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Metabonomics and its role in amino acid nutrition research

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1. ABSTRACT

Metabonomics combines metabolic profiling and multivariate data analysis to facilitate the high-throughput analysis of metabolites in biological samples. This technique has been developed as a powerful analytical tool and hence has found successful widespread applications in many areas of bioscience. Metabonomics has also become an important part of systems biology. As a sensitive and powerful method, metabonomics can quantitatively measure subtle dynamic perturbations of metabolic pathways in organisms due to changes in pathophysiological, nutritional, and epigenetic states. Therefore, metabonomics holds great promise to enhance our understanding of the complex relationship between amino acids and metabolism to define the roles for dietary amino acids in maintaining health and the development of disease. Such a technique also aids in the studies of functions, metabolic regulation, safety, and individualized requirements of amino acids. Here, we highlight the common workflow of metabonomics and some of the applications to amino acid nutrition research to illustrate the great potential of this exciting new frontier in bioscience.

2. INTRODUCTION

In the post-genomic era, a major challenge for biology is to understand how organisms function and adapt to the environment (1). In response to this challenge, systems biology, which is based on genomics, transcriptomics, proteomics, and metabonomics, is providing a new logical framework to explore biological functions and the complexity of the whole organisms and their interactions with genetic, dietary, disease, or other environmental factors in greater depth (2-4). Essentially, these "omics" approaches aim at understanding the biological systems at the levels of genes (DNA), transcripts (mRNA), proteins and metabolites as a whole rather than isolated events. Nowadays, these "omics" analysis of the biological systems, including plasma, fetal fluid, cells, tissues, organs and whole organisms, has now become the norm of bioscience research (5-7). Among these "omics", metabonomics play a central role in studying global systems biology since its birth in 1999, because biochemical metabolites represent the end-point products of biological processes. This is in contrast to genomes, transcriptomes and proteomes which would only help predict what might happen in a biological system (that may

or may not happen) (5). Metabonomics is a powerful top-down systems biology tool in nutritional research that can provide a direct biochemical explanation to understand metabolic regulation and to assess how metabolic balances may be disturbed by deficiencies or excesses of nutrients (8-9). Notably, amino acids not only serve as substrates for protein synthesis, but also regulate key metabolic pathways associated with metabolism, growth, and immunity, thereby enhancing the efficiency of food utilization, promoting growth and gut development, and improving health. Here, we review the common workflow of metabonomics and its widespread applications in amino acid nutrition research.

3. CONCEPT AND WORKFLOW OF METABONOMICS

The concept of metabonomics was initially defined as "the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications" (10). There is another related term "metabolomics", which was defined as "the quantitative measurement of all low molecular weight metabolites in an organism's cells at a specified time under specific environmental conditions" (5, 11-13). According to these definitions, metabonomics and its strategies are highly convergent because of their aims at detecting changes in the distribution and concentration of a broad range of metabolites in biological matrices (9). However, metabonomics is more reasonable and comprehensive as a systems biology approach since life process is a dynamic and integrated one. Metabonomics measures not only cellular and extracellular concentrations of endogenous metabolites but also dynamic perturbations of the metabolite complement in response to the internal or external stimuli, such as physiology, nutrition, pathology, and even the gut bacterial populations (14-15). Therefore, based on extensive research on method development and applications of metabolic profiling, Professor Tang and Wang broadly defined metabonomics as a branch of science concerned with the quantitative understandings of the metabolite complement in living systems and its dynamic responses to the changes of both endogenous factors (such as physiology and development) and exogenous factors (such as environment and xenobiotics) (5).

In addition to the conceptual development, many scientists have made a lot of concerted efforts to standardize metabonomic analysis. As an early representative step, the Standard Metabolic Reporting Structures working group reported a summary of draft recommendations and standards in metabolic profiling in 2005 (16). Subsequently, the Metabolomics Standards Initiative (MSI) was proposed as a part of community consultation (http://www.nature.com/nbt/consult/index.html) (17-19). The MSI has a certain degree of overlap with other existing initiatives in functional genomics, including the Human Proteome Organization Proteomics Standards Initiative (20) and the Microarray Gene Expression Data Society (21). As essential parts of systems biology approaches, these standards are maintained through close collaborations. Such collaborations can not only facilitate data integration across biological or cellular domains and instrumental equipment, but also avoid duplication of efforts and define common standards (17, 22). These will be the most effective ways to ensure that the generated output is pragmatic and the standards are both useful and widely accepted by the community. Therefore, considering the great potential and the social implications of metabonomics for nutritional research, American Society for Nutritional Sciences Long Range Planning Committee has defined the basic framework of metabonomics analysis in the specific field of nutrition (23). There are some publications to provide detailed explanations in aspects of the basic steps of nutritional metabonomics analysis (9, 24-26). As a kind of important nutrients, amino acids share the same metabonomics workflow with other nutrients, which includes the three successive steps: sample preparation, data acquisition, and data mining and interpretation (Figure 1). The basic workflow of metabonomics analysis is outlined as following:

