine, leucine and isoleucine degradation, riboflavin metabolism, and phenylalanine metabolism were enriched in the metabolomes of the fermented milk compared with milk

Figure 6. Metabolomic analysis of fermented milk (FM) and milk (M). (A) Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA), (B) permutation test (200 random permutations), (C) receiver operating characteristic (ROC) curve, and (D) S-plot of orthogonal partial least squares discriminant analysis (OPLS-DA) of FM and M metabolomes. Red squares indicate substances with variable importance in projection (VIP) value >1 . (E) Heatmap showing the relative abundance of differential metabolites between fermented milk and milk. In panel B, the y-axis represents the values of R and Q, and the x-axis is the similarity to the original model. TPR = true positive rate. POS = receiver operating characteristic (ROC) curve in positive [electrospray ionization (ESI)⁺] ion modes. The color scale represents the relative abundance of significant differential abundant metabolites: blue indicates the lowest abundance and red the highest. The relative abundance is preprocessed by median centering, $\log_2$ transformation, and Pareto scaling.

($P < 0.05$; Figure 9B). Pathways related to taurine and hypotaurine metabolism, pyrimidine metabolism, and lysine biosynthesis were enriched in the metabolomes of the fermented brown milk compared with fermented milk ($P < 0.05$; Figure 9C).

DISCUSSION

Fermented dairy products are popular worldwide, and they have good nutritional values. In recent years, new fermented milk products have been developed

(e.g., fermented brown milk, which has a unique flavor, rich aroma, and nutrition). To understand the chemical nature of fermented brown milk, this work explored the metabolomic differences between milk and brown milk to describe chemical changes that occurred during the Maillard reaction. Meanwhile, the metabolomic profiles between fermented milk and fermented brown milk were compared to identify differential metabolites responsible for the unique features of fermented brown milk.

Metabolomic Changes During the Maillard Reaction

The reconstituted milk was heated to 95°C for 3 h for browning to occur. Then, LC-MS was used to analyze differential metabolites between brown milk and milk samples. Significant changes in the milk metabolomes occurred after browning, and 21 significant differential metabolites were detected, which mainly belonged to the following: AA, peptides, bovine milk lipids, organic acids, nucleosides, vitamins, aldoketones, dithianes, pyrazines, pyrrolines, and amide compounds.

Significant changes occurred in the milk AA composition after the Maillard reaction took place. Eight free AA, including L-lysine, L-phenylalanine, L-asparagine, L-leucine, L-glycine, L-alanine, L-serine, and L-proline, significantly decreased in the brown milk compared

with milk (Table 3; Figure 4E). Milk is a highly perishable food material that easily undergoes physical, chemical, and nutritional changes upon processing. Heat treatment of milk leads to protein degradation, and chemical changes occur between the reducing sugars (mainly lactose, glucose, and galactose) and amino groups of the protein (mainly lysine in milk CN and whey proteins) in nonenzymatic Maillard browning (Gu et al., 2009). When both components are present, the reaction might occur. Particularly, the content of lysine significantly decreases in the Maillard browning process. The Maillard reaction is divided into the following 3 stages: primary, advanced, and final stages. During the early stage, lactose interacts with lysine and the reaction continues, forming a Schiff's base and undergoing Amadori rearrangement to constitute lactulosyllysine (Aalaei et al., 2019). The reactivity of free AA with glucose ranks as follows: lysine > glycine > tryptophan > tyrosine > histidine > arginine > cysteine (Ashoor and Zent, 1984).

