Advances in Metabolomics Research on Dairy Cow Nutrition and Milk Quality and Safety (Postprint)

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Abstract

Metabolomics is a discipline that detects changes in low-molecular-weight metabolites (typically with molecular mass less than 1,000 u) to investigate the composition and variation patterns of metabolites produced by organisms following pathological/physiological stimuli or genetic modifications. It is an emerging discipline developed in the post-genomic era and constitutes an important component of systems biology. Metabolomics has been widely applied across various fields including physiology, pathology, pharmacology, animal nutrition, zoology, and botany; however, its application in research on dairy cow nutrition and milk quality and safety remains relatively limited. This article reviews the current applications of metabolomics in dairy cow nutrition, disease, heat stress, milk quality, and dairy product safety, beginning with the fundamental concepts, research strategies, and methodologies of metabolomics.

Full Text

Research Progress on Metabolomics Application in Dairy Cow Nutrition and Milk Quality and Safety

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Abstract: Metabolomics is a discipline that detects changes in low-molecular-weight metabolites (typically <1,000 u) to study the composition and variation patterns of metabolites produced by organisms in response to pathological/physiological stimuli or genetic modifications. As an emerging field in the post-genomic era, metabolomics represents an important component of systems biology. While it has been widely applied in physiology, pathology, pharmacology, animal nutrition, zoology, botany, and other domains, its application in dairy cow nutrition and milk quality/safety research remains relatively limited. This review begins with the fundamental concepts, research approaches, and methodologies of metabolomics, then systematically summarizes current applications in dairy cow nutrition, disease, heat stress, milk quality, and dairy product safety.

Keywords: metabolomics; dairy cow; nutrition; milk; quality; safety

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Milk contains not only abundant proteins, carbohydrates, and lipids, but also numerous biologically active substances present in trace amounts yet serving critical physiological functions, such as immunoglobulins, nucleotides, and oligosaccharides. Many factors influence milk yield and composition, including genetics, feed composition, seasonal variations, processing methods, and animal health status. Consequently, by monitoring changes in milk components, we can assess not only product quality but also trace back to the physiological or pathological conditions of the producing cows. Although conventional biochemical and sensory indicators can monitor cow health and milk quality, their limited scope inevitably constrains the comprehensiveness of results. Moreover, when parameters lack interconnections, understanding the complete picture becomes more challenging. In living organisms, biochemical reactions are continuous and interconnected, forming metabolic networks rather than isolated pathways. This necessitates a holistic, systems-level approach to understanding metabolism. The emergence of systems biology has propelled life science research toward integrated and systematic investigation. With continuous innovation in research concepts and technologies, metabolomics offers new opportunities to comprehensively understand animal health and product quality/safety.

1 Basic Concepts, Classification, and Applications of Metabolomics

Metabolomics investigates the time-associated metabolite profiles produced by organisms following internal or external stimuli or genetic modifications. Its measured physiological parameters directly reflect nutritional status, stress conditions, or disease states, with metabolite responses occurring much faster than transcriptomic or proteomic changes. This provides a more direct analytical approach, representing a key distinguishing feature from other omics methods.

Additionally, the number of metabolite types is far smaller than that of genes and proteins, and the instruments and methods employed are more standardized, facilitating comparison across studies. These advantages have driven rapid metabolomics development and broad applications in nutrition, toxicology, disease diagnosis, and drug development.

1.2 Research Objectives, Instrumentation, and Analytical Methods

Metabolomics detects numerous small-molecular-weight metabolites, requiring highly sensitive, precise, and high-throughput instrumentation. Current data acquisition primarily employs mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, supplemented by efficient separation devices. Common platforms include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS or LC-MS/MS), high-performance liquid chromatography-NMR (HPLC-NMR), and HPLC-NMR-MS hybrid technologies. NMR is particularly suitable for analyzing abundant metabolites, requiring minimal sample preparation that preserves molecular structure and properties, thus ensuring excellent reproducibility and high sensitivity. NMR also allows experimental conditions within specific temperature and physiological buffer ranges, enabling more physiologically relevant real-time and dynamic monitoring. NMR analyzes isotopic nuclei, with ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR being most common. ^{1}H NMR detects hydrogen-containing compounds, allowing detection of most metabolites in biological samples including biofluids, cell extracts, tissue fluids, and living tissues.

