Advances in the Application of Metabolomics in Ruminant Nutrition Research: Postprint

Authors: Dai Tongtong, Li Yaokun, Liu DeWu, Liu Guangbin, Sun Baoli

Date: 2017-11-07T00:00:00+00:00

Abstract

Metabolomics is an emerging discipline dedicated to the study of all metabolites within biological systems. It enables the detection of changes in all metabolites at specific biological hierarchies in living systems and facilitates systematic quantification of these metabolites through its distinctive analytical and data analysis platforms. In recent years, owing to the broad applicability of its analytical techniques, metabolomics has been progressively applied to nutritional research in ruminant animals. This review summarizes the advances in the application of metabolomics to ruminant nutrition research from four perspectives: rumen metabolomics, liver metabolomics, mammary gland metabolomics, and blood metabolomics.

Full Text

Application Advance of Metabonomics to Nutrition Research of Ruminants

DAI Tongtong, LI Yaokun, LIU Dewu, LIU Guangbin, SUN Baoli*

Herbivores Research Laboratory, College of Animal Science, South China Agricultural University, Guangzhou 510642, China

Abstract: Metabonomics is an emerging discipline that studies all metabolites in biological systems. It can detect changes in all metabolites at a specific biological level in living systems and systematically measure these metabolites using its unique analytical technology platforms and data analysis platforms. In recent years, due to the broad applicability of its testing technologies, metabonomics has been gradually applied to nutrition research in ruminants. This paper reviews the advances in the application of metabonomics in ruminant nutrition research from four aspects: rumen metabonomics, liver metabonomics, mammary gland metabonomics, and blood metabonomics.

Key words: metabonomics; analysis platform; ruminant nutrition; rumen; liver; mammary gland; blood

*Corresponding author, associate professor, E-mail: SUNBAOLI221@126.com

---

Metabonomics is a branch of systems biology that uses population index analysis as its foundation and data modeling and system integration as its objectives. It reflects pathological and physiological processes by detecting the overall trajectory of changes in endogenous metabolites [1]. By employing its unique high-throughput detection and data analysis methods, metabonomics can simultaneously conduct qualitative and quantitative analysis of metabolites with relative molecular masses below 1,000 u in biological or cellular systems, screening for characteristic metabolites under different metabolic conditions to understand and grasp the overall metabolic state of the organism [2-4]. Currently, the main analytical technology platforms for metabonomics include nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and Fourier transform infrared spectroscopy (FT-IR) [5]. However, simply using analytical techniques to detect metabolites is insufficient. Various metabolite spectra contain large amounts of hidden data information, requiring a series of mathematical and biostatistical methods to extract and optimize useful information. The commonly used data analysis platforms mainly include principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) [6].

1. Metabonomics Analysis Platform

With the development of systems biology, exploration of entire biological systems (including cells, tissues, organs, and whole organisms) has become a key focus of contemporary research, and the rise and application of metabonomics represents a significant benefit for current studies [7]. The complete metabonomics analysis workflow includes sample preparation, data acquisition and processing, metabolic pathway prediction, and biological interpretation. Among these, the analytical technology platform and data analysis platform are critical to the success of this technology.

1.1. Analytical Technology Platform

The most widely used detection methods in metabonomics are NMR, LC-MS, GC-MS, and FT-IR. Each method has its own advantages and disadvantages, but a single detection method can rarely detect all substances. Therefore, to analyze as many metabolites as possible at different levels in various biological systems, we should select appropriate analysis platforms based on sample characteristics and research objectives and use them comprehensively in practice [8].

1.1.1. NMR NMR is a spectroscopic technique based on the spin properties of atomic nuclei that absorb radiofrequency radiation under an external magnetic field to produce energy transitions. It can be performed under near-physiological conditions and exhibits characteristics of being non-destructive, unbiased, highly sensitive, and rapid when determining compound structures [9]. Commonly used NMR techniques include proton NMR (^{1}H-NMR), carbon NMR (^{13}C-NMR), and phosphorus NMR (^{31}P-NMR), among which ^{1}H-NMR is the most widely applied. ^{1}H-NMR is a multi-parameter dynamic analysis method capable of comprehensive analysis of endogenous metabolites in a single run, with simple sample pretreatment and low sample requirements. However, ^{1}H-NMR has relatively low resolution and sensitivity, often resulting in extensive spectral overlap when analyzing certain complex organic mixtures, which can negatively impact experimental results.

