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Review

F3-metabolomics: Integrating feed, fluid, and food metabolomics in dairy production

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ABSTRACT

Metabolomics is increasingly recognized as a powerful approach to decode the biochemical complexity of dairy systems. In this review, we propose a novel integrative framework, F3-metabolomics, that systematically connects feed metabolomics (here described as the metabolomic profiling of different feedstuffs), fluid metabolomics (here newly defined as the metabolomic analysis of animal biofluids), and food metabolomics (the characterization of dairy products as a food category). This feed-fluid-food axis could enable researchers to trace the metabolic fate of nutrients, bioactive compounds, and environmental outputs from the ration, through the animal's systemic metabolism, to the final product. By formally introducing fluid metabolomics as the central analytical node, encompassing saliva, rumen fluid, blood, urine, milk, and feces, we highlight its role as a dynamic interface linking nutritional strategies to phenotypic and compositional outcomes. Anchored in this structured continuum, the F3-metabolomics framework provides a high-throughput basis to explore animal performance, feed efficiency, and product functionality, with a special focus on milk quality parameters. We critically evaluate recent methodological developments across each metabolomic layer, examine integrated case studies, and discuss practical applications in precision livestock farming, sustainability, and food traceability. Finally, we address current challenges in data harmonization, annotation confidence, and multi-omics integration, proposing a roadmap to accelerate the adoption of F3-metabolomics as a next-generation systems-level paradigm for dairy science.

Abbreviations: AA, amino acids; BMDB, Bovine Metabolome Database; CCS, collision cross section; DIABLO, Data Integration Analysis for Biomarker discovery using Latent cOmponents; FooDB, Food Database; GC-MS, Gas chromatography–mass spectrometry; HCA, hierarchical clustering analysis; HMDB, Human Metabolome Database; HS-SPME, head space-solid phase microextraction; HS, head space; HRMS, high-resolution mass spectrometry; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, liquid chromatography–mass spectrometry; LMDB, Livestock Metabolome Database; ML, Machine Learning; MB-PLS, Multiblock Partial Least Squares; NEFAs, non-esterified fatty acids; NMR, nuclear magnetic resonance; OPLS-DA, orthogonal PLS-DA; PCA, principal component analysis; PDO, Protected Designation of Origin; PLF, precision livestock farming. PLS-DA, partial least squares-discriminant analysis; QTOF, quadrupole time-of-flight; SESI, secondary electrospray ionization; UHPLC, ultra-HPLC; VOC, volatile organic compounds.

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1. Introduction

Modern dairy science integrates animal nutrition, physiology, microbiology, and food chemistry, yet these fields have often been studied separately (Oldham, 2017). Traditional ruminant nutrition focused on feed analysis and digestibility (Krizsan et al., 2012), while animal health and product traits were assessed independently. Such compartmentalization may miss the cascading metabolic effects of diet across the biological and productive continuum. The complexity of modern dairy systems, marked by dynamic interactions among feed composition, rumen and intestinal microbiota communities, host metabolism, and milk or cheese composition, calls for a transition toward integrative, systems-level methodologies (Xue et al., 2020; Wadood et al., 2025). Global pressures intensify this need: climate instability impacts feed availability and quality (Godde et al., 2021); regulatory and societal demands emphasize sustainability, traceability, and product healthfulness; and producers are increasingly required to balance productivity with environmental stewardship (Malik et al., 2024). In this context, metabolomics has emerged as a powerful tool to decipher biological responses to dietary, environmental, and physiological stimuli (Hajjar et al., 2023). With its capacity to provide high-resolution, time-sensitive metabolic profiles, metabolomics enables the monitoring of functional changes across biological compartments and production stages (Ashokan et al., 2023).

