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

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Abstract

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

Full Text

Application Advance of Metabonomics to Nutrition Research of Ruminants

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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 and data analysis platforms. In recent years, due to the broad applicability of its testing technologies, metabonomics has been gradually applied to nutritional research in ruminants. This paper reviews the application progress 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

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Metabonomics is a branch of systems biology that fundamentally relies on population index analysis and aims at data modeling and system integration, reflecting pathological and physiological processes by detecting the overall changes of 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 organisms or cells, screening 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 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 spectra of metabolites contain large amounts of 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 the focus of current research, and the rise and application of metabonomics can be considered a major benefit for contemporary research [7]. Currently, the complete metabonomics analysis process includes sample preparation, data acquisition and processing, metabolic pathway speculation, and biological interpretation. Among these, the analytical technology platform and data analysis platform are key to the success of this technology.

1.1 Analytical Technology Platform

The most widely used detection methods in metabonomics mainly include four types: NMR, LC-MS, GC-MS, and FT-IR. Each method has its own advantages and disadvantages, but a single detection method can hardly detect all substances. Therefore, to analyze all metabolites at different levels in various biological systems as much as possible, we should select appropriate analysis platforms and comprehensively utilize them according to sample characteristics and research purposes in actual operation [8].

1.1.1 NMR NMR is a spectroscopic technique based on the spin properties of atomic nuclei that absorb radiofrequency radiation under the action of an external magnetic field to produce energy transitions. It can conduct experiments under near-physiological conditions and exhibits characteristics of being non-destructive, unbiased, highly sensitive, and rapid when determining compound structures [9]. Currently, commonly used NMR includes hydrogen NMR (^{1}H-NMR), carbon NMR (^{13}C-NMR), and phosphorus NMR (^{31}P-NMR), among which ^{1}H-NMR is the most widely used. It is a multi-parameter dynamic analysis method capable of conducting holistic analysis of endogenous metabolites at one time, with simple sample pretreatment and small sample requirements. However, the resolution and sensitivity of ^{1}H-NMR are relatively low, often causing large-area overlapping of spectral information when determining certain complex organic mixtures, which negatively impacts experimental results.

1.1.2 GC-MS GC-MS features high resolution and sensitivity, and does not require derivatization treatment 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 with very low content [8]. GC-MS technology not only breaks the limitation of molecular mass range when detecting substances with gas chromatography (GC), expanding its detection range, but also can quickly and sensitively identify metabolites and conduct selective analysis of multiple metabolites. Moreover, GC-MS has a relatively complete database for retrieval and can simultaneously determine hundreds of compounds with different chemical properties, 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 metabolically unstable substrates or products that cannot be vaporized.

1.1.3 LC-MS LC-MS is a relatively mature qualitative analysis technology that combines the outstanding separation capability of liquid chromatography with the high sensitivity, rapid and accurate detection characteristics of mass spectrometry, becoming one of the most powerful chemical screening methods 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 mass 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, non-derivatizable substances with large relative molecular mass. However, LC-MS does not have a corresponding database for retrieval, which brings certain technical difficulties in data analysis. Additionally, 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 holistic 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 shortcomings, 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 human factors, can reflect the most authentic differences between different groups and the original state of data, and can help us understand and grasp the overall state of data, as well as discover 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 diagrams and score diagrams [13]. When establishing a PLS-DA model, it is necessary to first determine the principal components of the training set, as PLS-DA corresponding to different principal components is not exactly the same. PLS-DA is similar to PCA in that both strive to extract information that can reflect the maximum variation in data.

1.2.3 OPLS-DA OPLS-DA is a supervised model analysis method that can provide a many-to-many linear regression modeling method, especially when the number of variables in two groups is large and there are multiple correlations, but the number of observed data samples is small, OPLS-DA modeling is very applicable.

The data analysis process of metabonomics includes steps such as data extraction, data preprocessing, multidimensional statistical analysis of data, and identification of variables of major differential metabolites. Among these, multidimensional statistical analysis of data is mainly completed collaboratively by PCA, PLS-DA, and OPLS-DA. Only through comprehensive utilization of analytical statistical methods to conduct deep-level information mining of metabolic profiles can we comprehensively understand and recognize experimental conclusions and obtain precious biological significance of samples [14]. It is precisely this advantage that compensates for the defect of traditional research methods that cannot deeply excavate deep-level information inside animal tissues or organs, breaks the limitation of traditional research that can only stay on the surface, and opens a new chapter for metabonomics in ruminant research.

