Metabolomics in Dairy Cow Nutrition and Milk Quality and Safety: Research Advances (Post-print)

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Abstract

Metabolomics is a discipline that detects changes in low molecular weight metabolites (typically with molecular weight less than 1 000 u) to investigate the composition and variation patterns of metabolites produced by organisms following pathological/physiological stimuli or genetic modifications. Developed in the post-genomic era, it represents an important component of systems biology. While metabolomics has been widely applied across various fields including physiology, pathology, pharmacology, animal nutrition, zoology, and botany, its utilization 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 approaches, 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 a novel discipline emerging in the post-genomic era, metabolomics constitutes an important component of systems biology. While widely applied in physiology, pathology, pharmacology, animal nutrition, zoology, botany, and other fields, 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 summarizes current applications in dairy cow nutrition, disease, heat stress, milk quality, and dairy product safety.

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

Milk contains not only abundant proteins, carbohydrates, and lipids, but also numerous trace bioactive substances that perform critical physiological functions, such as immunoglobulins, nucleotides, and oligosaccharides [1-2]. Many factors influence milk yield and composition, including genetic factors, feed composition, seasonal variations, dairy processing procedures, and animal health status [2-5]. Therefore, by monitoring changes in milk components, we can assess both milk product quality and trace the physiological or pathological conditions of dairy cows [6-7]. While conventional indicators based on biochemistry or sensory evaluation can monitor cow health or milk quality, these simple detection parameters inevitably yield limited results. Moreover, when parameters lack correlation, understanding the complete picture becomes more challenging. In living organisms, biochemical reactions are not only continuous but also interconnected through metabolic pathways that exist as metabolic networks. Consequently, a holistic and systematic research philosophy is required to understand organismal metabolism. The birth of systems biology has elevated life science research thinking, methods, and technology to a stage of holistic and systematic investigation [8]. With innovations and advances in research concepts and technologies, metabolomics provides a new opportunity to comprehensively understand animal health and product quality/safety [8-9].

1 Basic Concepts, Classification, and Application Scope of Metabolomics

Metabolomics studies the numerous metabolites produced by organisms over time sequences in response to internal/external stimuli or genetic modifications [8,10]. The physiological parameters it measures can directly reflect nutritional, stress, or disease states, with metabolite responses occurring much faster than those in transcriptomics or proteomics. Metabolomics therefore offers a more direct analytical approach, representing its key distinction from other omics methods. Additionally, the number of metabolite types is far smaller than that

of genes and proteins, and the instruments and methods used in research 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 [10].

1.2 Research Objectives, Instrument Types, and Analytical Methods

The characteristics of metabolomics detection require instruments with high sensitivity, precision, and throughput to analyze these small-molecule metabolites [8,10]. Currently, data acquisition primarily employs mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy as core analytical technologies, supplemented by efficient separation devices to form integrated metabolomics platforms. These include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS or LC-MS/MS), high-performance liquid chromatography-nuclear magnetic resonance (HPLC-NMR), and HPLC-NMR-MS technologies [8,10].

NMR technology is particularly suitable for analyzing high-abundance metabolites, requiring only simple sample preparation that preserves sample structure and properties, thus offering excellent experimental reproducibility and high sensitivity. NMR also allows experimental conditions to be selected within certain temperature and physiological buffer ranges, enabling measurements closer to physiological states and facilitating real-time dynamic monitoring [8,10]. NMR detects isotopic atomic nuclei, with the most common analyses being proton NMR (^{1}H NMR), carbon NMR (^{13}C NMR), and phosphorus NMR (^{31}P NMR). These spectroscopic methods can detect biological samples including biofluids, cell extracts, tissue fluids, and living tissues, with ^{1}H NMR responding to hydrogen-containing compounds in biological samples, enabling detection of most metabolites [8,10].

GC-MS offers high sensitivity for analyzing volatile organic compounds or those that become volatile after derivatization, and benefits from extensive searchable standard mass spectral libraries such as the National Institute of Standards Technology database, enabling accurate compound identification [8,10,11]. Current technology can simultaneously determine hundreds of chemically diverse metabolites, including organic acids, most amino acids, sugars, sugar alcohols, aromatic amines, and fatty acids [8,10,11]. LC-MS differs from GC-MS by distinguishing and identifying metabolites based on mass-to-charge ratio differences. Since LC-MS analysis requires no derivatization, the sample scope is not limited by compound properties, making it suitable for analyzing unstable, non-volatile polar and low-polarity components that are difficult for GC-MS. However, LC-MS metabolite identification lacks standard databases for comparison and requires online databases such as the Human Metabolome Database (HMDB) for compound identification [12].