However, despite the growing number of omics-based studies in livestock science (Hao et al., 2021), integration across biological layers and production phases remains an approach still limited. In particular, the use of the term "Feedomics" introduced by Sun and Guan (2018), while conceptually promising, has been sporadically applied, often referred to generic mechanisms involved in biological processes dealing with animal productivity, product quality, and health, but without a unified framework, considering that the term has been explicitly mentioned in few additional published works (Sun et al., 2019; Liu et al., 2022). Moreover, animal biofluid analysis, though increasingly common, has lacked a dedicated conceptual identity within the omics landscape. To address these gaps, this review introduces a new conceptual and operational framework: F3-metabolomics, which encompasses three vertically integrated omics domains: a) feed metabolomics, mainly referred to the chemical and functional profiling of feedstuffs and dietary strategies using metabolomic tools; b) fluid metabolomics, introduced here, at the best of our knowledge, for the first time in the context of

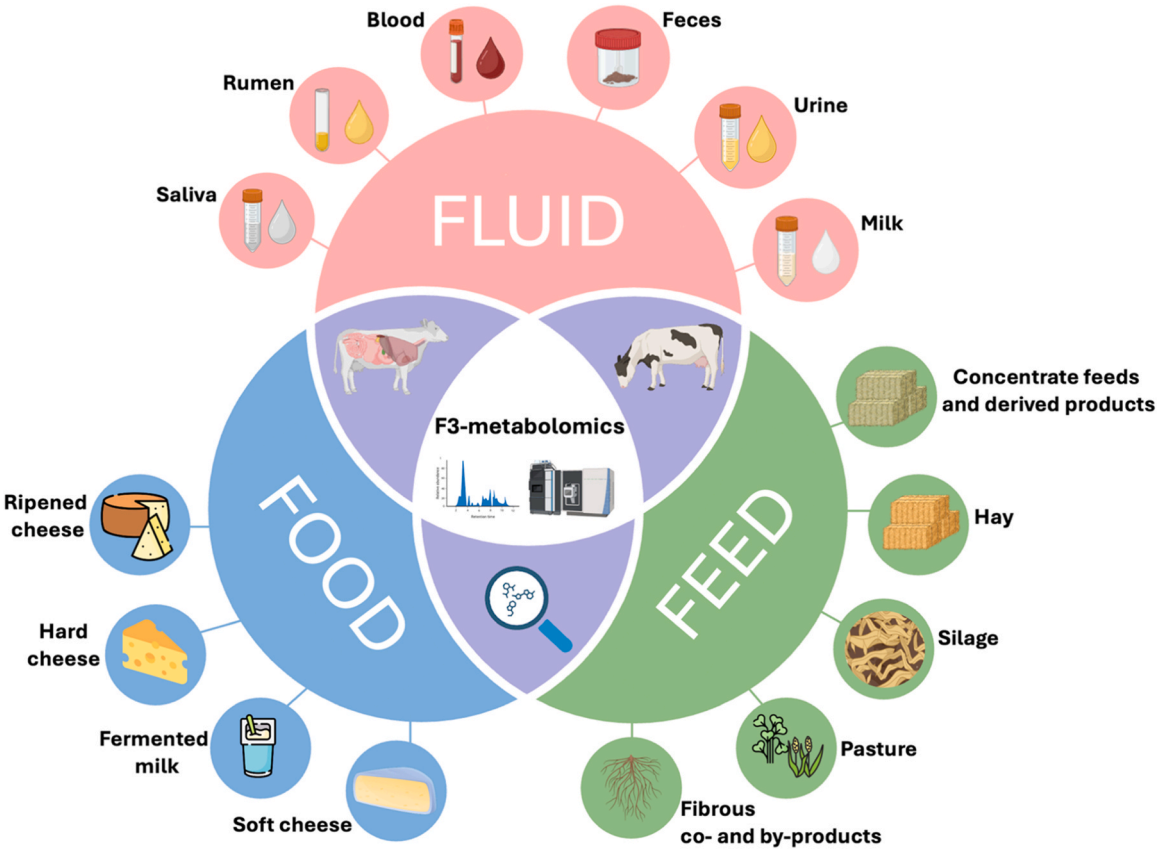

Fig. 1. Conceptual framework illustrating the F3-metabolomics continuum in dairy systems. Feed metabolomics involves the metabolomic profiling of feedstuffs, identifying bioactive and contaminant molecules (e.g., polyphenols, terpenoids, mycotoxins). These compounds undergo transformation through digestion, absorption, and systemic metabolism, processes captured by fluid metabolomics via the analysis of biofluids such as saliva, rumen, blood, feces, urine, and milk. The final output is characterized by food metabolomics, which applies metabolomics to dairy products, linking dietary inputs to product quality, functionality, and traceability.