Moreover, our data showed that there were more AA derivatives in brown milk compared with milk, including acrylamide, carboxymethyl lysine, 4-hydroxypropyl-leucine, glutamylcysteine, and 6-aminotryptophan. Acrylamide can be released by thermal treatment of certain AA (e.g., asparagine), particularly in combination with reducing sugars, and Maillard-driven

Table 5. Significant differential metabolites between fermented milk and milk¹

Metabolite no.	Compound	Formula	Actual mass	RT (min)	VIP value	P-value	log2(FC) (FM/M)	AUC
1	L-Valine	C ₅ H ₁₁ NO ₂	117.15	2.01	1.08962	2.86E-07	5.2715	—
2	L-Isoleucinamide	C ₆ H ₁₃ NO ₂	131.10	4.99	1.09822	02.12E-17	7.7715	1
3	L-Methionine	C ₅ H ₁₁ NO ₂ S	149.21	5.25	1.09008	2.66E-14	7.7744	1
4	L-Phenylalanine	C ₉ H ₁₁ NO ₂	165.19	5.47	1.03694	2.66E-14	7.6175	1
5	Ile-Lys-Gly-Pro	C ₁₉ H ₃₅ N ₅ O ₅	431.44	2.45	1.04081	4.90E-14	7.4721	—
6	Pro-Trp	C ₁₆ H ₁₉ N ₃ O ₃	337.37	1.45	1.09061	9.77E-05	3.5098	1
7	Val-Ala-Pro-Phe	C ₂₂ H ₃₂ N ₄ O ₅	432.53	0.64	1.0180	1.09E-02	-5.2016	0.88889
8	Trp-Arg	C ₁₇ H ₂₄ N ₆ O ₃	360.42	5.01	1.08899	1.19E-06	11.8020	1
9	Ile-Pro	C ₁₁ H ₂₀ N ₂ O ₃	228.29	2.35	1.0747	2.75E-16	8.5665	—
10	Pyrimidinedione	C ₄ H ₄ N ₂ O ₂	112.0269	2.56	1.08955	3.16E-07	7.8676	1
11	Glyceric acid	C ₃ H ₈ O ₁₀ P ₂	266.04	0.65	1.0172	1.03E-02	-1.8823	—
	1,3-biphosphate							
12	Lysophosphatidylcholine	C ₂₆ H ₅₄ N ₇ O ₇ P	523.70	2.30	1.0165	4.46E-02	10.5896	1
13	Ethyl acetate	C ₄ H ₈ O ₂	88.1051	0.62	1.02010	1.27E-02	-10.1082	—
14	L-Carnitine	C ₇ H ₁₅ NO ₃	161.20	3.26	1.09045	1.45E-16	7.9116	1
15	Glycerophosphocholine	C ₈ H ₂₁ NO ₆ P	258.23	4.83	1.07942	1.04E-16	8.9907	1
16	Uric acid	C ₅ H ₄ N ₄ O ₃	168.11	2.07	1.03100	5.91E-13	9.9755	1
17	Benzoic acid	C ₇ H ₆ O ₂	122.12	5.00	1.06336	3.59E-15	7.3239	1
18	Lactic acid	C ₃ H ₆ O ₃	90.0317	0.89	1.03101	02.66E-15	12.188	1
19	Xanthine	C ₅ H ₄ N ₄ O ₂	152.11	4.69	1.09069	8.21E-18	9.6390	—
20	Paraxanthine	C ₇ H ₈ N ₄ O ₂	180.16	0.59	1.02018	1.08E-02	1.5746	0.88889
21	Vitamin A	C ₂₀ H ₃₀ O	286.45	3.82	1.08103	1.07E-16	8.4480	1
22	Riboflavin	C ₁₇ H ₂₀ N ₄ O ₆	376.36	2.99	1.0338	2.46E-05	-1.0070	1
23	Nicotinamide-N-oxide	C ₆ H ₆ N ₂ O ₂	138.12	3.62	1.0864	6.18E-08	3.1340	1

¹RT = retention time; VIP = variable important in the projection; FC = fold-change; FM = fermented milk; M = milk; AUC = area under the curve (only metabolites with AUC value > 0.8 are shown).

acrylamide generation influences the flavor and color in thermally processed milk (Mottram et al., 2002). Some compounds formed during the Maillard reaction

have caused debates related to health concerns (e.g., carboxymethyl lysine, an Amadori degradation compound; Aalaei et al., 2019).