1.1.2. GC-MS GC-MS features high resolution and sensitivity and does not require derivatization for non-volatile metabolites. Additionally, the separation function of chromatography and the identification function of mass spectrometry enable GC-MS to rapidly measure metabolites, even those at very low concentrations [8]. GC-MS technology not only overcomes the molecular weight limitations of gas chromatography (GC) detection, expanding its detection range, but also can quickly and sensitively identify metabolites and conduct selective analysis of multiple metabolites. GC-MS has relatively complete databases for retrieval and can simultaneously determine hundreds of chemically different compounds, including organic acids, amino acids, sugars, fatty acids, and aromatic amines. Unfortunately, GC-MS technology has certain requirements for thermal stability and volatility of substances and cannot analyze metabolites or substrates that are thermally unstable or non-volatile.

1.1.3. LC-MS LC-MS is a relatively mature qualitative analysis technology that combines the excellent separation capability of liquid chromatography with the high sensitivity, rapid and precise detection characteristics of mass spectrometry, becoming one of the most powerful chemical screening tools in the field of natural products [7]. LC-MS can be used to analyze, separate, and identify trace metabolites in samples, especially polar compounds with large relative molecular masses and poor thermal stability [10]. Compared with GC-MS, LC-MS has higher sensitivity, a wider dynamic range, simpler sample processing, and lower detection costs. Compared with NMR, LC-MS is more suitable for identifying thermally unstable, non-volatile, difficult-to-derivatize, and large relative molecular mass substances. However, LC-MS lacks corresponding databases for retrieval, creating certain technical difficulties in data analysis. Additionally, its liquid phase separation efficiency is not high, detection costs are high, and sample detection requires further separation and verification.

1.1.4. FT-IR FT-IR is widely used in high-throughput detection and overall analysis of biological systems due to its advantages of not requiring sample

preparation, short time consumption, non-destructiveness, and simple operation. It is a metabolite fingerprint analysis method [11]. However, FT-IR also has some limitations, as its water absorption peak is very strong and must be eliminated through dehydration or data processing.

1.2. Data Analysis Platform

1.2.1. PCA PCA is an unsupervised model analysis method that does not impose any artificial factors, reflecting the most authentic differences between different groups and the original state of the data. It can help us understand and grasp the overall state of the data, as well as identify and remove unqualified products to improve model accuracy [12].

1.2.2. PLS-DA PLS-DA is a supervised model analysis method that uses a set of samples with known categories as a training set to plot sample loading and score diagrams [13]. When establishing a PLS-DA model, it is necessary to first determine the principal components of the training set, as different principal components correspond to different PLS-DA models. PLS-DA is similar to PCA in that both strive to extract information that can reflect the maximum variation in the data.

1.2.3. OPLS-DA OPLS-DA is a supervised model analysis method that provides a multi-to-multi linear regression modeling approach, particularly suitable when the number of variables in two groups is large and multicollinearity exists, but the number of observed samples is small.

The metabonomics data analysis process includes steps such as data extraction, data preprocessing, multivariate statistical analysis of data, and identification of variables representing the main differential metabolites. Among these, multivariate statistical analysis of data is primarily accomplished through the collaborative use of PCA, PLS-DA, and OPLS-DA. Only through comprehensive utilization of statistical analysis methods to conduct deep-level information mining of metabolic profiles can we fully understand and recognize experimental conclusions and obtain valuable biological significance from samples [14]. It is precisely this advantage that compensates for the defect of traditional research methods that cannot delve into animal tissues or organs to mine deep-level information, breaks the limitation of traditional research that can only stay at the surface level, and opens a new chapter for metabonomics in ruminant research.