animal science, as the study of metabolic fingerprints in biofluids (starting from saliva and through rumen fluid, blood, milk, urine, and feces), serving as dynamic indicators of host responses; c) food metabolomics, referred to the characterization of dairy products (e.g., fermented milk, ripened and fresh cheese) in relation to their biochemical composition, functional quality, and traceability. This tripartite framework can better enable researchers to trace the metabolic fate of feed-derived compounds, from ingestion, through transformation and circulation in the animal, to their final deposition in dairy products. Unlike conventional multi-omics approaches that integrate data within a single matrix (e.g., genomics and transcriptomics in tissues), F3-metabolmics proposes a cross-matrix, cross-phase model, rooted in biological causality and applicable across the feed-fluid-food axis. Such a perspective has profound implications. For instance, feed interventions (e.g., polyphenol-rich silages or nitrogen-reduced rations) can alter ruminal fermentation, modulate systemic metabolic pathways, and eventually shape the volatile, lipid, or peptide profiles of milk and cheese (Vasta et al., 2019; Maggira et al., 2023). Fluid metabolomics acts as the biochemical bridge, capturing *in vivo* transformations of feed-derived compounds and providing early biomarkers of efficiency, health, and nutritional status. Dairy food metabolomics, in turn, completes the loop, offering a molecular readout of the entire production system, while also supporting traceability, authenticity, and consumer communication. Thus, the F3-metabolomics paradigm is not simply a conceptual innovation: it represents a pragmatic research strategy capable of informing precision nutrition, environmental modeling, and value-added product development. The integrative vision of F3-metabolomics is summarized in Fig. 1, which illustrates its continuum from feed inputs to final dairy outputs, highlighting the central role of biofluid metabolism as a dynamic interface between nutrition and product quality. The following sections illustrate the potential of this approach through a structured review of current literature across the three omic domains, supported by critical reflection and future perspectives.

2. Methodological framework

This review was structured around a novel conceptual framework, termed for the first time here “F3-metabolomics”. This term defines how integrated metabolomic information from three interconnected layers of the dairy production system can be combined to trace, interpret, and predict the transfer of nutrients, bioactive compounds, and metabolic signals across the production chain. In particular, the layers are feed metabolomics (feed matrices), fluid metabolomics (animal biofluids), and food metabolomics (final dairy products). The aim was not only to summarize the existing metabolomic evidence in each domain but also to critically assess their interrelations, gaps, and translational potential within a systems biology perspective. Relevant literature was identified through targeted searches in PubMed, Scopus, and Web of Science, using combinations of keywords such as 'metabolomics', 'dairy cattle', 'milk', 'cheese', 'rumen', 'biofluids', 'feed analysis', and 'multi-omics. Interestingly, according to databases searching (August 2025), the number of scientific publications dealing with metabolomics applied to dairy science has dramatically increased over a short period,