Figure 7. Metabolomic analysis of fermented brown milk (FBM) and fermented milk (FM). (A) Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA), (B) permutation test (200 random permutations), (C) receiver operating characteristic (ROC) curve, and (D) S-plot of orthogonal partial least squares discriminant analysis (OPLS-DA) of FBM and FM metabolomes. Red squares indicate substances with variable importance in projection (VIP) value >1. (E) Heatmap showing the relative abundance of differential metabolites between fermented brown milk and fermented milk. In panel B, the y-axis represents the values of R and Q, and the x-axis is the similarity to the original model. TPR = true positive rate. POS = receiver operating characteristic (ROC) curve in positive [electrospray ionization (ESI)⁺] ion modes. The color scale represents the relative abundance of significant differential abundant metabolites: blue indicates the lowest abundance and red the highest. The relative abundance is preprocessed by median centering, Log2 transformation, and Pareto scaling.

Our results showed that there were generally more flavor components in the brown milk than in milk, including aldehydes, ketones, alcohol, esters, organic acids, and furan compounds. The 2,3-enolization route or 1-deoxyosone-pathway occurs under neutral conditions in the advanced stage of the Maillard reaction and produces 1-deoxyketo aldose, which further produces reducing ketones and dehydro-reducing ketones, such as β -pyranone, 3-furanone, reductones, α -dicarbonyls, cyclopentenone, galactosylisomaltol, and acetylpyrrole (Aalaei et al., 2019). These are common chemical reactions that happen in dairy products of near-neutral pH in the presence of lactose. Flavor components, such as acetaldehyde, 2-furaldehyde, and furfural, are produced during the browning stage, and they provide the characteristic flavor of these products (O'Brien, 2009). Our study did not detect the entire spectrum of flavor substances mentioned above. However, significantly more aldehydes and ketones were found in the brown milk, including 1-(4-propoxy-phenyl)-pyrrole-2,5-dione, 1-(3-pyridyl)-1-butanone, and 5-ethyl-4-hydroxy-3(2H)-furanone. The contribution of the detected compounds of the brown milk remains to be explored.

Apart from aldehydes, ketones, lactones, and nitrogen-containing compounds, the flavor compounds in dairy products might also include sulfur-containing compounds. Higher levels of sulfur compounds (2-heptyl-1,3-dithiane and 2,2,4-trimethyl-1,3-dithiane) were detected in brown milk compared with milk. The carbonyl intermediates of the Maillard reaction might

react with sulfur compounds released in heated milk (Yu et al., 2012); however, the interactions between sulfhydryl compounds formed in milk and the intermediates derived from the Maillard reaction are not well-characterized. The strong odor of low-molecular-weight sulfur compounds might also impart special organoleptic properties to brown milk (O'Brien, 2009). Other typical sulfur-containing flavor compounds in milk and dairy products are methyl mercaptan, dimethyl disulfide, and dimethyl sulfide. Although the 2 sulfur-containing compounds detected in this study (i.e., 2-heptyl-1,3-dithiane and 2,2,4-trimethyl-1,3-dithiane) have not been reported in literature, they likely contribute to the unique flavor of brown milk.

Distinction Between Fermented Brown Milk and Fermented Milk Metabolomes

Different from traditional drinking yogurt, the production of brown dairy products requires a high-temperature Maillard reaction that alters the milk components before fermentation. Thus, the starter bacteria interact with milk and brown milk differently to impart the unique color and aroma to fermented brown milk (Ni et al., 2017). It was anticipated that the 2 types of fermented milk products would have rather different metabolomic profiles. Our LC-MS analysis identified 8 types of significant differential metabolites or potential discriminatory biomarkers between the 2 types of fermented milk, involving pathways related to

Table 6. Significant differential metabolites between fermented brown milk and fermented milk¹