(1) Sample preparation includes experimental design, biological experiment and collection of biological samples. Nutritional metabonomics often quantitatively measure subtle dynamic perturbations of metabolic pathways in organisms in response to relatively low doses of bioactive food substances or supplements. This is different from metabonomics in toxicological studies where effects are generally linked to relatively high doses of chemicals. The subtle biochemical response to nutritional factors could be affected by differences in diet, diurnal changes, age, gender and estrus cycle, health status, and stage of embryonic/fetal development. Thus, a careful experimental design is required for studies involving nutritional metabonomics.

(2) Data acquisition needs to detect and characterize metabolites by chemical analysis to acquire the quanlitative and quantitative information of metabolites. The main analytical techniques that are employed for metabonomics studies are based on nuclear magnetic resonance (NMR) spectroscopy (27), mass spectrometry (MS) (28), Fourier transform infrared spectroscopy (29), and Raman spectroscopy (30). Nowadays, both MS and NMR are gradually developed to be the mainstream techniques for metabonomics analysis because they can give complementary information despite their different analytical advantages and weaknesses. The ultimate starting point of a metabonomics experiment is to quantify all of the metabolites in a biological sample (e.g., plasma, cell or tissue). Ideally, metabonomics should be non-biased but at best it can be thought of as a 'non-targeted' analysis for metabolites. However, currently this cannot be achieved because the main challenges are the chemical complexity and heterogeneity of metabolites, the dynamic range of the analytical technique, the throughput of the measurements, and the extraction protocols. Moreover, another challenge is associated with the relative quantification of metabolites and the de novo identification of unknown metabolites.

(3) Data mining and interpretation needs data processing and statistics. In metabonomics, advanced

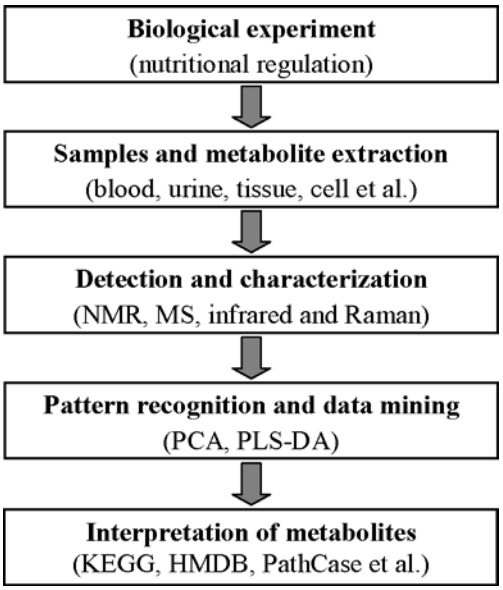

Figure 1. Common workflow of metabonomics analysis in amino acid nutrition. Abbreviations used: HMDB, The Human Metabolome Database; KEGG, Kyoto Encyclopedia of Genes and Genomes; MS, mass spectrometry; PCA, principle component analysis; PLS-DA, partial least square discriminant analysis.

statistical and bioinformatic tools are employed to maximize the recovery of information and aid in the interpretation of the very large datasets that are generated by chemical analysis. Firstly, statistical analysis is used to extract information about metabolic changes in groups. The key point in metabonomics studies is to identify the different groups of substances, as they become the potentially complex set of biomarkers that define the biological context and help explain the mechanisms related to biological functions. Secondly, all metabonomics studies result in complex multivariate data sets that require visualization software and chemometric and bioinformatics methods for interpretation.

Thus, metabonomics is a typical interdisciplinary science which integrates the principles and knowledge of analytical chemistry, biology and statistics (chemometrics). Expertise of investigators in these areas of research should be capitalized on in experimental design, data analysis and interpretation, and drawing of conclusions. Team work is most desirable to achieve this goal.

