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Serum metabolome profiling revealed potential biomarkers for milk protein yield in dairy cows

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ABSTRACT

Milk yield (MY) and milk protein (MP) content are crucial milk performance traits of dairy cows that directly affect the dairy profits. This study first proposed milk protein yield (MPY) by considering MY and MP content together. Forty multiparous cows were selected from the 348 Holstein dairy cows, which fed the same diet under the same management condition, to investigate the serum metabolome profiles and to identify key metabolites associated with MPY. Among them, 20 cows with a higher MPY (MY > 34.5 kg/d and MP > 3.2%. i.e., MPY > 1.11 kg/d) were defined as the HH group, and 20 cows with a lower MPY (MY < 31 kg/d and MP < 2.9%, i.e., MPY < 0.87 kg/d) as the LL group. The GC-TOF/MS and the ultra HPLC-MS/MS platforms were used to identify metabolites and quantify biomarkers, respectively. Orthogonal partial least squares discriminant analysis of serum metabolomes revealed a clear separation between the 2 groups. Thirty-six significantly different metabolites were identified, which mainly involved in valine, leucine and isoleucine biosynthesis and glycine, serine and threonine metabolism. With biomarker analysis and validation, hippuric acid, nicotinamide and pelargonic acid may serve as key metabolites associated with MPY.

Biological significance: This study reports the application of serum metabolomics to identify biomarkers related to MPY and to reveal the biological pathways affecting milk protein synthesis. Three novel serum biomarkers were discovered to reflect the MPY variation of dairy cows, which may be useful in quality control in dairy cow production and for optimizing industrial production of dairy products. This study confirms that individual physiological and metabolic differences contribute to the variations in MPY and provides directions for further improving the MPY of dairy cows.

1. Introduction

Milk protein is a valuable nutritional component used to assess milk quality and is an important protein source for humans [1]. Milk protein content (MP, %) and milk yield (MY, kg/d) are two key economical traits for evaluating the lactation performance of dairy cows, but they are negatively correlated [2]. To date, most studies have focused on enhancing MP or MY through nutritional strategies such as altering diet ingredients [3], balancing amino acids (AAs) profiles in feed [4], and adding extra bio-available ingredients to feed [5]. Meanwhile, breeding and management strategies have also been applied to improve milk protein production [6, 7]. However, the above researchers only focused on either MP or MY, which could not meet the requirements for both traits. In this study, a new measure, milk protein yield (MPY, here defined as MP × MY) is introduced as a way to consider MY and MP at the same time, which will assist in maximizing benefits for the dairy industry.

It is known that even when animals are fed the same diet, they can have divergent milk performance [8]. Many factors can affect such variation, including genetics, digestion and absorption processes, stress status, health, etc. [9]. We speculate that individual metabolic and physiological variations can contribute to differences in MPY. Milk production and milk protein synthesis are complex biological processes that involve many metabolic pathways. A comprehensive understanding of their regulatory mechanisms is limited, which prevents its further improvement through nutritional and management strategies.

Metabolomics, as a vital part of system biology, aims to investigate relevant mechanisms by analyzing metabolic profiles of cells, biofluids or tissues. Recent studies have revealed that blood metabolites used as physiological biomarkers are able to reflect metabolic dysfunction, health and performance in vivo [10]. Our previous study showed that two key urine metabolites were associated with MY when cows consumed different quality forages [11], suggesting that metabolites could serve as potential biomarkers for milk synthesis. Here, we further

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hypothesized that serum metabolites of dairy cows with different MPY could serve as potential biomarkers for milk production and milk protein synthesis through their different metabolic functions.

In this study, to reflect the physiological mechanisms relating to MPY, we selected dairy cows with extremely high MPY and extremely low MPY fed the same diet to characterize serum metabolome profiles. Moreover, biomarker analysis and quantitative validation were performed to identify key metabolites as potential biomarkers regarding MPY, which can provide high reliability and are feasible parameters for selection of high performance dairy cows.

2. Materials and methods

2.1. Animals and management

All the experimental protocols were approved by the Animal Care Committee at the Zhejiang University (Hangzhou, P. R. China), and the animal study was conducted according to the University's guidelines for animal research. With high reliability of a power analysis (Supplementary Table S1), 40 multiparous Holstein dairy cows with days in milk of 160.0 (± 28.7 SD) and parity of 3.3 (± 1.5 SD) were selected from among 348 dairy cows housed at Hangjiang Dairy Farm (Hangzhou, China). They were kept in individually tethered stalls in a barn with good ventilation and were milked and fed 3 times daily at 06:30, 14:00, and 20:00 h. The cows were fed an identical total mixed ration with a concentrate-to-forage ratio of 57:43 (DM basis) and had free access to water.

2.2. Sample collection and measurement

Diets were sampled and collected at 05:30 and 19:00 h before feeding, and then stored at −80 °C in sealed plastic bags until later analysis of chemical composition (detailed methods are supplied in the Supplementary file). The ingredients and chemical composition are listed in Supplementary Table S2.