Metabolite no.	Compound	Formula	Actual mass	RT (min)	VIP value	P-value	log ₂ (FC) (FBM/FM)	AUC
1	L-Lysine	C ₆ H ₁₄ N ₂ O ₂	146.1055	5.59	1.18265	1.02E-02	-8.2062	0.88883
2	(R)-4'-Phosphopantothenoyl-L-cysteine	C ₁₂ H ₂₃ N ₂ O ₉ PS	402.0862	2.14	1.50885	7.18E-05	-4.8768	—
3	Carboxymethyl lysine	C ₈ H ₁₆ N ₂ O ₄	204.2236	3.21	1.35892	1.38E-03	6.4056	—
4	L-Methionine S-oxide	C ₅ H ₁₁ NO ₃ S	165.046	5.25	1.51815	5.04E-05	-3.6014	—
5	Glutaurine	C ₇ H ₁₄ N ₂ O ₆ S	254.0573	10.46	1.66636	5.96E-11	12.8077	1
6	L-Glutamyl-L-cysteinyl-glycin	C ₁₀ H ₁₇ N ₃ O ₆ S	307.0838	1.87	1.48246	1.24E-04	-3.18	—
7	Methylglyoxal	C ₃ H ₄ O ₂	72.0211	1.56	1.35892	1.38E-03	6.4056	1
8	Glyoxal	C ₂ H ₂ O ₃	74.0004	1.79	1.48157	1.35E-04	3.1393	1
9	2,3-Pentanedione	C ₅ H ₈ O ₂	100.1158	2.12	1.39181	7.88E-04	4.787	1
10	Glycerophosphocholine	C ₈ H ₂₁ NO ₆ P	258.1106	2.50	1.48157	1.35E-04	-3.1393	—
11	N ⁶ -Acetyl-L-2,6-diaminopimelate	C ₉ H ₁₆ N ₂ O ₅	232.1059	1.44	1.19731	8.94E-03	-2.3998	—
12	N-Acetylmuramic acid 6-phosphate	C ₁₁ H ₂₀ NO ₁₁ P	373.0774	0.70	1.66803	2.73E-11	7.6504	—
13	3-Hydroxybutanoic acid	C ₄ H ₈ O ₃	104.0473	3.07	1.18142	1.02E-02	8.1007	0.88883
14	Butyryl-CoA	C ₂₅ H ₄₂ N ₇ O ₁₇ P ₃ S	837.1571	5.21	1.41188	5.64E-04	-8.3105	—
15	Cinnabarinic acid	C ₁₄ H ₈ N ₂ O ₆	300.0382	2.15	1.67405	2.14E-14	-8.3073	—
16	Spermidine	C ₇ H ₁₉ N ₃	145.1579	2.07	1.54912	1.69E-05	-6.3363	—

RT = retention time; VIP = variable important in the projection; FC = fold-change; FBM = fermented brown milk; FM = fermented milk; AUC = area under the curve (only metabolites with AUC value > 0.8 are shown).

Figure 8. Five significant differential metabolites between fermented brown milk (FBM) and fermented milk (FM). (A) Differences in relative abundance of identified differential metabolites. The box indicates the concentrated range of the data. The horizontal line in the middle of the box represents the median of the distribution. Whiskers indicate the extent to which the data extend up and down. Circles represent outliers. The red horizontal line indicates the optimal cutoff. (B) Multivariate receiver operating characteristic (ROC) curves of 5 significant differential metabolites.

pyrimidine metabolism, arginine biosynthesis, butanoate metabolism, lysine biosynthesis, glycine, serine and threonine metabolism, arginine and proline metabolism, and taurine and hypotaurine metabolism. These pathways were mainly related to Maillard reaction-induced metabolic differences.

Amino Acids and Their Derivatives. Significant differences were found in the profile of AA-related compounds between the fermented brown milk and fermented milk metabolomes. Significantly less L-lysine, (R)-4'-phosphopantothienoyl-L-cysteine, and L-methionine S-oxide were detected in the fermented brown milk. As fermentation progressed, the starter culture *L. bulgaricus* used the milk proteins to sustain bacterial growth (Dong et al., 2017). Lysine reacts with reducing

sugars during Maillard browning, which could explain the lower lysine level in the brown milk. We found that the L-lysine level in the fermented brown milk was much higher than that in brown milk, which could be caused by milk protein degradation by the starter bacteria during fermentation (Dong et al., 2017).