2. Applications of Metabonomics in Ruminant Nutrition Research

2.1. Rumen Metabonomics

The most obvious physiological characteristic of ruminants is their complex stomach digestion, which includes the rumen, reticulum, omasum, and abomasum. The first three stomachs are "true" stomachs that primarily conduct

microbial digestion, with the rumen microbial system being the most important. Under the action of rumen microorganisms, 50% of dietary crude fiber and 70-85% of digestible dry matter (DM) are fermented and degraded in the rumen into volatile fatty acids, peptides, amino acids, ammonia, carbon dioxide (CO), and other components. Meanwhile, these carbon and nitrogen sources are utilized by microorganisms to synthesize proteins, vitamins, and other nutrients that are further absorbed and utilized by the animal [15]. The rumen also has its own balance mechanisms: saliva secretion and rumination, periodic rumen contraction, entry of endogenous nutrients into the rumen, eructation, and an effective buffering system. Once this balance mechanism is disrupted, animal health is threatened. Therefore, it is important to monitor rumen metabolic activities in ruminants in actual production. However, in practice, we cannot artificially simulate a truly reliable rumen environment, nor can we culture rumen microorganisms in vitro. Therefore, traditional research methods cannot deeply understand rumen metabolic mechanisms, and only high-throughput analysis methods like metabonomics can make such research possible [2].

Currently, metabonomics is mainly applied to study the effects of high-concentrate diets on the metabolic mechanisms of rumen fluid, rumen microorganisms, and rumen epithelial tissue. Research has shown that high-grain diets can lead to rumen metabolic disorders, abnormal metabolites, and increased incidence of nutritional metabolic diseases in ruminants, and high-concentrate diets often cause subacute rumen acidosis. Therefore, using metabonomics methods to evaluate whether dietary nutrition levels are reasonably set is an ideal approach in production [16-17]. Ametaj et al. [18] fed 46 Holstein dairy cows with diets containing four barley grain addition levels (0, 15%, 30%, 45%). At the end of the experiment, they detected rumen fluid from these cows using both NMR and LC-MS methods, identifying 46 characteristic rumen metabolites. They found no significant difference between the 0% and 15% grain addition groups, but compared with the 30% addition level, the 45% addition level showed significantly increased harmful metabolites (primarily methylamine and endotoxin) in the rumen with increasing grain levels. The presence of methylamine affects semicarbazide-sensitive amine oxidase activity in the body. This experiment was the first to discover a certain degree of correlation between grain intake and harmful rumen products. Huo [16] fed goats with high-grain diets containing different corn addition levels (0%, 25%, 50%) and used GC-MS metabonomics analysis and Pyrosequencing-based high-throughput sequencing to detect rumen microorganisms and metabolites in goats. Combined with PCA and PLS-DA analysis of these metabolites, 78 characteristic metabolites were identified, including endotoxin and biogenic amines. The study found that high-grain diets significantly affected rumen fermentation and reduced rumen microbial diversity, preliminarily revealing the connection between diet, microorganisms, and metabolites, while also providing experimental basis and theoretical foundation for further research on rumen microbial mechanisms. Bertram et al. [19] selected four milk replacer addition levels (3.10, 4.84, 6.60, and 8.10 kg/d; dry matter content in milk

replacer was 123 g/kg) to feed Holstein bull calves in Experiment 1 (n =8, divided into 4 groups of 2 each) and two types of calf starter concentrates (low-starch concentrate: 319 g/kg; high-fiber concentrate: 68 g/kg) to feed Holstein bull calves in Experiment 2 (n =7, randomly divided into 2 groups: control group with 4 calves, experimental group with 3 calves). At the end of the experiments, rumen epithelial tissue was collected from these calves and the tissue extracts were detected using ^{1}H-NMR. Combined with PCA analysis, the study found that in Experiment 1, as milk replacer intake increased, the contents of acetate, propionate, choline, unsaturated fatty acids, leucine, isoleucine, valine, and glutamine in the rumen significantly decreased. In Experiment 2, rumen acetate content significantly decreased while propionate content significantly increased. The study also found that as milk replacer intake decreased and concentrate intake increased, rumen epithelial tissue activity became more vigorous. More importantly, this was the first study to use metabonomics to identify the metabolic mechanisms through which milk replacer and concentrate supplementation affect calf rumen epithelial tissue activity and its metabolites. The above studies demonstrate that when feeding ruminants, concentrate addition levels should be strictly controlled below 50%. If concentrate levels are too high, animals will produce more harmful metabolites (primarily methylamine, endotoxin, and propionate) during feed digestion and absorption in the rumen, which can affect certain ureases in the body and hinder metabolic activities.