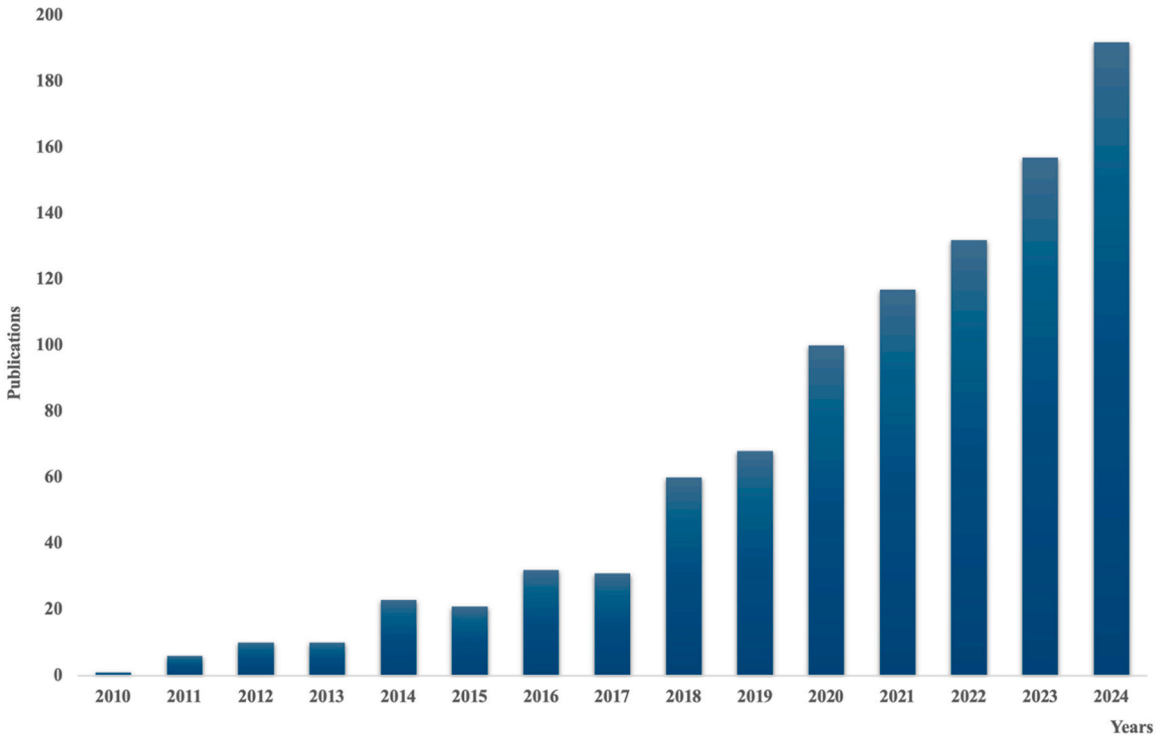

Fig. 2. Reported scientific publications by year (2010–2024) dealing with the keywords 'metabolomics' and 'dairy science' (source: PubMed, Web of Science, and Scopus).

moving from 1 article in 2010 up to 192 articles in the sole 2024 (Fig. 2). To ensure consistency and avoid redundancy, all records retrieved from the three databases were merged into a single reference library. Duplicate entries were identified and removed using a combination of automated de-duplication (based on DOI and metadata) and manual verification, and only unique publications were retained for screening. In this review, preference was given to targeted and untargeted studies (2010–2025) employing high-resolution mass spectrometry (HRMS) or nuclear magnetic resonance (NMR), particularly those that: a) involved productive ruminants (dairy cows, buffaloes, goats, and sheep), b) reported identified metabolites or annotated pathways, c) examined dietary effects, production traits, or feed-milk-rumen interactions. The extracted 70 studies were then categorized across the F3-metabolomics levels based on the sample type, analytical platform, and study objectives. For each axis, representative studies were summarized in tables detailing sample matrices, compound classes, analytical methods, key findings, and references. These tables supported the subsequent discussion of strengths, limitations, and future directions. The review also emphasizes integrative studies linking multiple omic layers (e.g., feed-fluid, fluid-food) to highlight the translational potential of systems-level metabolomics in precision livestock farming.