Some common changes in the AA profiles were observed in both fermented milk and fermented brown milk. For example, the levels of some free AA significantly increased after fermentation, such as L-isoleucine, L-phenylalanine, L-methionine, and L-valine, which were also reported previously in fermented milk produced by *L. bulgaricus* (Zhou et al., 2012). A high level of branched-chain AA in dairy products (e.g., L-isoleucine and L-threonine) act synergistically with calcium to ac-

Figure 9. Mummichog pathway activity predicted between different milk sample pairs. (A) Fermented brown milk and brown milk; (B) fermented milk and milk; and (C) fermented brown milk and fermented milk.

celerate weight loss via adiposity attenuation (Zemel, 2004). Free AA also contribute to flavor by themselves or as precursors in the formation of other compounds (Hillmann and Hofmann, 2016). Thus, the rich variety of AA in the fermented brown milks not only serves as important nutrients and main taste contributors but also health-promoting bioactive components of these products.

Peptides. The content of peptides differed greatly between the fermented brown milk and fermented milk metabolomes. Fermented brown milk contained a much higher level (7,169.6 times higher) of glutathione than fermented milk. Glutathione is an active tripeptide formed by the condensation of glutamic acid, cysteine and glycine through peptide bonds, and it contains cysteine residues of sulfhydryl groups, making it the main intracellular antioxidant that serves important physiological functions in organisms (Zhu 2008). Maillard products were fermented by *Lactobacillus gasseri* H10 and *Lactobacillus fermentum* H9, and the overall preventive cardiovascular activity was improved. Also, they

identified the production of small-molecule compounds from fermented Maillard reaction products (MRP) that probably had greater cardiovascular preventive effects than nonfermented (MRP) (Oh et al., 2014). These beneficial effects could be related to the antioxidation activity of glutathione in regulating cholesterol synthesis and metabolism (Oh et al., 2016). However, the biological function of the fermented brown milk produced in this study remains to be experimentally confirmed.

During fermentation, the starter bacteria (*L. bulgaricus*) actively hydrolyzed the milk protein into polypeptides and AA (Zheng et al., 2012). A total of 5 identified differential metabolites were peptides in nature. The contents of 4 peptides, Ile-Lys-Gly-Pro, Ile-Pro, Trp-Arg, and Pro-Trp were significantly higher in fermented milk compared with fermented brown milk ($P < 0.05$), whereas significantly ($P < 0.05$) less Val-Vla-Pro-Phe was detected in fermented milk. Three of the 4 peptides that were found to be increased, Ile-Pro, Pro-Trp, and Trp-Arg, are human dipeptidyl peptidase IV (hDPP-IV) inhibitors that could reduce blood glu-

cose concentration and ameliorate type 2 diabetes (Lan et al., 2015).

Fermentation of brown milk also increased the content of other peptides. Some of these peptides might contribute to the unique sensory quality of fermented brown milk (Li et al., 2020). Some other peptides identified in this study have been reported to influence the product taste (e.g., Gly-Gly-Pro and Gly-Gln-Ala-Arg were found to relate to sweetness and sourness, respectively; Li et al., 2020). Glu-Glu and Asp-Trp-Asp-Ser were found to be umami (Li et al., 2020). The functional properties and beneficial effects of short peptides identified in the 2 types of fermented milks produced by ND02 need to be further investigated.

Aldehydes and Ketones. The levels of aldehyde and ketone metabolites were higher in fermented brown milk than in fermented milk. The levels of methylglyoxal, glyoxal, and 2,3-pentanedione were 87.80 times, 8.81 times and 27.61 times higher in the fermented brown milk than the fermented milk, respectively. Aldehydes are another important class of flavor substances in yogurt that have a relatively low flavor threshold. Previous studies have found that acetaldehyde, the main flavor substance in yogurt, is produced in fermentation by *L. bulgaricus* (Dan et al., 2012). Methylglyoxal and glyoxal could be produced during the *Lactobacillus* growth (O'Brien, 2009). Ketones are important flavor components of yogurt, which are produced by the oxidation and thermal degradation of polyunsaturated fatty acids, microbial metabolism, and AA degradation. 2,3-Pentanedione is associated with a creamy aroma and buttery aroma (Li et al., 2013b).