Metabonomics methods have shown clear advantages in studying rumen activities in ruminants, breaking the limitations of conventional methods and advancing to the molecular level of rumen research, bringing new opportunities for ruminant rumen studies. Metabonomics transforms rumen metabolic information into regular patterns of graphics and data output, making the research traceable. Meanwhile, we have also found that high-concentrate diets can damage animal bodies to a certain extent, so excessive use of concentrates should be avoided in actual production to reduce unnecessary losses.

2.2. Liver Metabonomics

The liver is the hub organ of material metabolism in the body, with multiple functions including detoxification, digestion, absorption, material metabolism, energy metabolism, and immunity. It is the main site for carbohydrate, fat, amino acid, vitamin, and hormone metabolism, primarily maintaining body metabolism and internal environment stability [20]. Throughout the material metabolism process, the liver mainly participates in regulating the composition and metabolism of nutrients in peripheral blood. After ruminants ingest feed, the feed ferments in the rumen, and the resulting nutrients are absorbed in the digestive tract, transported to the hepatic portal vein, and then enter the liver for necessary material and energy metabolism before entering the circulatory system to participate in new substance formation [21].

Currently, liver metabolism research mainly focuses on identifying key regula-

tory points of liver material metabolism, the mechanisms by which harmful substances produced by rumen fermentation affect liver nutrient metabolism, and excavating liver material metabolic pathways. Traditionally, we often use the multi-vascular fistula method to understand liver metabolic mechanisms. However, multi-vascular fistula installation is complex with high surgical difficulty. It cannot completely maintain fistula patency nor avoid tissue and organ irritation caused by the fistula. If liver metabolic disorders and functional damage occur, using blood samples obtained through multi-vascular fistula technology as evaluation standards yields incomplete detection results and provides limited help for studying liver physiological status and metabolic processes. Metabonomics can detect all metabolites in tissue samples and describe their flow processes in the body in the form of metabolic pathways, avoiding result one-sidedness and providing strong support for disease diagnosis and metabolic pathway excavation [22]. Wang et al. [23] intraperitoneally injected two groups of Guanzhong dairy goats with similar age and body weight with endotoxin solutions of 20 and 40 μg/kg BW, respectively, with the same basal diet formula. At the end of the experiment, liver tissue was collected and metabolites were detected using ^{1}H-NMR. Combined with PLS-DA, nine differential metabolites between groups were identified, revealing that endotoxin mainly affects liver metabolic status by influencing liver carbohydrate, fat, and amino acid metabolism.

The application of metabonomics in ruminant liver research has only emerged in recent years and has not yet been widely popularized and used. After reviewing extensive literature, we found that metabonomics research in animals mainly focuses on small animals, particularly mice. There are few related experiments on large animals like ruminants. On one hand, there are few reference materials; on the other hand, using ruminants as experimental materials is costly, thus limiting the initiation of related research. However, this also represents an opportunity. Some researchers have already applied metabonomics to ruminant liver metabolism research and obtained significant results, demonstrating that this direction has high research value and provides a new research direction and 思路 for animal nutrition studies.

2.3. Mammary Gland Metabonomics

The mammary gland is an important organ for lactation metabolism in dairy cows, primarily conducting synthesis and secretion of milk components, including milk protein, milk fat, and lactose synthesis in mammary alveoli [24]. Amino acids and glucose are the two major nutrients for mammary gland metabolism. Glucose is the precursor for lactose synthesis, which not only maintains milk osmotic pressure but also influences milk quality through its synthesis amount and efficiency [25]. Amino acids mainly participate in milk protein synthesis in ruminants, and dietary amino acid levels and balance significantly affect milk protein synthesis and milk yield in lactating dairy cows [26-27]. Therefore, studying mammary gland material metabolism and metabolic mechanisms is