4. APPLICATION OF METABONOMICS TO AMINO ACID NUTRITION

Nowadays, metabonomics has been developed as a powerful analytical tool and hence found successful applications in amino acid nutrition research. Thus, knowledge of protein metabolism and areas of investigations have been rapidly expanded by the advance of this discovery technology. These areas include: a) profiles and characteristics of dietary and body-fluid amino acids; 2) digestion, absorption and metabolism of amino acids as well as their metabolism, regulation and functions in growth and health; 3) individualized requirements of amino acids; and 4) safety and toxicity of amino acids.

4.1. Composition and profiles of dietary amino acids

Amino acids belong to one of the most important classes of natural compounds because they participate in many key biological processes. Analysis of amino acids in tissues and cells is a major method to assess protein nutritional status and the balance of amino acids in the body. Thus, the composition and profiles of amino acids is of great importance in food, biological fluids and fermentation products (31-32). The composition and profiles of amino acids can be determined using NMR, MS, or HPLC (33-34). Plasma amino acid profiles can be applied for identification of biomarkers and diagnosis of liver or intestinal disease (35). Notably, a recent study involving NMR-based metabonomics revealed that the effects of heat stress on the metabolites of mouse myocytes are related to the differences in composition of some amino acids (e.g., leucine, valine, isoleucine, alanine, and phenylalanine) and creatine (36). This study could be used as an excellent reference for metabonomics studies of food aging and storage. In addition, NMR-based metabonomics was useful to identify the grape varieties and production areas by the metabolite profile of wines (37). Some metabolites, including valine, alanine, and proline, were good indicators of characteristic grape varieties and production areas (e.g., Australia, Korea, France, and United States). Most recently, tomato paste samples from Italian and Chinese manufacturers have been successfully analyzed using NMR-based metabonomics (38). The significant discrimination between these sources of samples was associated with amino acids, organic acids and glucose. Of particular interest, LC-MS-based metabonomics was applied to study Japanese fermented food, whose metabolites (including arginine, lysine, glutamate, glutamine, aspartate, and phenylalanine) varied with different stages of ripeness (39). These metabonomics studies will greatly expand our knowledge about the nutritional values and potential biological functions of food and reasonably guide the consumption and intake of food in daily life.

4.2. Digestion, absorption and metabolism of amino acids in the gastrointestinal tract

Nutritional value of food nutrients and other functional elements are related with not only their own composition and content, but also their digestion, absorption and metabolism in the gastrointestinal tract (40-43). The gut microbiota plays an important role in the regulation of energy and lipid metabolism in the host (44-46) as well as the efficiency of utilization of dietary nutrients (47). Recent studies indicated that the intestinal microbiota from children differed between a modern western diet and a rural diet (48). It was hypothesized that bacteria in the lumen of the intestine coevolved with the polysaccharide-rich diet of individuals from rural African villages, allowing them to maximize energy intake from fibers. Certainly, the gut microbiota can modulate digestion, absorption and metabolism of amino acids in the