Each technique has distinct advantages and inevitable limitations, but combining multiple analytical approaches can compensate for individual shortcom-

ings—an important trend in metabolomics research [8,10]. In HPLC-NMR and HPLC-NMR-MS studies, high-throughput HPLC separation efficiently fractionates metabolites and simplifies compositional complexity. NMR provides molecular weight and fragment ion information for metabolites, offering high selectivity and sensitivity for qualitative and quantitative analysis, while one- and two-dimensional NMR spectra can determine molecular structures [13].

Metabolomics data analysis methods include unsupervised and supervised approaches. Unsupervised methods comprise principal components analysis (PCA), hierarchical cluster analysis (HCA), and self-organizing maps (SOMs). Supervised methods include discriminant analysis (DA), partial least squares (PLS), partial least squares-discriminant analysis (PLS-DA), orthogonal partial least squares-discriminant analysis (OPLS-DA), soft independent modeling of class analogy (SIMCA), and artificial neural network (ANN) [8,10].

1.3 Classification and Advantages/Disadvantages

With advances in experimental techniques and data analysis methods, metabolomics-based approaches have been successfully applied in nutrition, toxicology, pathology, pharmacology, disease diagnosis, animal models, microbiology, botany, biomedicine, and environmental science [8,10]. Despite varying research directions, metabolomics studies can be broadly categorized into four types: targeted metabolomics analysis, untargeted metabolomics analysis, metabolic fingerprinting analysis, and metabolite profiling. As small-molecule compounds, metabolites represent highly promising technical means for identifying and quantifying metabolites in milk and dairy products [8,15-18]. Because different milk types possess distinct nutritional and economic values, and milk composition varies significantly among dairy species, biomarkers are needed to effectively differentiate between milks from different species during actual detection processes [14]. Milk quality and safety issues have become major public concerns, particularly regarding prohibited substances in milk and dairy products—an area where targeted metabolomics can play an important role, such as in NMR-based metabolomics analysis.

2.1 Application in Dairy Product Processing Performance Testing

Raw milk quality is crucial to the dairy industry as it directly affects milk processability and the economic value of final products [20-21]. Poor processability in raw milk inevitably results in low-quality dairy products [20-21]. Approximately 40% of European raw milk production is used for cheese manufacturing, primarily through enzymatic coagulation, making rennet-induced coagulation properties a critical quality indicator [20]. Factors affecting milk coagulation characteristics include species, breed, seasonality, and intrinsic composition [20], with the coagulation process involving numerous biochemical changes important to dairy processing. Key cheese-making steps include rennet coagulation

and syneresis of the curd. Rennet coagulation involves enzymatic hydrolysis of casein to produce para- -casein, hydrophilic caseinomacropeptide, and glycomacropeptide—a process dependent on the glycosylation degree of -casein molecules [20-21]. These biochemical processes involve many intermediate or terminal metabolites, though the specific changes remain poorly understood.

Some researchers have made valuable explorations in this field. Sundekilde et al. [17] used NMR to analyze metabolic profiles of milk with different coagulation properties from different cow breeds, seeking intrinsic relationships between milk metabolites and breed/process characteristics. Their analysis revealed that milk metabolite profiles correlated with breed and coagulation properties, with carnitine and lactose variations serving as breed classification indicators. Changes in citrate, choline, carnitine, and lactose were associated with milk coagulation properties. LC-MS/MS analysis of easily coagulating versus non-coagulating milk further identified oligosaccharides as key factors affecting coagulation performance [21].

Another critical factor affecting dairy processing performance is raw milk heat stability. Thermal processing sometimes requires temperatures exceeding 130 °C, causing biochemical changes such as protein coagulation, whey protein denaturation, and casein modifications (dephosphorylation, -casein hydrolysis) [22-23]. Metabolomics can dynamically analyze and monitor component changes during processing. For instance, ^{31}P NMR can detect phosphorus-containing compound changes in ultra-high-temperature (UHT) milk during storage [23].