GC-MS offers high sensitivity for volatile or derivatized organic compounds and benefits from extensive searchable standard mass spectral libraries such as the NIST database, enabling accurate compound identification. Current GC-MS methods can simultaneously quantify hundreds of chemically diverse metabolites including organic acids, most amino acids, sugars, sugar alcohols, aromatic amines, and fatty acids. LC-MS distinguishes metabolites by mass-to-charge ratio differences without requiring derivatization, enabling analysis of unstable, non-volatile, polar, and low-polarity compounds that are difficult for GC-MS. However, LC-MS lacks standardized databases for metabolite identification, requiring online resources like the Human Metabolome Database (HMDB). Each technique has distinct advantages and limitations, but combined applications can compensate for individual shortcomings, representing an important trend in metabolomics research. HPLC-NMR and HPLC-NMR-MS integrate high-throughput HPLC separation with NMR' s molecular weight and fragment information for highly selective and sensitive qualitative and quantitative analysis, while one- and two-dimensional NMR spectra enable molecular structure determination.

Metabolomics data analysis encompasses unsupervised and supervised methods. Unsupervised approaches include principal component analysis (PCA), hierarchical cluster analysis (HCA), and self-organizing maps (SOMs). Supervised methods comprise discriminant analysis (DA), partial least squares (PLS),

PLS-discriminant analysis (PLS-DA), orthogonal PLS-DA (OPLS-DA), soft independent modeling of class analogy (SIMCA), and artificial neural networks (ANN).

1.3 Classification and Characteristics

Metabolomics research can be categorized into four main types: targeted analysis, untargeted analysis, metabolic fingerprinting, and metabolic profiling. Targeted analysis validates identified biomarkers but requires standards for quantification, typically limiting analysis to a finite number of compounds across large sample sets. Untargeted analysis compares metabolomes between control and experimental groups to identify differential metabolites. Metabolic profiling requires researchers to hypothesize specific metabolic pathways for in-depth investigation. Metabolic fingerprinting analyzes mass spectral peaks of individual metabolites to elucidate compound structures and develop comprehensive identification methods.

1.4 Advantages and Necessity of Milk and Dairy Products as Detection Matrices

Modern dairy nutrition research and milk quality/safety monitoring require real-time, continuous, rapid, and non-invasive sampling techniques. Traditional sample types such as blood, saliva, urine, or feces are difficult to collect at regular intervals or frequently, with unstable yields and sometimes only trace amounts, preventing continuous and precise analysis. Milk and dairy products offer unique advantages: first, timed sampling is feasible, especially on large farms with dedicated milking equipment and fixed schedules; second, large sample volumes are obtainable (several to over ten kilograms per cow per milking); third, animals adapt to regular milking disturbances, minimizing stress-induced artifacts; and fourth, diverse product types are available. As a crucial human nutrient source, raw milk and its processed products (various milk powders, liquid milk, butter, cheese, whey, cream, yogurt) can all serve as analytical samples to assess quality, safety, and trace back to animal nutritional status, breed, and species.

Milk provides high-quality proteins and lipids, plus vitamins, minerals, and immunoglobulins vital for human health. Different processing methods yield varying nutrient profiles with distinct health impacts, underscoring the importance of quality and safety research. Metabolomics can detect small molecules, making it a powerful tool for identifying and quantifying milk metabolites. Due to nutritional and economic value differences among milk types, composition varies significantly between species, necessitating biomarkers to distinguish them. Metabolite profiles reflect integrated outputs of mammary epithelial cells, peripheral blood, and microbial gene expression, carrying species-specific signatures. Targeted metabolomics can effectively detect prohibited substances, such as through NMR-based analysis.

2 Applications of Metabolomics

2.1 Dairy Product Processing Performance Assessment

Raw milk quality directly affects processability and economic value. Poor processability leads to inferior dairy products. Approximately 40% of European raw milk is used for cheese production, primarily through enzymatic coagulation, making rennet-induced coagulation properties crucial quality indicators. Factors affecting coagulation include species, breed, seasonality, and composition. The coagulation process involves numerous biochemical reactions critical to dairy processing. Rennet coagulation hydrolyzes -casein to produce para- -casein, caseinomacropeptide, and glycomacropeptide, depending on -casein glycosylation. While many intermediate and final metabolites are involved, their specific changes remain poorly understood.

Sundekilde et al. used NMR to analyze metabolic profiles of milk from different breeds with varying coagulation properties, identifying relationships between metabolites, breed, and technological characteristics. Carnitine and lactose could differentiate breeds, while citrate, choline, carnitine, and lactose correlated with coagulation properties. LC-MS/MS analysis revealed oligosaccharides as key factors affecting coagulation. Heat stability represents another critical processing parameter, as temperatures exceeding 130°C can cause protein denaturation (whey protein unfolding, casein dephosphorylation, -casein hydrolysis). ^{31}P NMR can monitor phosphorus-containing component changes in UHT milk during storage.