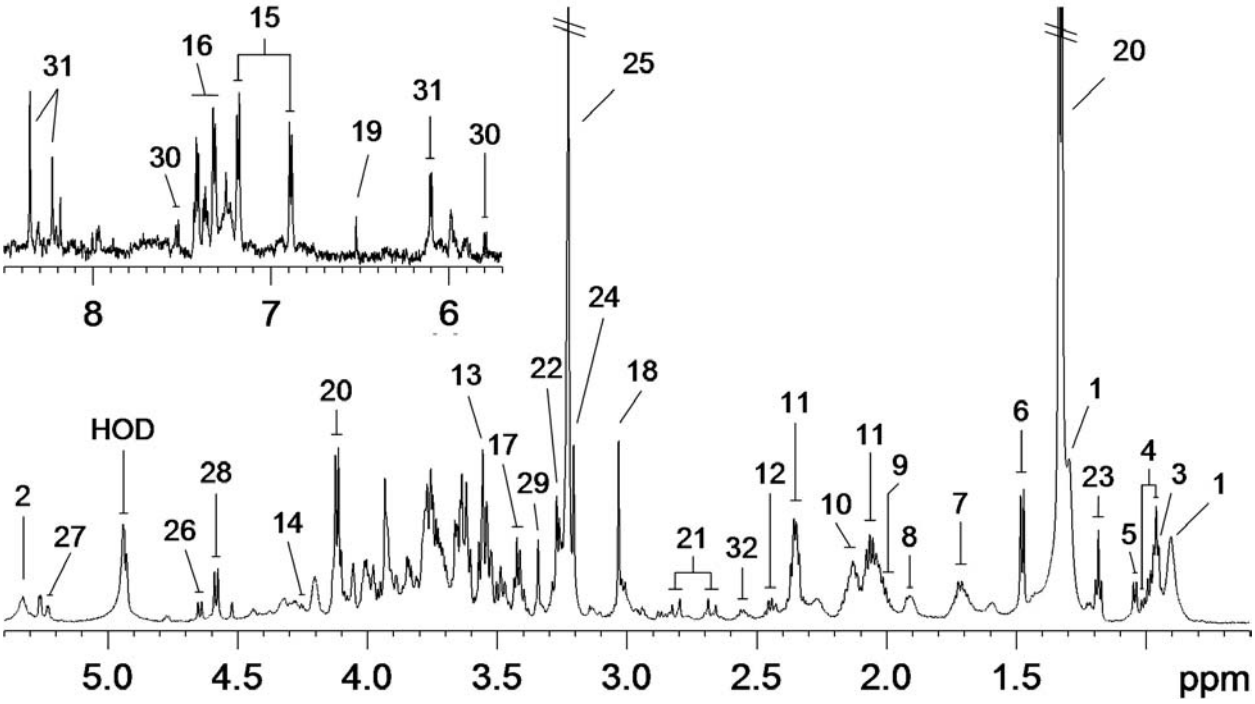

Figure 2. Typical 600 MHz CPMG ^{1}H HR-MAS NMR spectrum of the jejunum of piglets on age of 21 days. The spectra in the aromatic region (δ 5.7-8.5) were magnified 4 times compared to the aliphatic region (δ 0.6-5.4). About 32 metabolites were unambiguously assigned in the jejunum. Key: 1, lipids; 2, unsaturated lipids; 3, leucine; 4, isoleucine; 5, valine; 6, alanine; 7, lysine; 8, arginine; 9, proline; 10, methionine; 11, glutamate; 12, glutamine; 13, glycine; 14, threonine; 15, tyrosine; 16, phenylalanine; 17, taurine; 18, creatine; 19, fumarate; 20, lactate; 21, citrate; 22, trimethylamine-N-oxide; 23, ethanol; 24, choline; 25, glycerophosphorylcholine; 26, β-Glucose; 27, α-Glucose; 28, D-galactose; 29, *scyllo*-inositol; 30, uracil; 31, inosine; 32, glutathione

diet, which can influence the catabolism and de novo synthesis of amino acids (49-52). The extensive catabolism of dietary amino acids by the intestine or by luminal microbes may affect the nutritional efficiency of dietary protein and supplemental amino acids (53-56). However, knowledge about digestion, absorption and metabolism of amino acids and epithelial-cell transport of amino acids in the gastrointestinal tract remains incomplete (57-58). Metabonomic technology facilitates the high throughout analyses of large amounts of metabolites in tissues and luminal content of the gastrointestinal tract. Specially, high resolution magic-angle spinning (HR-MAS) NMR can provide a direct *in situ* detection to the intact gastrointestinal tissues *ex vivo* to avoid sample destruction (59). This new technique has been used to study the metabolic signatures of topographical variation in human and murine gastrointestinal tissues (60-62). Recently, we applied HR-MAS NMR spectroscopy to characterize metabolic signatures of the jejunal tissues obtained from piglets at 21 days of age (Figure 2). The HR-MAS NMR spectroscopy can detect about 32 metabolites, including leucine, isoleucine, valine, alanine, lysine, arginine, proline, methionine, glutamate, glutamine, glycine, threonine, tyrosine, phenylalanine, taurine and creatine. The HR-MAS NMR-based metabonomics can be a promising method to investigate the *in situ* processing of digestion and absorption of amino acids in the gastrointestinal tract. It can be anticipated that this post-genomics approach will advance our knowledge about the physiological function of intestinal bacteria and their interactions with dietary amino acids (63).

4.3. Metabolic functions and signatures of amino acids

Amino acids have many critical functions in physiological processes. One important function of amino acids is as the building block of proteins. Amino acids also play versatile roles in metabolism, growth, and immunity. Metabonomics has been proved as a very promising approach to study functionalities of foods and nutrients via the simultaneous measurements of multiple metabolic endpoints in complex organisms (9). The understanding of the complex relationships between human or animal metabolism and diets have been thought as the keys to decipher the mechanisms of actions of foods to promote health. Lenz *et al.* (64) applied the NMR-based metabonomics technology to identify that urinary levels of trimethylamine-N-oxide (TMAO) was significantly higher in Swedish population than in British population due to the higher consumption of fish. This study suggests that the endogenous urinary or blood profiles of metabolites are subject to profound dietary influences or other lifestyles. For example, fish consumption led to elevated levels of TMAO in urine, while drinking wine resulted in increased concentrations of alcohol in blood (65). Furthermore, vigorous exercise was associated with increased levels of lactic acid in urine (65). Different compositions of dietary