3. Overview of metabolomics and multi-omics tools in animal science

Metabolomics has rapidly become a pivotal tool in animal and dairy science, offering a window into the biochemical phenotype of feeds, animals, and derived products (Goldansaz et al., 2017; Suh, 2022; Ashokan et al., 2023). Depending on the research question and matrix under study, different analytical platforms are employed, each with distinct strengths and limitations (Rocchetti and O'Callaghan, 2021; Becchi et al., 2025). Nuclear magnetic resonance (NMR) spectroscopy provides high reproducibility and minimal sample preparation, making it suitable for routine and comparative analyses. However, it suffers from limited sensitivity and a relatively narrow metabolite coverage (Rocchetti and O'Callaghan, 2021). Gas chromatography-mass spectrometry (GC-MS), particularly after derivatization, is ideal for profiling volatile and thermally stable compounds, which are highly relevant in feed volatilome studies and cheese flavor characterization (Rocchetti and O'Callaghan, 2021; Tunick, 2014). Headspace-based approaches such as HS-SPME-GC-MS and HS-GC-MS represent also powerful tools for the profiling of volatile organic compounds (VOC), enabling the detection of aroma-related markers in both feed matrices (e.g., silages, hays) (Sigolo et al., 2023; Chen et al., 2025) and dairy products (Nzekou et al., 2019; Di Donato et al., 2021; Lee-Rangel et al., 2022) with high sensitivity and minimal sample preparation. These techniques are particularly suited for capturing low-abundance volatiles that contribute to sensory quality, spoilage markers, or bioactive signatures, making them highly relevant in both feed quality assessment and food authenticity studies. In contrast, liquid chromatography–mass spectrometry (LC-MS), especially when coupled with high-resolution mass spectrometers (HRMS), such as QTOF or Orbitrap systems, has become the gold standard for untargeted and semi-targeted metabolomics due to its versatility in detecting a wide spectrum of molecules (Rocchetti and O'Callaghan, 2021), from amino acids (AA) and biogenic amines (Jia et al., 2011; Moniente et al., 2022;) to lipophilic compounds like lipids (Ren et al., 2023), terpenoids and phenolics (Rocchetti et al., 2024), and mycotoxins (Gallo et al., 2024; Lapris et al., 2024). In parallel with advancements in analytical technologies, the development of curated metabolomics and food-related databases has significantly improved metabolite annotation, functional interpretation, and data standardization across dairy studies. Several resources are particularly valuable for researchers operating within the F3-metabolomics framework. The Milk Composition Database, developed by Foroutan et al. (2019), provides detailed compositional data on hundreds of compounds in milk from various species, supporting nutritional modeling and food authenticity assessments. The Bovine Metabolome Database (BMDB) (Foroutan et al., 2020) and the Livestock Metabolome Database (LMDB) (Goldansaz et al., 2017) represent two of the most comprehensive repositories for metabolite annotations in ruminant biofluids and tissues, offering information thousand metabolites including physicochemical properties, concentration ranges, and associated pathways, crucial for fluid metabolomics applications. For feed-derived compounds, the Phenol-Explorer database (Neveu et al., 2010) provides extensive coverage of polyphenols, their glycosidic forms, and biotransformation products, supporting the identification of plant-derived bioactives in both feed- and food-metabolomic contexts. Similarly, ContaminantDB (https://contaminantdb.ca/), developed within food safety and security initiatives, includes curated information on mycotoxins, processing contaminants, and other chemical hazards, facilitating untargeted screenings in feed and milk metabolomics. The Food Database (FooDB; https://foodb.ca/), part of the Human Metabolome Database (HMDB; latest update by Wishart et al., 2022) infrastructure, extends its utility to dairy science by cataloging food-derived metabolites with links to sensory, nutritional, and functional attributes. Together, these databases not only enhance metabolite identification rates in untargeted workflows, but also support downstream biological interpretation, inter-study comparability, and hypothesis generation, particularly when coupled with cheminformatics tools and pathway enrichment algorithms. However, their full potential is realized when raw MS data are accompanied by high-quality metadata, highlighting the importance of FAIR (Findable, Accessible, Interoperable, Reusable) data practices within the metabolomics community (Zulfiqar et al., 2024).