crucial. Through metabolic pathways, we can understand how dietary glucose and amino acid content affect milk quality, providing a consideration standard for further improving milk fat and milk protein content and consequently increasing milk yield. Wang [21] collected mammary tissue from high milk quality group cows (n=9) at 3 months of lactation, low milk quality group cows, and dry-period cows (1 month), and detected these tissue samples using ^{1}H-NMR. Analysis using PLS-DA and OPLS-DA showed that compared with the low milk quality group, the high milk quality group had significantly increased contents of creatine, lactate, methionine, lysine, leucine, glycine, and phenylalanine, revealing that increased amino acid levels can improve milk protein content in dairy cows to a certain extent. In actual production, certain amino acids can be intentionally supplemented to animals to increase milk protein content and consequently increase milk yield. Sun et al. [28] used GC-MS-based metabonomics methods to conduct metabonomics detection on four sample types (body fluid, milk, serum, plasma) from lactating dairy cows (n=16, divided into 2 groups of 8 each) fed two different roughages (alfalfa hay and corn straw). After 80 days of the experiment, these four sample types were collected for high-throughput detection and multivariate statistical analysis. The results showed that the main metabolic pathways involved in this experiment were glycine metabolism, serine metabolism, threonine metabolism, tyrosine metabolism, and phenylalanine metabolism. These metabolic pathways can directly serve as important indicators for evaluating dairy cow milk production performance and milk protein content.

Mammary gland metabonomics is a research field that primarily uses milk and mammary tissue as research materials to identify important indicators related to milk production performance and milk protein quality, thereby discovering key factors in animals that can affect milk production performance. It can also evaluate the feeding value of diets and identify feeding standards that can increase milk yield during the lactation period for reference by the dairy farming industry. Additionally, dairy farming in southern China is still in its infancy, and heat stress is particularly severe in summer in the south, seriously affecting dairy cow milk yield. To reduce the adverse effects of heat stress, farms also make some changes to dairy cow diets, striving to increase diet palatability while ensuring that milk production performance is not inhibited. Metabonomics methods are very applicable in this context and can serve as an auxiliary tool for diet evaluation.

2.4. Blood Metabonomics

Blood metabonomics includes serum metabonomics and plasma metabonomics. Overall, blood metabonomics research in ruminants mainly focuses on disease diagnosis, such as ketosis, postpartum anestrus, heat stress, milk fever, ovarian quiescence, acute foot rot, postpartum negative energy balance, and fatty liver in dairy cows. Li et al. [29] used ^{1}H-NMR metabonomics methods to detect plasma from dairy cows with Type I and Type II ketosis, finding that ab-

normalities in carbohydrate metabolism (blocked tricarboxylic acid cycle), lipid metabolism (occurrence of negative energy balance), and amino acid metabolism (glucogenic amino acids entering other metabolic pathways) led to ketosis occurrence. Wang et al. [30] used ^{1}H-NMR-based metabonomics methods to detect plasma from dairy cows with postpartum anestrus and identified the key factors for postpartum anestrus occurrence, mainly the suppression of energy, amino acid, fat, and choline metabolic pathways, leading to reproductive hormone secretion disorders and resulting in postpartum anestrus. Tian [31] used LC-MS and ^{1}H-NMR metabonomics methods to detect plasma from heat-stressed dairy cows, finding that heat stress response mainly occurred because carbohydrate, amino acid, and fat metabolism became disordered, leading to negative energy balance in the body. Zheng et al. [32] used ^{1}H-NMR metabonomics methods to detect serum from dairy cows with acute foot rot, finding that acute foot rot was mainly caused by blocked pathways of carbohydrate (gluconeogenesis), carbohydrate (glycerol and succinic acid), and fat metabolism (fat mobilization).

Blood circulates throughout the body around major tissues and organs, participating in most of the body's metabolic processes and serving as the "pipeline" for maintaining life activities. Even tiny fluctuations in the content of substances or information mediators in the body can be well reflected in the blood. For example, when disease occurs in the body, certain physiological activities are inhibited or promoted, and the substances involved in these activities differ from their states when the body is healthy, either being transformed into harmful substances or changing in content to levels that prevent the body from maintaining normal life activities. By excavating these substances and following this information for deep analysis, we can discover what causes the disease and its symptoms in the body, which is extremely helpful for finding treatment plans and preventive measures. Blood consists of serum and plasma, with the main difference being that plasma contains fibrinogen while serum does not. However, both can reflect the dynamic metabolic state of the body and can serve as analytical materials for metabonomics.