amino acids (e.g., meat, milk, and vegetables) may explain the distinct functions of diets or food that induce remarkably different metabolite signatures in humans or animals. Stella *et al.* (66) used the NMR spectroscopy in combination with multivariate statistical analysis to characterize the effects of three diets: "vegetarian", "low meat", and "high meat" on the metabolite signature of human subjects. Twelve healthy male participants consumed each of these diets, in a randomized order, for continuous 15-day-periods with an intervening washout period between each diet of 7 days duration. Each participant provided three consecutive 24-hour urine collections on days 13, 14, and 15 of each dietary period, and NMR spectra of all samples were acquired. Pattern recognition analysis showed that three kinds of dietary patterns leaded to different characteristic metabolic signatures. Urinary levels of creatine, carnitine, acetylcarnitine, and trimethylamine-N-oxide were significantly elevated in the high-meat consumption period. Orthogonal projection to latent structure discriminant analysis indicated that the *p*-hydroxyphenylacetate level (a microbial mammalian metabolite) of urine was higher in the vegetarian than in meat diet samples (66). Furthermore, this metabonomics study revealed that the different effects of "vegetarian", "low meat", and "high meat" diet on human metabolites were related to the differences in composition of dietary animal protein and plant (vegetable) protein. These findings underscore the efficacy of the metabonomic approach in determining the influence of dietary amino acids on human metabolism.

4.4. Safety and toxicity of amino acids

Metabonomics and metabolic profiling offer many advantages regarding toxicological assessment and biomarker discovery (67-68). Metabonomics technology has the potential to explore mechanisms of homeostatic control and to assess how metabolic balances may be disturbed by deficiencies or excesses of amino acids. Metabolic profiles of body fluids or tissues can be regarded as important indicators of normal phenotype, physiological, or pathological states, giving a unique opportunity to discover new biomarkers of excesses or deficiencies of amino acids (69-70). Obvious toxicity occurs in animals which consume excesses of amino acids, such as methionine and arginine (71-72).

Available evidence supports the view that metabonomics is a useful tool to study amino acid adequacy and safety in animals and humans. For example, Noguchi *et al.* (8) found that the analysis of a metabonomic subset, such as amino acids, may yield important information, and that correlation-based analyses could be useful in the analysis of metabonomic data to determine which metabolites may be responsible for the effects of excessive intakes of amino acids. Moreover, Matsuzaki *et al.* (73) applied metabonomics and transcriptomics approach to investigate the metabolic changes induced by dietary excess of L-leucine on rats. In this study, rats were fed for 2 wk on a basal diet or diets supplemented with 1.5., 5, 10, 15, or 30% L-leucine. Metabonomics analysis indicated that the variables associated with excess nitrogen clustered together with leucine and α-ketoisocaproate. Consistent with the metabonomic results, there were marked changes in hepatic expression of genes associated with nitrogen metabolism and other pathways downstream of leucine catabolism. Of particular interest, urea or α-ketoisocaproate could be an early marker for an upper limit of adequate intake of leucine. Thus, the rapid development of biomarkers offers the potential to rationalize the risk assessment of amino acids, and to refine the extrapolation of data from animals to humans (74).

Recently, Bohus *et al.* (75) applied NMR-based metabonomics approach to investigate the time-related metabolic responses to L-arginine-induced exocrine pancreatic toxicity. At a dose of 1 g/kg body weight, arginine administration resulted in no histopathological damage. However, at a dose of 4 g/kg body weight, arginine caused pancreatic acinar degeneration and necrosis and characteristic metabolic trajectory profiles. The initial trajectory phase involved changes in the urea cycle enzyme and transamination, which reflected a homeostatic response to detoxify excess ammonia generated from arginine catabolism. Subsequently, there was a marked increase of the excretion of the gut microbial metabolites, including phenylacetylglycine, 4-cresol-glucuronide and 4-cresol-sulfate, indicating that changes in gut microbiotal metabolism may contribute to hepatic and pancreatic toxic responses. Thus, the NMR-based metabonomics can be successfully applied to readily accessible biological fluids, such as urine, feces and plasma. This technique offers great potential for the identification of toxicologically relevant biomarkers in animal and studies of amino acid nutrition.