Microbial contamination during production, processing, and storage causes milk spoilage. While severe microbiological changes can be detected through odor and color, subtle biochemical processes in slightly spoiled products are difficult to identify through conventional sensory or biochemical analysis. Metabolomics can illuminate metabolite changes during spoilage. One study inoculated milk with *Pseudomonas* and identified several metabolites indicative of spoilage, demonstrating that exogenous microorganisms affect analytical results [24]. Sample collection thus becomes problematic in such studies, as contaminating microbes may interfere with final results.

2.2 Application in Milk Nutrition Research

Milk nutrient content and composition are critical concerns for both producers and consumers [1]. NMR has become a powerful tool in food analysis due to its minimal sample destruction and rapid detection capabilities. Comparative studies of human milk, cow milk, and infant formula revealed that cow milk contains significantly more metabolite types, with composition varying across lactation stages [25-26]. Comparison of human and macaque milk showed higher oligosaccharide and amino acid content in human milk, while macaque milk contained more glycerophosphocholine, hippuric acid, and trimethylamine-N-oxide [27].

Choline is essential for constructing liver and brain cell membrane phospholipid

layers, and water-soluble acetylcholine serves as an important neurotransmitter with vital physiological functions. Human colostrum contains lower total choline than milk collected days postpartum, and human breast milk likely cannot meet infant growth and development requirements, necessitating supplementation from infant formula [28-29]. Besides NMR detection, hydrophilic interaction liquid chromatography (HILIC LC-MS/MS) can effectively detect choline and its metabolites, including acetylcholine, betaine, glycerophosphocholine, and lysophosphatidylcholine. Research indicates phosphocholine is the main choline form in early lactation cow milk, with levels similar to human milk but decreasing exponentially thereafter. Conversely, phosphatidylcholine dominates mid- and late-lactation, increasing with lactation duration [30]. Phospholipid particles participate in numerous biological functions and constitute key cell membrane components. Milk phospholipids form the skeleton of milk fat globules and benefit brain development, making phospholipid content an important nutritional evaluation indicator.

Milk contains various elements beyond nutrients. For example, ^{31}P NMR can compare phosphorus-containing compounds across different milk types [30]. However, most milk nutrition studies are limited to single-isotope NMR spectroscopy (^{1}H, ^{13}C, ^{31}P), with few multi-isotope NMR metabolomics studies. While lipid metabolomics can employ ^{1}H NMR for sensitive and rapid results, ^{13}C NMR is more suitable for lipid qualitative and quantitative analysis as it provides more information [31-32]. More multi-isotope NMR studies, particularly combined with mass spectrometry, are therefore needed.

2.3 Application in Milk and Dairy Product Biomarkers

Although cow milk dominates global production, minor dairy species (buffalo, yak, camel, horse) produce milk valued by nutritionists as functional foods that promote health and prevent disease [33]. Due to their high nutritional and economic value, fraudulent adulteration or substitution is common, yet difficult to detect through sensory or conventional methods, creating regulatory challenges. Rapid and accurate techniques are urgently needed to identify species-specific biomarkers [34].

Milk metabolites represent the integrated expression of mammary epithelial cells, peripheral blood, and microbial genes, conferring species-specific metabolic signatures. NMR can identify metabolic differences among cow breeds (Danish, Holstein, Jersey), with carnitine, choline, and citrate serving as potential biomarkers for breed identification [27-29]. GC-MS analysis indicates valine and glycine can differentiate cow and goat milk [35]. Combined LC-MS and NMR effectively distinguish cow, goat, buffalo, yak, camel, and horse milk. Holstein milk shows 68, 74, 54, 58, 77, and 91 differential metabolites compared to Jersey, buffalo, yak, goat, camel, and horse milk, respectively. Holstein milk contains significantly higher lactate, acetylcholine, succinate, and pyruvate, but lower carnitine, uridine, and pyroglutamic acid than other species [14].

For lactose-intolerant or milk-allergic consumers, lactose-free dairy or plant-based alternatives (soy, oat, grain) are available. ^{1}H NMR can detect quality in lactose-free beverages by quantifying lactose content using nicotinamide as an internal standard, making it suitable for rapid analysis of milk and dairy substitutes [36].