Despite the growing availability of specialized metabolomics databases, the process of compound identification, mainly in untargeted metabolomics, remains one of the most limiting steps. Matching experimental spectra to database entries still captures only a portion of the chemical diversity present in complex matrices such as milk, rumen fluid, or cheese and other milk derived-products. This bottleneck reflects both the vast metabolic complexity of biological systems and the current limitations in spectral libraries, particularly for compounds of microbial, feed-derived, or fermentation origin. Importantly, in livestock research, most spectral databases are still largely human-oriented. As a consequence, untargeted workflows often rely on cross-species comparisons, which may introduce uncertainties and lead to putative annotations that are not always fully applicable or biologically meaningful in animal production contexts. Given that a large proportion of the studies reviewed rely on untargeted metabolomics, this limitation should be explicitly acknowledged as it can affect the confidence and interpretability of differential metabolite signatures reported across dairy systems. To address this, a structured framework of annotation confidence levels has been established and widely adopted by the metabolomics community (Salek et al., 2015). These levels range from Level 0, which denotes full structural elucidation including

stereochemistry from an isolated pure compound to Level 4, which refers to unidentified features with unknown structure. Intermediate levels reflect increasing degrees of ambiguity in spectral matching, retention time alignment, and mass fragmentation interpretation (Blaženović et al., 2018). For metabolomic studies in dairy systems, it is strongly recommended that annotation levels be clearly reported for each feature or metabolite, thereby enhancing transparency, reproducibility, and downstream biological interpretation. While untargeted metabolomics provides a wide-angle view of metabolic diversity, it often yields a high proportion of features with uncertain identity or function. To complement this, targeted metabolomics offers high selectivity and quantitative precision by focusing on predefined metabolites. These analyses rely on optimized workflows that include validated transitions, internal standards, and matrix-specific calibrations (Roberts et al., 2012; Cao et al., 2020). In dairy science, targeted approaches are especially valuable for monitoring known bioactives (e.g., mycotoxins, AA, terpenes, sphingolipids) under controlled dietary or physiological conditions and allow the rapid generation of actionable insights. Looking ahead, a promising strategy involves integrating untargeted discovery-driven studies with targeted validation pipelines, enabling comprehensive profiling followed by the reliable quantification of biologically or technologically relevant metabolites. This dual-level approach is essential for advancing metabolomics from descriptive surveys to predictive and mechanistic tools in F3-metabolomics research.

The metabolomic workflow extends far beyond compound detection and annotation. Rigorous data preprocessing, including baseline correction, peak detection and deconvolution, signal drift correction, and normalization (e.g., total ion current, probabilistic quotient normalization), is essential to minimize technical noise and preserve biological signal (García-Pérez et al., 2024). Statistical analyses typically begin with unsupervised approaches like principal component analysis (PCA) and hierarchical clustering analysis (HCA) for exploratory data visualization and outlier detection. Supervised methods such as partial least squares-discriminant analysis (PLS-DA) and orthogonal PLS (OPLS-DA) are widely used to identify discriminant features; yet, they must be interpreted cautiously and validated through cross-validation, permutation testing, and external datasets to avoid overfitting, an inherent risk in high-dimensional, low-replication metabolomics datasets (García-Pérez et al., 2024). Also, machine learning (ML)-based tools (Freire et al., 2025) are emerging to offer a better understanding and optimization process by analyzing large amounts of data. ML has the potential to unravel and quantify complex processes involved in dairy production, then being able to revolutionize the dairy industry. Once significant metabolites are identified, pathway enrichment and network analysis tools (e.g., MetaboAnalyst, KEGG Mapper, Mummichog, Cytoscape) help contextualize findings within broader biological systems. This enables researchers to connect diet-induced changes to specific metabolic pathways, such as AA turnover, lipid oxidation, or microbial fermentation. Such insights are particularly valuable in dairy systems, where dietary manipulations directly affect rumen microbial activity, host metabolism, and milk composition.