2. Application 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 mainly conduct microbial digestion, with the rumen microbial system being the most important. Under the action of rumen microorganisms, 50% of crude fiber and 70%-85% of digestible dry matter (DM) in feed are fermented and degraded in the rumen into volatile fatty acids, peptides, amino acids, ammonia, carbon dioxide (CO_2), and other components. Meanwhile, these carbon and nitrogen sources are utilized by microorganisms to synthesize proteins, vitamins, etc., which are further absorbed and utilized by the ruminant as nutrients [15]. At the same time, the rumen has its own balance mechanism: saliva secretion and rumination, periodic rumen contraction, entry of endogenous nutrients into the rumen, eructation, and an effective buffer system. Once this balance mechanism is destroyed, animal health is threatened. Therefore, it is important to pay attention to the metabolic activities of the rumen 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 the metabolic mechanism of the rumen. Only metabonomics, a high-throughput analysis method, can make this 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 adverse consequences such as rumen metabolic disorders, abnormal metabolites, and frequent nutritional metabolic diseases in ruminants, and high-concentrate diets often lead to the occurrence of subacute rumen acidosis. Therefore, using metabonomics methods to evaluate whether dietary nutrition levels are reasonably set is an ideal production method [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 the rumen fluid of these cows using both NMR and LC-MS methods, identifying 46 characteristic rumen metabolites. They found that the detection data from the 0% and 15% grain addition groups were not significantly different, but compared with the 30% addition level, the content of harmful metabolites (mainly methylamine and endotoxin) in the rumen of animals in the 45% addition level group increased significantly with the increase of grain addition level. The presence of methylamine affects the activity of semicarbazide enzymes in the body. Moreover, this experiment first discovered a certain degree of correlation between grain intake and harmful products in the rumen. Huo Wenjie [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 of goats. Combined with PCA and PLS-DA analysis of these metabolites, 78 characteristic metabolites including endotoxin and biogenic amines were identified. The study found that high-grain diets could significantly affect rumen fermentation and reduce rumen microbial diversity, preliminarily revealing the connection among diet, microorganisms, and metabolites, and providing experimental basis and theoretical foundation for further research on rumen microbiological mechanisms. Bertram et al. [19] selected four addition levels of milk replacer (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_1=8, divided into 4 groups with 2 calves per group), and two types of calf concentrate supplements (low-starch concentrate: 319 g/kg; high-fiber concentrate: 68 g/kg) to feed Holstein bull calves in Experiment 2 (n_2=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 experimental calves and the tissue extracts were detected with ^{1}H-NMR. Combined with PCA analysis, it was found that in Experiment 1, with the increase of milk replacer intake, the contents of acetic acid, propionic acid, choline, unsaturated fatty acids, leucine, isoleucine, valine, and glutamine in the rumen of calves all decreased significantly. In Experiment 2, the content of acetic acid in the rumen of calves decreased significantly, but the content of propionic acid increased significantly. Meanwhile, the experiment also found that with the decrease of milk replacer intake and increase of concentrate supplement intake, the activity of rumen epithelial tissue would become more active. More importantly, this experiment was the first to use metabonomics to identify the metabolic mechanisms through which milk replacer and concentrate supplements affect the activity of rumen epithelial tissue and its metabolites in calves. The above studies prove that when feeding ruminants, the concentrate addition level should be strictly controlled below 50%. If the concentrate addition level is high, animals will produce more harmful metabolites (mainly methylamine, endotoxin, and propionic acid) during feed digestion and absorption in the rumen, which will affect the activity of certain ureases in the body and hinder metabolic activities.

Metabonomics methods have shown obvious advantages in studying rumen activities of ruminants, breaking the limitations of conventional methods and delving into the molecular level of rumen research, bringing new opportunities for rumen research in ruminants. Metabonomics transforms rumen metabolic information into graphics and data output according to certain rules, making it traceable for us. At the same time, we have also found that high-concentrate diets can cause certain damage to animal bodies. Therefore, in actual production processes, excessive use of concentrate should be avoided to reduce unnecessary losses.

2.2 Liver Metabonomics

The liver is the hub organ of substance metabolism in the body, with multiple functions including detoxification, digestion, absorption, substance 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 process of substance metabolism, the liver mainly participates in regulating the composition and metabolism of nutrients in peripheral blood. After ruminants ingest feed, the feed is fermented in the rumen, and the produced nutrients are absorbed in the digestive tract, transported to and converge in the hepatic portal vein, then enter the liver for necessary substance and energy metabolism, and finally enter the circulatory system to participate in the formation of new substances [21].