Due to the complexity of biological organisms, conventional detection technologies are often greatly limited when exploring potential molecular mechanisms in the body, and deep-level information is difficult to clearly excavate. On one hand, metabonomics can cleverly amplify the life information contained in biological systems, making metabolic pathways clearly visible and greatly reducing the exploration and analysis workload for researchers. On the other hand, metabonomics grasps and explains the dynamic responses of organisms to various internal and external factors from the perspective of small-molecule metabolites, which is very helpful for exploring the mechanisms of action of certain nutrients or active substances on the body. Metabonomics methods can not only present all small-molecule metabolites participating in the dynamic response to stimuli in a specific part of the body at a specific time on the same spectrum but also describe the composition and content of these metabolites and potential biomarkers. Currently, the application of metabonomics in ruminant nutrition mainly involves liver metabonomics, rumen metabonomics,

mammary gland metabonomics, and blood metabonomics, aiming to identify nutrient metabolic pathways and the metabolic mechanisms of certain feed ingredients (or additives) in animals. For example, evaluating whether a diet formula is reasonable requires understanding the entire reaction process and changes in metabolic substances in the animal body to make an objective evaluation. Traditional research methods mostly obtain samples through slaughter, but for large animals like ruminants, the cost and loss of slaughter are substantial. Metabonomics only requires collection of simple samples such as blood, tissue fluid, or urine, or random selection of a few animals for live slaughter to obtain tissue samples, which is more convenient than traditional methods. Moreover, the data obtained from metabonomics are more comprehensive and systematic, providing high reference value for experiments. However, metabonomics still has some limitations, as its databases are not yet complete. Therefore, when using metabonomics technology, we must overcome data-related problems based on our own capabilities and experience.

References

[1] Xia JF, Liang QL, Hu P, et al. New progress in research strategies and methods of metabonomics [J]. Chinese Journal of Analytical Chemistry, 2009(01):136-143.

[2] Sun HZ, Liu JX. Nutritional metabonomics and its application in dairy cow nutrition research [J]. Chinese Journal of Animal Science, 2014,50(11):81-85.

[3] Sun L, Guo Y, Fan Y, et al. Metabolic profiling of stages of healthy pregnancy in Hu sheep using nuclear magnetic resonance (NMR) [J]. Theriogenology, 2017,(92):121-128.

[4] Dan Y, Zhang Y, Cheng L, et al. Hepatitis B virus X protein (HBx)-induced abnormalities of nucleic acid metabolism revealed by 1H-NMR-based metabonomics [J]. Scientific Reports, 2016,6:24430.

[5] Dunn WB, Ellis DI. Metabolomics: current analytical platforms and methodologies [J]. Trac-Trends in Analytical Chemistry, 2005,24(4):285-294.

[6] Nie CX, Zhang WJ. Metabonomics and its application in animal nutrition research [J]. China Animal Husbandry & Veterinary Medicine, 2011,38(1):108-112.

[7] Tang H, Wang Y. Metabonomics: a revolution in progress [J]. Progress in Biochemistry & Biophysics, 2006,33(5):401-417.

[8] Zhou QX, Yu XB, Tu GQ, et al. Research progress and application of metabonomics [J]. Biotechnology Bulletin, 2013(1):49-55.

[9] Wang ST, Li XN, Wang J, et al. Metabonomics and its analysis technologies [J]. Journal of Pharmaceutical Analysis, 2010(9):1792-1799.

[10] You R. Study on the mechanism of Guangdong herbal tea based on metabonomics [D]. PhD Thesis. Guangzhou: South China University of Technology, 2012.

[11] Lu HM, Liang YZ. Metabonomics analysis technology and data processing technology [J]. Journal of Instrumental Analysis, 2008,27(3):325-332.

[12] A JY. Metabonomics data processing method—principal component analysis [J]. Chinese Journal of Clinical Pharmacology and Therapeutics, 2010,15(5):481-.

[13] Ke CF, Wu XY, Li K. Comparison of four diagnostic statistics for PLS-DA model in metabonomics application [J]. Chinese Journal of Health Statistics, 2014,31(3):403-406.