Geographical origin imparts distinct social, cultural, and economic value to dairy products. With urbanization, improved transportation, and expanded human activity, locally-produced foods are available far from their origins, raising questions about authenticity and quality consistency. Rapid, reliable techniques are needed for verification. Sacco et al. [37] combined NMR, high-performance ion chromatography (HPIC), inductively coupled plasma atomic emission spectroscopy (ICP-AES), isotope ratio mass spectrometry (IRMS), and chemometrics to differentiate milk from southern Italy versus Central/Eastern Europe, finding significantly higher lactose in Central/Eastern European milk. Integrated analysis of NMR and IRMS data revealed clear differentiation based on metabolite profiles. ^{1}H high-resolution magic-angle spinning NMR (^{1}H HRMAS-NMR) can assess mozzarella cheese quality and verify buffalo milk origin from Campania. Integrated metabolomic and microbiological analysis revealed high microbial diversity but low psychrotrophic bacterial diversity in buffalo mozzarella, with *Streptococcus thermophilus* and elevated galactose and phenylalanine. Orotic acid was the only metabolite associated with species differentiation [38].

3 Application of Metabolomics in Monitoring Dairy Cow Metabolic Diseases and Heat Stress

Negative energy balance (NEB) occurs during early lactation due primarily to insufficient feed intake. While most studies have examined NEB from blood biochemistry and physiological perspectives, such approaches poorly characterize the comprehensive metabolic features of early lactation NEB [18,26]. Metabolomics, however, can understand this physiological period from a systems biology perspective.

Klein et al. [26] used NMR and MS to detect milk metabolites during early and late lactation to identify relationships between milk composition and cow metabolic status. Results showed acetone and β-hydroxybutyrate closely correlated with mammary metabolic state during early lactation. NMR analysis revealed that besides β-hydroxybutyrate and acetoacetate as acute ketosis biomarkers, the glycerophosphocholine-to-phosphocholine ratio in first-month lactation milk may serve as a potential prognostic marker for ketosis health assessment and could be used in animal breeding to select metabolically stable animals [39-40]. Additionally, plasma concentrations of glucose, pyruvate, lactate, and alanine significantly decreased in NEB cows, while β-hydroxybutyrate exceeding subclinical ketosis thresholds indicated insufficient glucose supply and reliance on ketone bodies for energy. Increased plasma glycine reflected excessive protein catabolism and potential future vitamin B6 deficiency [41-42].

During peak lactation postpartum, cows inevitably mobilize body fat to compensate for energy deficits when intake cannot meet lactation demands. Current research indicates triglycerides from adipose tissue are the primary energy source, with hydrolyzed non-esterified fatty acids directly serving as metabolic fuel to compensate for glucose deficiency. While the alternative pathway mechanism has been studied from single-metabolite perspectives, the overall metabolic pathways and patterns remain unclear [18,26]. Targeted metabolomics using high-performance liquid chromatography-electrospray tandem mass spectrometry (ESI-LC-MS/MS) can provide more comprehensive metabolite information, offering a more complete picture of excessive lipolysis during early lactation [41]. Phosphatidylcholines involved in hepatic very-low-density lipoprotein synthesis are key lipid metabolism metabolites. Lipid metabolomics analysis revealed that high-lipolysis animals exhibited excessive fat breakdown, impaired insulin sensitivity, and altered acylcarnitine profiles. Thirty-seven key metabolites were identified across these pathways, including sphingomyelins, phospholipids, and lysophospholipids [41,43]. However, these studies are limited to dairy cows, and research in minor dairy species is needed to better understand metabolic adjustments during NEB in livestock [32,41].

Bovine mastitis involves not only infected cow health and welfare but also drug residues in milk, threatening milk quality and safety [43]. Identifying mastitis biomarkers is therefore critically important. Studies suggest haptoglobin, serum amyloid A, and ATPase as potential markers [43], but these are limited to single or few metabolites. Mastitis involves numerous metabolic processes producing diverse endogenous and exogenous molecules including peptides, amino acids, nucleic acids, sugars, organic acids, vitamins, tea polyphenols, and alkaloids, making comprehensive understanding difficult from limited data. Mass spectrometry and NMR enable monitoring of numerous metabolites [44]. Prostaglandins are important inflammatory mediators in mastitis. Monitoring related metabolic pathways revealed increased arachidonic acid metabolites including thromboxane A2, prostaglandin E2, and prostacyclin in milk from infected animals, indicating their important mediating role in the pathophysiological inflammatory response triggered by *E. coli* endotoxin release [45]. Hettinga et al. [46] used GC-MS to identify significant differences in volatile metabolites (ethyl acetate, acetic acid) between mastitic and healthy cow milk, demonstrating that milk metabolite profiling can trace animal health status. Subsequent studies showed these volatile metabolites were primarily pathogen-induced and formed by blood esterases. Detection of esterases in mastitic milk suggested increased blood-mammary barrier leakage, explaining the abundant detection of esterase-mediated metabolites [47]. However, some studies show inconsistent results regarding relationships between blood and milk metabolomics parameters in metabolically altered cows [39,43], necessitating further work to clarify distinctions and connections between different body fluids under various physiological and pathological states.