Lipid Metabolites. Lipid metabolites are another chemical group associated directly with the taste and flavor quality of fermented products. Bovine milk lipids mainly consist of triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, phospholipids, and sterols. Four compounds related to lipid metabolism were identified between different milk metabolomes, including L-carnitine, lysophosphatidylcholine, glycerophosphocholine, and glyceric acid 1,3-biphosphate. The former 3 metabolites were initially present in milk in low or undetected levels, which increased gradually during the fermentation process. L-Carnitine and glycerophosphocholine were not detected in milk, but were detected both in fermented milk and fermented brown milk. Consistent results were reported in a previous study of dairy metabolomics that showed the content of L-carnitine increased during the fermentation process (Le Boucher et al., 2013). L-Carnitine is an AA metabolite that accelerates the transformation of fatty acids into energy, facilitating weight reduction; it is indispensable for glucose and fatty acid metabolism and

hormonal regulation. Significantly more glycerophosphocholine was found in fermented milk than fermented brown milk. Significantly more lysophosphatidylcholine was detected in fermented milk than milk, which is an indicator of impaired glucose tolerance and type 2 diabetes. Lysophosphatidylcholine is known to activate natural killer cells, which could promote bacterial clearance and tumor rejection, as well as quell autoimmune disease pathology (Fox et al., 2009). Thus, it would be of interest to further investigate the role of these biomolecules in human health and their metabolism and interactions with LAB.

Organic Acids. Five significantly differential abundant organic acids or organic acid salts (i.e., cinnabarinic acid, butyryl-CoA, N6-acetyl-LL-2,6-diaminopimelate, N-acetylmuramic acid 6-phosphate, and 3-hydroxybutanoic acid) were identified between fermented brown milk and fermented milk. The amount of N6-acetyl-LL-2,6-diaminopimelate in fermented brown milk was about 16-fold lower than in fermented milk. In contrast, the levels of N-acetylmuramic acid 6-phosphate and 3-hydroxybutanoic acid were significantly higher in fermented brown milk ($P < 0.05$), and the amount of 3-hydroxybutanoic acid was even 274.51-fold higher than in fermented milk.

Organic acids are the primary products of carbohydrate catabolism of LAB. N6-Acetyl-LL-2,6-diaminopimelate is involved in the lysine synthesis pathway, and its decrease in fermented brown milk could be related to the loss of lysine in milk due to the Maillard reaction. On the other hand, fermentation by the starter bacteria could increase protein degradation and subsequently increase in fermented dairy products.

Only a small amount of lactic acid is normally present in milk. However, a large quantity of lactic acid is produced as nonvolatile flavor substance via carbohydrate metabolism of LAB during fermentation (Akalin et al., 2002; Izco et al., 2002). Apart from serving as a major flavor that contributes to the unique flavor of fermented milk, it also extends the shelf life of fermented milk, imparts acidity and a refreshing taste to the product, makes it easier to digest the protein, promotes gastrointestinal peristalsis, improves digestion, and improves absorption of calcium, phosphorus, iron, and other minerals present in fermented milk (Chen et al., 2007). Some other organic acids also serve both as natural flavor compounds and biopreservatives of fermented products. For example, benzoic acid is an antimicrobial compound that is a significant differential abundant metabolite detected in both fermented brown milk and fermented milk, which is likely produced by *L. bulgaricus* via transforming hippuric acid naturally present in milk (Park et al., 2017).

Nucleotides and Related Metabolites. No significant differential nucleotide-related metabolites were found between the metabolomics of fermented brown milk and fermented milk. However, 5 differentially abundant nucleotides and related metabolites were detected between fermented milk and milk, including uric acid, xanthine, hypoxanthine, uracil, and purine. All 5 compounds were significantly increased in fermented milk ($P < 0.05$). Uridine increased 861-fold by the end of brown milk fermentation.