[14] Zhu C, Liang QL, Wang YM, et al. Integrated development and new progress of metabonomics [J]. Chinese Journal of Analytical Chemistry, 2010,38(7):1060.

[15] Zhang GF. Effects of different concentrate-to-forage ratios on liver amino acid and glucose metabolism in lactating dairy goats [D]. Master' s Thesis. Nanjing: Nanjing Agricultural University, 2014.

[16] Huo WJ. Study on the microbiological mechanism of high-grain diet altering goat rumen metabolism [D]. PhD Thesis. Nanjing: Nanjing Agricultural University, 2014.

[17] Ma JL, Dong GZ, Wu JB. Changes in rumen fluid metabonomics of dairy cows caused by high-concentrate diets and their impact on cow health [J]. Chinese Journal of Animal Nutrition, 2016,28(1):27-34.

[18] Ametaj BN, Zebeli Q, Saleem F, et al. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows [J]. Metabolomics, 2010,6(4):583-594.

[19] Bertram HC, Kristensen NB, Vestergaard M, et al. Metabolic characterization of rumen epithelial tissue from dairy calves fed different starter diets using 1H-NMR spectroscopy [J]. Livestock Science, 2009,120(1):127-134.

[20] Xu GY, Ge WH. Progress in metabonomics and liver metabonomics [J]. Inner Mongolia Journal of Traditional Chinese Medicine, 2011,30(6):115-116.

[21] Wang XY. Study on nutrition metabonomics related to lactation in Chinese Holstein dairy cows [D]. Master' s Thesis. Harbin: Northeast Agricultural University.

[22] Cao Y, Sun LW, Bao K, et al. Application of metabonomics technology in animal nutritional metabolic diseases [J]. Modern Animal Husbandry and Veterinary Medicine, 2016(1).

[23] Wang LF, Jia SD, Yang GQ, et al. The effects of acute lipopolysaccharide challenge on dairy goat liver metabolism assessed with 1H-NMR metabonomics [J]. Journal of Animal Physiology & Animal Nutrition, 2016.

[24] Zhao K, Liu HY, Liu JX. Research progress on glucose absorption, metabolism and regulation in lactating dairy cow mammary gland [J]. China Dairy Cattle, 2009(8):26-30.

[25] Qi LZ, Yan SM, Ao CJ. Research progress on the effects of milk component precursors on milk component synthesis in dairy cow mammary gland [J]. Chinese Journal of Animal Nutrition, 2011,23(12):2077-2083.

[26] Fu Y. Effects of limiting essential amino acids on lactation performance and mammary amino acid metabolism in dairy goats [D]. Master' s Thesis. Jinan: Shandong Agricultural University, 2011.

[27] Li XY, Wang JQ, Wei HY, et al. Absorption and metabolism of amino acids in ruminant mammary gland [J]. China Dairy Cattle, 2011(2):11-14.

[28] Sun H, Wang D, Wang B, et al. Metabolomics of four biofluids from dairy cows: potential biomarkers for milk production and quality [J]. Journal of Proteome Research, 2015,14(2):1287-98.

[29] Li Y, Xu C, Xia C, et al. Analysis of plasma metabolic profiles of Type I and Type II ketosis in dairy cows based on 1H-NMR technology [J]. Scientia Agricultura Sinica, 2015,48(12):2449-2459.

[30] Wang G, Xiao XH, Fan ZL, et al. Metabonomics analysis of plasma from postpartum anestrus dairy cows based on 1H nuclear magnetic resonance technology [J]. Chinese Journal of Biologicals, 2016,29(9):922-927.

[31] Tian H. Study on metabonomics of heat-stressed dairy cows based on LC-MS and 1H-NMR methods [D]. PhD Thesis. Beijing: Chinese Academy of Agricultural Sciences, 2016.

[32] Zheng JS, Zhang HY, Xia C, et al. Metabonomics analysis of serum from dairy cows with acute foot rot based on 1H-NMR technology [J]. Chinese Journal of Veterinary Medicine, 2016(6):7-9.

Note: Figure translations are in progress. See original paper for figures.