Currently, liver metabolism research mainly focuses on identifying key sites regulating liver substance metabolism, the mechanism of action of harmful substances produced by rumen fermentation on liver nutrient metabolism, and excavating liver substance 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 not only cannot completely maintain the patency of vascular fistulas but also cannot avoid the stimulation of fistulas to tissues and organs. If the liver experiences metabolic disorders and functional damage, using blood samples obtained by multi-vascular fistula technology as evaluation standards, the detection results are not comprehensive enough and are not very helpful for research on the physiological state and metabolic processes of the liver. Metabonomics can detect all metabolites in tissue samples and describe their flow processes in the body in the form of metabolic pathways, avoiding the one-sidedness of results and providing strong support for disease diagnosis and metabolic pathway mining [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, their liver tissues were collected and metabolites were detected with ^{1}H-NMR. Combined with PLS-DA, nine differential metabolites between groups were identified, and it was found that endotoxin mainly affected the metabolic state of the liver by influencing carbohydrate, fat, and amino acid metabolism.

The application of metabonomics in ruminant liver research has only emerged in recent years and has not been widely popularized and used. After reviewing a large amount of literature, we found that metabonomics research in animals mainly focuses on small animals, especially mice. There are few related experiments on large animals like ruminants. On the one hand, there are few reference materials; on the other hand, the cost of using ruminants as experimental materials is high, thus limiting the initiation of related research work. However, this is also an opportunity. Some researchers have already applied metabonomics to liver metabolism research in ruminants and have obtained significant results, indicating that this direction is very valuable for research 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, mainly conducting synthesis and secretion of milk components, and synthesizing milk protein, milk fat, and lactose 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 the osmotic pressure of milk but also affects milk quality through its synthesis amount and efficiency [25]. Amino acids mainly participate in milk protein synthesis in ruminants. The level and balance of amino acids in feed have a great impact on milk protein synthesis and milk yield in lactating dairy cows [26-27]. Therefore, studying mammary gland substance metabolism and metabolic mechanisms is crucial. Through metabolic pathways, we can understand how glucose and amino acid content in feed affect milk quality, providing a consideration standard for further improving milk fat and milk protein content and thus increasing milk yield. Wang Xiaoyan [21] collected mammary tissue from dairy cows in the high milk quality group and low milk quality group at 3 months of lactation, as well as from dry-period cows at 1 month (n=9), and detected these tissue samples with ^{1}H-NMR. Combined with PLS-DA and OPLS-DA analysis, the results 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 thus increase milk yield. Sun et al. [28] used a GC-MS-based metabonomics method to conduct metabonomics detection on four samples (body fluid, milk, serum, plasma) from lactating dairy cows fed two different roughages (alfalfa hay and corn straw) (n=16, divided into 2 groups with 8 cows per group). After 80 days of the experiment, these four samples 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 an important indicator for evaluating milk production performance and milk protein content in dairy cows.

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

2.4 Plasma 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 Ying et al. [29] used ^{1}H-NMR metabonomics to detect plasma from dairy cows with Type I and Type II ketosis, finding that abnormalities 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 the occurrence of ketosis. Wang Gang et al. [30] used ^{1}H-NMR-based metabonomics to detect plasma from dairy cows with postpartum anestrus and identified the key factors for the disease, mainly the inhibition of energy, amino acid, fat, and choline metabolic pathways, which disrupted the secretion of reproductive hormones and led to postpartum anestrus. Tian He [31] used LC-MS and ^{1}H-NMR metabonomics 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. Zheng Jiasan et al. [32] used ^{1}H-NMR metabonomics 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 spreads throughout all major tissues and organs, participating in most metabolic processes and serving as the "oil pipeline" for maintaining life activities. Tiny fluctuations in the content of substances or information mediators in the body can be well reflected in blood. For example, when disease occurs, certain physiological activities are inhibited or promoted, and the substances participating in these activities differ from those in a healthy state, either being transformed into harmful substances or changing in content to a level where the body cannot maintain normal life activities. By excavating these substances and deeply analyzing this information, 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 biggest difference being that plasma contains fibrinogen while serum does not, but both can reflect the dynamic metabolic state of the body and can be used as analysis 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 excavate clearly. On the one hand, metabonomics can cleverly amplify the life information contained in biological systems, making metabolic pathways clear and greatly reducing the workload of scientific researchers in exploration and analysis. 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 stimulating factors in a specific part of the body at a specific time on the same map, but also describe the composition and content of 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, to evaluate whether a diet formula is reasonable, we can only make an objective evaluation of the diet by understanding the entire reaction process in the animal body and the changes in metabolic substances. Traditional research methods mostly obtain samples through slaughter, but for large animals like ruminants, the cost and loss of slaughter are significant. Metabonomics only requires collection of simple samples such as blood, tissue fluid, and 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 by metabonomics are relatively comprehensive and systematic, providing high reference value for experiments. However, metabonomics still has some defects, as its database is not yet complete. Therefore, when using metabonomics technology, we need to overcome data problems based on our own ability and experience.

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