Microbial contamination during production, processing, and storage causes spoilage. While severe changes are detectable by sensory evaluation, subtle biochemical processes in mildly spoiled milk require metabolomic analysis. Researchers inoculating milk with Pseudomonas identified several spoilage-indicator metabolites, though sample collection presents challenges as exogenous microorganisms may confound results.

2.2 Milk Nutrition Research

Nutrient content and composition are primary concerns for producers and consumers. NMR' s minimal sample destruction and rapid analysis make it powerful for food testing. Comparative studies revealed more metabolite diversity in bovine milk than in human milk or infant formula, with composition varying across lactation stages. Human milk contained higher oligosaccharides and amino acids than rhesus monkey milk, while monkey milk had higher glycerophosphocholine, hippuric acid, and trimethylamine-N-oxide. Choline is essential for liver and brain cell membrane phospholipids and as a neurotransmitter. Human colostrum contains less total choline than bovine milk collected days postpartum, suggesting human milk may not fully meet infant requirements, necessitating supplementation from formula.

Besides NMR, hydrophilic interaction liquid chromatography (HILIC LC-

MS/MS) effectively detects choline metabolites including acetylcholine, betaine, glycerophosphocholine, and lysophosphatidylcholine. Phosphocholine dominates early lactation, decreasing exponentially, while phosphatidylcholine becomes predominant in mid-to-late lactation, increasing over time. Phospholipids participate in numerous biological functions and are key membrane components, with milk phospholipids forming the milk fat globule membrane skeleton and supporting brain development, making them important nutritional assessment indicators.

^{31}P NMR can compare phosphorus-containing components across milk types. However, most nutrition studies focus on single isotope spectra (^{1}H, ^{13}C, ^{31}P NMR), with few employing multi-isotope approaches. While ^{1}H NMR sensitively detects lipid metabolites, ^{13}C NMR provides superior qualitative and quantitative information. More multi-isotope NMR and MS-integrated studies are needed.

2.3 Biomarker Identification in Milk and Dairy Products

Although cow milk dominates global production, niche milks (buffalo, yak, camel, mare) are valued for functional properties. High nutritional and economic values drive adulteration and counterfeiting, which are difficult to detect through sensory or conventional analysis, creating regulatory challenges. NMR can differentiate metabolite profiles among Holstein, Danish, and Jersey breeds, with carnitine, choline, and citrate as potential biomarkers. GC-MS identified valine and glycine as specific markers distinguishing cow and goat milk. Combined LC-MS and NMR effectively differentiated cow, goat, buffalo, yak, camel, and mare milks, revealing 68, 74, 54, 58, 77, and 91 differential metabolites between Holstein milk and Jersey, buffalo, yak, goat, camel, and mare milk, respectively. Holstein milk contained significantly higher lactate, acetylcholine, succinate, and pyruvate but lower carnitine, uridine, and pyroglutamic acid.

For lactose-intolerant or milk-allergic consumers, lactose-free milk or plant-based alternatives are available. ^{1}H NMR can assess quality of lactose-free beverages, using nicotine as an internal standard for lactose quantification. Different dairy products carry distinct socio-cultural and economic values based on origin. With urbanization and improved transportation, verifying authenticity and quality consistency of geographically distant products requires reliable analytical methods. Sacco et al. combined NMR, HPIC, ICP-AES, IRMS, and chemometrics to differentiate Italian and Central/Eastern European milks, finding higher lactose in the latter. Integrated NMR-IRMS data clearly distinguished geographical origins. ^{1}H HRMAS-NMR can assess mozzarella cheese quality and verify buffalo milk origin. Integrated metabolomic and microbiological analysis revealed higher microbial diversity but lower psychrotroph diversity in buffalo mozzarella, with *Streptococcus thermophilus* and elevated galactose and phenylalanine. Orotic acid was the only species-discriminating metabolite.

3 Metabolomics in Dairy Cow Disease and Heat Stress Monitoring

Early lactation negative energy balance (NEB) results primarily from insufficient feed intake. While blood biochemistry studies have investigated NEB characteristics, they fail to provide comprehensive understanding. Metabolomics offers a systems biology perspective on this physiological state.

Klein et al. used NMR and MS to identify relationships between milk composition and metabolic status, finding acetone and β-hydroxybutyrate closely associated with early lactation mammary metabolism. NMR analysis revealed that besides β-hydroxybutyrate and acetoacetate as acute ketosis markers, the glycerophosphocholine-to-phosphocholine ratio in first-month milk could serve as a prognostic health assessment biomarker and breeding selection criterion for metabolic stability. Plasma glucose, pyruvate, lactate, and alanine concentrations decreased significantly in NEB cows, while elevated β-hydroxybutyrate indicated glucose deficiency requiring ketone bodies as alternative energy sources. Increased plasma glycine reflected excessive protein mobilization and potential vitamin B6 deficiency.