4.5. Individualized amino acid requirements

Metabonomics techniques can be used to identify the metabolic differences closely associated with the effects of the excess or lack of a single amino acid (such as arginine, glutamate and glutamine). This will, in turn, help define the adequate range of dietary intakes of the nutrient in animals and humans (8, 76). For example, He *et al.* (62) applied the NMR-based metabonomics approach to the study of metabolic changes in serum of growing pigs. In this work, 16 growing pigs were randomly assigned to one of two groups, representing supplementation with 0 or 1.0 % L-arginine to corn- and soybean meal-based diets. The results indicated that dietary arginine supplementation not only improved protein accretion and growth performance, but also decreased fat deposition in the body. Principal component analysis showed that serum concentrations of low density lipoprotein, very low density lipoprotein, and urea were lower, but concentrations of creatinine, tricarboxylic acid cycle metabolites, ornithine, lysine and tyrosine were greater in arginine-supplemented than in control pigs. Additionally, the arginine treatment affected serum concentrations of nitrogenous and lipid signaling molecules (glycerophosphorylcholine and *myo*-inositol) and intestinal bacterial metabolites (formate, ethanol, methylamine, dimethylamine, acetate, and propionate). These findings indicate that the current recommendation for dietary intake of arginine is grossly inadequate for growing-finishing pigs. Although traditional methods, including carbon oxidation model, nitrogen balance measurement, and plasma amino acid response, had long

been used to determine amino acid requirements (77-78), metabonomics can help further refine the values and expand the necessary database. This will lead to the development of paradigms that take the complexities of metabolism into account, culminating in the establishment of scientifically based assessment strategies for the adequate range of amino acid intakes. Moreover, compared with proteomics and transcriptomics, metabonomics has the advantage of low cost and high throughput and, therefore, holds promise to provide a convenient and desirable method for the determination of individualized requirements of amino acids (79-81).

5. SUMMARY AND PERSPECTIVE

Although still in its infancy, metabonomics has greatly expanded the research areas of amino acid nutrition, including a) profiles and characteristics of dietary amino acids; b) digestion, absorption and metabolism of amino acids; c) metabolic functions of amino acids; and d) safety and toxicity of amino acids. This advanced technique also holds great promise for refining individualized requirements of amino acids and identifying new biomarkers for the nutritional status of amino acids. Despite its wide applications to bioscience research, metabonomics faces three major challenges. Firstly, improvements should be made to resolve some technical and methodological problems in identifying low-abundance metabolites. For example, new chemical analytical techniques (e.g., two-dimensional chromatograph, capillary electrophoresis, and combination of NMR and MS) for qualitative and quantitative analysis of metabolites need to be developed and optimized for laboratory use. In addition, advanced chemometric methods are required to assess the accuracy and quality of experimental data. Secondly, because metabonomics is concerned with the small-molecule metabolites, metabonomics data alone would not be sufficient to satisfactorily explain the complex biological problems if the corresponding genomic and proteomic information is absent. Therefore, it is a real challenge to integrate the metabonomics data with other "omics" data to have a complete view of what are happening in a biological process at different levels. Thirdly, the community of the gut micro-organisms (microbiota) and the host (human or animal) together form a symbiotic system due to their billions of years of co-evolution (82). Thus, the functional genomes in host consist of both the host genome and the microbiota genome (microbiome). Considering microorganisms in the gastrointestinal tract is physiologically and nutritionally important because even the human genome alone only represents approximately 10% of the total genomes in the human (83-84). However, because metabonomic analysis provides information on metabolism in the host and microbiota as well as their interactive metabolic pathways (85), we should resolve a new issue of how to differentiate metabolites of the host from those of the gut microbiota in a symbiotic system (5). Nevertheless, metabonomics approach will offer unique global information at the metabolic level. Integrated with other advanced technologies (genomics, transcriptomics, and proteomics) and traditional biological technologies (86), metabonomics will be very efficient in enhancing our knowledge of: a) the complex mechanisms responsible for the essential of amino acids in nutrition, growth, development, health and disease; and b) the extensive interactions between amino acids and other nutrients. We anticipate that metabonomics will become an increasingly important global systems-biology tool in amino acid research, thereby contributing to improved growth and health in both animals and humans.

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Abbreviations: HR-MAS, high resolution magic-angle spinning; MS, mass spectrometry; MSI, the Metabolomics Standards Initiative; NMR, nuclear magnetic resonance; TMAO, trimethylamine-N-oxide

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