Heat stress during hot summer weather damages animal health, reduces milk yield and quality, and causes substantial economic losses. Traditional meth-

ods using temperature-humidity index to determine heat stress are convenient but inaccurate, and the physiological mechanisms and metabolic markers of heat stress remain unclear [16]. Plasma targeted metabolomics using LC-MS identified 41 heat stress markers in lactating dairy cows. Thirteen metabolites—including trimethylamine, glucose, lactate, betaine, creatine, pyruvate, acetoacetate, acetone, β-hydroxybutyrate, C16 sphingosine, lysophosphatidylcholine, phosphatidylcholine, and arachidonic acid—showed high sensitivity and specificity for diagnosing heat stress and may serve as potential biomarkers. These markers participate in carbohydrate, amino acid, lipid, and gut microbial metabolic pathways, indicating that heat stress affects multiple metabolic pathways [48]. Tian et al. [15-16] compared and integrated LC-MS and NMR data from milk samples under heat-stressed and non-heat-stressed conditions, identifying 53 diagnostic biomarkers involved in carbohydrate, amino acid, lipid, and gut microbial metabolism. Comparison with existing results revealed significant correlations between milk and plasma for lactate, pyruvate, creatine, acetone, β-hydroxybutyrate, trimethylamine, oleic acid, linoleic phosphatidylcholine, and lecithin during heat stress, suggesting enhanced blood-milk barrier permeability. While progress has been made in heat stress milk metabolomics, these studies have only screened metabolite changes across body fluids. Further experiments are needed to validate whether these markers reliably indicate metabolic changes during heat stress, clarify similarities and differences across animal groups and sample types, assess biomarker reliability in practical applications, and elucidate the physiological mechanisms underlying biomarker effects.

4 Summary and Outlook

Metabolomics has been recognized as a discipline for less than two decades, making it an emerging field. As a modern analytical technology featuring high throughput, sensitivity, and precision, it has seen extensive application and research philosophy advances, yet numerous limitations remain. For instance, metabolite identification via NMR and LC-MS is challenging due to the lack of standard reference databases. Inconsistent sample preparation methods and loading matrices also compromise comparability across studies. Furthermore, the complexity of metabolite types, structures, and compositions makes comprehensive detection impossible with single methods. Most importantly, mature research frameworks derived from metabolomics approaches are still lacking, making it difficult to address fundamental scientific questions.

Nevertheless, systems biology thinking drives continuous experimental method and analytical technique improvements, enabling comprehensive life understanding from subcellular to whole-organism levels. Traditional animal nutrition research typically discusses issues based on few indicators and their correlations, failing to fully reflect metabolic patterns or enable dynamic monitoring —limitations that represent metabolomics' strengths. By measuring numerous small-molecule metabolites, metabolomics provides metabolic profiles reflecting complete physiological or pathological states, offering more comprehensive in-

formation on animal metabolic regulatory mechanisms. Metabolomics is thus becoming an important systematic method in animal nutrition research.

With rapid economic and social development and improving living standards, animal product quality and safety receive increasing attention. Metabolomics can comprehensively analyze milk composition and composition, offering unique advantages in both metabolic fingerprinting and profiling compared to other methods, and thus holds promising application prospects in milk quality/safety research and monitoring. In summary, metabolomics' combination of macro-level high throughput and micro-level detection can help us more comprehensively understand and master information on dairy cow nutrition, health status, milk quality, processing performance, and safety. Continuous improvement in metabolomics detection methods and data analysis, along with integration and fusion with other omics approaches, will provide greater intellectual support for livestock development represented by dairy farming.

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