The integration of metabolomics with other omics layers (the so-called multiomics approach), namely transcriptomics, metagenomics, proteomics, has expanded the functional interpretability of metabolic data, although it introduces significant computational and conceptual complexity (Becchi et al., 2025; Zhu et al., 2022; Li et al., 2025). Multi-omics studies can, for example, correlate transcript levels of hepatic enzymes with serum metabolite abundances (Ping et al., 2024) or link microbial taxa to fecal or milk metabolite profiles (Wang et al., 2024b). In this context, metabolomics acts as both a downstream readout and a functional integrator across biological hierarchies. In ruminant science, metabolomic applications must also contend with practical constraints: biological variability across breeds and individuals, dynamic physiological states (e.g., lactation, transition period), and the logistical challenges of sample collection under farm conditions. This has encouraged the use of biofluids like milk, urine, and feces as minimally invasive matrices (Jorge-Smeding et al., 2025), increasingly preferred for longitudinal monitoring and biomarker discovery. However, matrix-specific considerations remain, differences in metabolite recovery, matrix effects, and analytical sensitivity all influence interpretation and comparability. Ultimately, the true value of metabolomics lies in its ability to support predictive and mechanistic models of animal performance, health, and product quality (Choudhary et al., 2024). When embedded within a systems biology framework, such as the F3-metabolomics axis proposed in this review, metabolomics becomes not just a descriptive tool but a strategic lever for sustainable and precision-oriented dairy production.

4. Defining a new paradigm under the F3-metabolomics perspective

4.1. Feed metabolomics: characterizing the nutritional and chemical complexity of dairy rations

Although the adoption of metabolomics in dairy science is increasing (Fig. 2), the already known term Feedomics requires a broader contextualization within animal science, linking it to the chemical characterization and comprehensive profiling of feedstuffs. Integrating metabolomics, proteomics, metagenomics, and transcriptomics under this umbrella could strengthen its role in the dairy science lexicon (Sun and Guan, 2018). Here, we suggest defining feed metabolomics as the omics-based chemical characterization of feedstuffs, dietary ingredients, and nutritional strategies, being a cornerstone for interpreting and optimizing ruminant diets. Unlike traditional approaches based on proximate composition and digestibility, it captures the full biochemical complexity of rations, including secondary metabolites, bioactives, and contaminants. Within the F3-metabolomics framework, feed metabolite data can act as both nutritional descriptors and functional drivers of systemic and product-level phenotypes.

Advances in HRMS-based targeted and untargeted workflows have expanded profiling of forages, silages, pasture, and feed additives (Rocchetti et al., 2023; Lapris et al., 2024; Ahsin et al., 2025), enabling detection of polyphenols, terpenoids, VOC, and mycotoxins for a more functional evaluation of feed quality. Phenolics in legumes such as alfalfa, red clover, and ryegrass can modulate rumen ecology, improve protein utilization, and exert antioxidant effects (Rapisarda and Abu-Ghannam, 2023). Terpenoids in pasture grasses contribute to dairy product aroma and serve as markers of botanical origin and seasonal feeding (Valdivielso et al., 2017).

Beyond these conventional plant-derived compounds, an emerging frontier in feed metabolomics concerns the incorporation of

novel feed ingredients such as microalgae, macroalgae, and marine-derived lipid supplements (e.g., fish oil). These feeds are increasingly used to enhance the omega-3 fatty acid content of milk and dairy products and introduce unique bioactive lipids, pigments, and antimetabolite compounds that interact with rumen fermentation pathways (Altomonte et al., 2018; Stamey et al., 2012). Algal supplementation, in particular, has been shown to modulate rumen biohydrogenation, shift volatile fatty acid profiles, and generate distinctive metabolite signatures detectable in rumen fluid (Choi et al., 2021), blood (Flaga et al., 2025), and milk (Bernanrd et al., 2020). Their inclusion therefore expands the metabolic diversity of dairy rations and represents a key area where feed metabolomics can elucidate carry-over mechanisms affecting milk nutritional value, oxidative stability, and the broader sensory and functional properties of dairy products.