Nucleotides are an important class of nitrogen-containing compounds in the body. Milk nucleotides play a pivotal role in regulating the intestinal microflora, promoting growth and development, enhancing immunity, and improving lipid metabolism. *Lactobacillus bulgaricus* can use purine as a carbon, nitrogen, and energy source for growth. In the process of purine decomposition, adenine is decomposed by adenine dehydrogenase to form hypoxanthine, which is further converted to xanthine by hypoxanthine oxidase. In addition, guanine can also generate xanthine via catalysis by guanine dehydrogenase. Finally, xanthine is catalyzed to form uric acid by the action of xanthine oxidase (Suzuki et al., 1986). The increase in the levels of these nucleotide-related compounds in fermented milk and fermented brown milk can thus be explained by the active metabolism of the starter bacteria in the fermentation process.

Vitamins. No significant differential vitamin metabolites were found between the metabolomes of fermented brown milk and fermented milk. However, the present study found an 8.78-fold enrichment of nicotinamide-*N*-oxide in fermented milk compared with milk. Nicotinamide-*N*-oxide is an intermediate product of vitamin B12 synthesis by LAB (Moore and Warren, 2012). An in vitro screen found that 8 of 16 tested LAB strains, including one *Lactobacillus*, had the ability to produce vitamin B12 (Masuda et al., 2012). On the other hand, less riboflavin was present in the fermented milk compared with milk, which could be explained by the intrinsic lack of some other vitamin biosynthetic pathways in LAB, making these bacteria nutritionally fastidious. Thus, they had to utilize certain vitamins in milk for growth, such as riboflavin and pyridoxal (Le Boucher et al., 2013).

The level of vitamin A increased significantly in both kinds of fermented milk (349-fold increase in fermented milk compared with milk; 231-fold increase in fermented brown milk compared with brown milk; $P < 0.05$ in both cases), suggesting that the *L. bulgaricus* ND02 strain used in this study was potentially producing vitamin A. This is supported by Panfili et al. (1998), who observed extensive retinol isomerization in cheese. However, only little is known about the metabolic path-

ways of vitamin A synthesis by *L. bulgaricus*, which is worthy of investigation.

CONCLUSIONS

In this work, we used a metabolomic approach to analyze chemical and nutritional differences among 4 products, milk, brown milk, fermented milk, and fermented brown milk. Significant differences were detected in the abundances of various groups of metabolites, including peptides, AA, aldehydes, ketones, organic acids, vitamins, and nucleosides. Our data showed that the Maillard browning reaction significantly increased a wide spectrum of flavor compounds such as short peptides, aldehydes, ketones, organic acids, sulfur, and furan compounds. They might together contribute to the unique flavor of brown milk. However, Maillard browning also resulted in significant increase in carboxymethyllysine, which might have negative health impacts. On the other hand, fermenting brown milk with the starter culture, *L. bulgaricus* ND02, effectively countered such effect. Finally, 6 differential abundant metabolites were identified between fermented milk and fermented brown milk metabolomes, including L-lysine, methylglyoxal, glyoxal, 2,3-pentanedione, and 3-hydroxybutanoic acid. Some of these biomolecules might account for the different nutritional qualities between these 2 types of fermented products.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China, Beijing (grant no. 31771954), National Natural Science Foundation of China, Beijing (grant no. 31922071), Inner Mongolia Autonomous Region Science and Technology Achievement Transformation Project, Hohhot (2019CG027), and Natural Science Foundation of Inner Mongolia Autonomous Region of China, Hohhot (2021ZD08). Ji-angying Peng and Wenyi Zhang participated in project design; Tiansong Sun supervised the project; Jiangying Peng and Liqing Ma performed the experiments; Jiangying Peng analyzed and interpreted the data and drafted the manuscript. Lai-Yu Kwok extensively revised the manuscript. All authors have read and approved the content of the manuscript. The authors have not stated any conflicts of interest.

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