Milk samples were collected at a proportion of 4:3:3 corresponding to the morning, noon and evening milking with a bronopol tablet (milk preservative, D & F Control Systems, San Ramon, CA), and then stored at 4 °C before the infrared analysis of milk components and somatic cells using a spectrophotometer (Foss-4000, Foss, Hillerød, Denmark) within 2 days of collection [12]. Milk yields were recorded using milk-sampling devices (Waikato Milking Systems NZ Ltd., Waikato, Hamilton, New Zealand) for 3 consecutive days.

Blood samples (10 mL) were collected using two 5 mL pro-coagulation tubes each from the jugular vein between 04:00 to 06:00 h. Then, the samples were centrifuged at 3000 × *g* 4 °C for 15 min to obtain the corresponding serum within 30 min of collection, which was then stored at −80 °C for later analysis of biochemical parameters (details are specified in the Supplementary file) and metabolome profiles.

2.3. GC-TOF/MS detection and analysis

2.3.1. Identification and quantification of compounds detected by GC-TOF/MS

Serums were prepared and loaded into the GC-TOF/MS system as depicted in the Supplementary file. The GC-TOF/MS analysis was performed using an Agilent 7890 GC system (Agilent 7890B, Agilent, USA) coupled with a Pegasus HT time-of-flight mass spectrometer (LECO Chroma TOF PEGASUS HT, LECO, USA). The system was equipped with a DB-5MS capillary column coated with 5% diphenyl that was cross-linked with 95% dimethylpolysiloxane (30 m × 250 μM inner diameter, 0.25-μM film thickness; J&W Scientific, Folsom, CA, USA). Procedures of serum compounds separation, identification and relative quantification are described in the Supplementary file.

2.3.2. Identification of significantly different metabolites and pathways between HH and LL cows

The SIMCA 14.1 software package (V14.1, Sartorius Stedim Data Analytics AB, Umea, Sweden) was applied to perform pattern recognition multivariate analysis, which included principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), with the log transformation and unit variance scaling conversion mode.

The variable importance for the projection (VIP) > 1.0 and *P* value < .05 was used to define the significantly different metabolites (SDMs) [13]. The SDMs were further identified and validated by searching the online Kyoto Encyclopedia of Genes and Genomes (KEGG) and Bovine Metabolome Database (BMDB). Metaboanalyst 3.0 (http://www.metaboanalyst.ca/) was employed to obtain relevant pathways. The *Bos taurus* (cow) pathway library was applied in this procedure.

2.3.3. Identification of biomarkers reflecting MPY

Effective peaks data with similarity > 400 were imported into Metaboanalyst 3.0 to manually select the minimum number of SDMs as biomarkers to represent and explain the physiological difference between HH and LL cows, based on the area under the curve (AUC), predicted class probabilities, and cross validation (CV) prediction (Specified procedures are depicted in the Supplementary file).

2.4. Biomarkers validation by ultra HPLC-MS/MS (UHPLC-MS/MS)

2.4.1. Standard solution and calibration curves

Hippuric acid (HUA, 98%) from Sigma-Aldrich (St. Louis, MO), nicotinamide (NAA, ≥99.5%) from Standards (Shanghai, China), and pelargonic acid (PEA, ≥90%) from Tokyo Chemical Industry (Tokyo, Japan) were separately dissolved or diluted at 10 mmol/L as stock solutions. An aliquot of each stock solution was transferred to form a mixed working standard solution. Then, a series of calibration standard solutions were prepared by stepwise dilution, which were subjected to UHPLC-MS/MS analysis to form the calibration curves (Supplementary Table S3).

2.4.2. UHPLC-MS/MS detection and analysis

The UHPLC separation was performed using an Agilent 1290 Infinity II series UHPLC System (Agilent Technologies) with a Waters ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm). An Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies), equipped with an AJS electrospray ionization interface, was applied for MS analysis. Details of metabolites extraction, quantitative parameters optimization, UHPLC separation and MS identification processes are specified in the Supplementary file.

2.4.3. LOD and LOQ

The calibration standard solution was diluted stepwise then subjected to UHPLC-MRM-MS analysis. The lower LOD (LLOD) and lower LOQ (LLOQ) were defined as those peaks with S/N of 3 and 10, respectively [14].

2.4.4. Precision and accuracy

The quantitation precision was evaluated by RSD, determined by injecting analytical replicates of a QC sample (quality control: the mixed sample of HH and LL cows). The quantitation accuracy was determined by the analytical recovery of the QC sample. The percent recovery was calculated as [(mean observed concentration)/(spiked concentration)] × 100%.

2.5. Data analysis

The SAS MIXED model was used to analyze milk yield, milk composition and blood biochemical parameters. *P* < .05 was defined as a