During peak lactation, cows mobilize body fat to compensate for energy deficits, primarily through adipose tissue triglyceride lipolysis producing non-esterified fatty acids as metabolic fuel. While this alternative pathway has been studied through single metabolites, the integrated metabolic network remains unclear. Targeted metabolomics using ESI-LC-MS/MS provides comprehensive metabolite information on early lactation lipolysis. Phosphatidylcholines are key lipid metabolites in hepatic very low-density lipoprotein synthesis pathways. Lipidomic analysis of over-lipolytic cows revealed characteristics of excessive fat mobilization, impaired insulin sensitivity, and altered acylcarnitine profiles, identifying 37 key metabolites including sphingomyelins, phospholipids, and lysophospholipids. However, these studies are limited to dairy cows, with research on other minor dairy species needed to better understand metabolic adaptations to NEB.

Mastitis affects cow health and welfare while posing milk safety risks from treatment residues. While haptoglobin, serum amyloid protein A, and ATPase have been proposed as markers, these studies focus on single or few metabolites. Mastitis involves numerous metabolic processes producing diverse endogenous and exogenous molecules (peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids), requiring comprehensive metabolomic monitoring. Prostaglandins are important mastitis inflammatory mediators; monitoring revealed increased thromboxane A2, prostaglandin E2, and prostacyclin in milk from infected animals, indicating their pathophysiological role in *E. coli*-induced inflammation. Hettinga et al. used GC-MS to identify significant differences in ethyl acetate and acetic acid between healthy and mastitic cows, demonstrating that milk metabolite profiling can trace animal health status. These volatile metabolites are pathogen-induced and formed

by blood esterases, with their detection in mastitic milk suggesting increased blood-milk barrier permeability. However, conflicting results exist regarding blood-milk metabolomic correlations, necessitating further research to clarify relationships across physiological and pathological states.

Heat stress during summer hot weather impairs health, reduces milk yield and quality, and causes substantial economic losses. Traditional temperature-humidity index assessment is convenient but inaccurate, with unclear physiological mechanisms and metabolic markers. LC-MS-based plasma targeted metabolomics identified 41 heat stress biomarkers in lactating cows, with 13 metabolites (trimethylamine, glucose, lactate, betaine, creatine, pyruvate, acetoacetate, acetone, β-hydroxybutyrate, C16 sphingosine, lysophosphatidylcholine, phosphatidylcholine, and arachidonic acid) showing high diagnostic sensitivity and specificity. These markers participate in carbohydrate, amino acid, lipid, and gut microbial metabolism pathways, indicating comprehensive metabolic impacts. Tian et al. integrated LC-MS and NMR data from heat-stressed and non-stressed cows, identifying 53 diagnostic biomarkers. Correlations between milk and plasma for lactate, pyruvate, creatine, acetone, β-hydroxybutyrate, trimethylamine, oleic acid, linoleic acid, phosphatidylcholine, and lecithin suggested increased blood-milk barrier permeability during heat stress. While progress has been made, further validation of these markers is needed, along with studies comparing different species and sample types and assessing biomarker reliability in practical applications.

4 Summary and Outlook

Metabolomics is an emerging discipline, recognized for less than two decades. As a high-throughput, sensitive, and precise analytical technology, it has achieved significant progress across numerous fields, yet limitations remain. Metabolite identification via NMR and LC-MS is challenging without standard reference databases. Variations in sample preparation and matrices reduce comparability across studies. The complexity of metabolite classes, structures, and compositions prevents comprehensive detection by single methods. Most importantly, mature research concepts derived from metabolomics are still lacking, limiting its ability to address fundamental scientific questions.

Nevertheless, systems biology thinking drives continuous methodological advancement, enabling comprehensive understanding from subcellular to whole-organism levels. Traditional animal nutrition research relies on limited indicators that cannot fully reflect metabolic patterns or enable dynamic monitoring—limitations where metabolomics excels. By quantifying numerous small-molecule metabolites, metabolomics provides metabolic maps reflecting complete physiological or pathological states, offering comprehensive insights into metabolic regulation. Consequently, metabolomics is becoming an essential systematic approach in animal nutrition research. With rising socioeconomic development and living standards, animal product quality and safety receive increasing attention. Metabolomics' comprehensive compositional analysis offers

unique advantages for metabolic fingerprinting and profiling in dairy quality and safety research. In summary, metabolomics' combined macro-scale high-throughput and micro-scale detection capabilities enable comprehensive understanding of dairy cow nutrition, health, product quality, processing properties, and safety. Continuous improvement in analytical techniques, data analysis methods, and integration with other omics approaches will provide substantial intellectual support for livestock development.

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