VOC profiling (alcohols, ketones, aldehydes, esters) supports silage fermentation monitoring and spoilage detection (Sigolo et al., 2023; Liu et al., 2024), with HS-SPME-GC-MS and HS-GC-MS providing sensitive early diagnostics. Feed metabolomics also enhances feed safety by enabling untargeted mycotoxin detection, with LC-HRMS allowing simultaneous quantification of compounds such as deoxynivalenol, fumonisins, and zearalenone, plus masked derivatives (Jensen et al., 2019; Lapris et al., 2025). Feed metabolite variations influence biological responses: tannin-rich forages can alter ruminal fermentation, reduce methane emissions, and modulate host metabolism (Min et al., 2020), while omega-3-rich diets can change milk fatty acid composition and nutritional quality (Bragaglio et al., 2015). From a broader systems perspective, enteric methane is a direct metabolic output shaped by feed characteristics and rumen microbial activity (Morgavi et al., 2023). In a previous study by Bica et al. (2022), methane emissions and rumen metabolite concentrations (from ^{1}H NMR metabolomics) were evaluated in cattle fed two different silages, namely red clover and grass silage. The authors showed how the changes in CH_4 emissions observed were more related to the extent of fermentations rather than the type of fermentation, revealing six metabolites showing significant differences between diets (acetate, propionate, butyrate, valerate, 3-phenylpropionate, and 2-hydroxyvalerate). Therefore, integrating greenhouse gas-related traits into the F3-metabolomics approach could expands its scope, allowing the exploration of how feed-derived metabolites influence rumen fermentation pathways, methane

Table 1
Recent applications of feed metabolomic approaches for the chemical characterization of different animal feedstuffs.

Feed matrix	Classes of compounds	Analytical platform	Study type	Key findings	References
Alfalfa hay	Volatile organic compounds	HS-SPME-GC-MS	Targeted	Ketones increase during harvest/ storage reflect alfalfa quality loss.	Yuan et al. (2022)
Pasture grasses	Phenolic compounds	LC-ESI-QTOF-MS/MS	Untargeted	Winter/spring pastures show higher polyphenol content and antioxidant activity.	Amrit et al. (2023)
Red clover species	Phenolic compounds	UPLC-MS/MS	Targeted	Isoflavones (biochanin A, formononetin) identified as red clover marker compounds.	Verhulst et al. (2024)
Corn silage (with or without *Lactiplantibacillus plantarum*)	Mycotoxins, masked toxins	SPME-GC-MS; GC-TOF-MS; LC-QE-MS/MS	Untargeted	Ensiling increases key AA and bioactives; phenylacetaldehyde marks fermentation	Su et al. (2023)
Corn silage	Mycotoxins and fungal metabolites	LC-MS/MS	Targeted	Samples were co-contaminated by 23 (safe) up to 43 mycotoxins (highly contaminated), concomitantly.	Gallo et al. (2021)
Alfalfa silage added with *Lycium barbarum* by-products	Flavonoids and other classes of compounds	UHPLC-HRMS	Untargeted	*Lycium barbarum* by-products enhance flavonoid-related functional pathways in silage.	Zhang et al. (2025)
High-moisture ryegrass silage (inoculated with lactic acid bacteria)	Phenolic acids, isoflavonoids, flavonoids, and other classes of metabolites	LC-MS	Targeted / Untargeted	Lactic acid bacteria inoculation increases organic acids, flavonoids, and dipeptides in ryegrass silage.	Xia et al. (2023)
Alfalfa silage (added with four sugar sources)	Bioactive peptides and terpenoids	UHPLC-TOF-MS	Untargeted	Fructose/pectin additives raise small peptides, reduce triterpene glycosides in silage.	Wang et al. (2020)
Alfalfa silage	Volatile organic compounds (VOC)	HS-GC-MS	Untargeted	Alfalfa silage VOC dominated by esters and terpenoids, key for aroma profiles.	Liu et al. (2024)
Whole-plant corn silage (WPCS)	Volatile organic compounds (VOC)	HS-SPME-GC-MS; HS-GC-IMS	Untargeted	Bitter almond VOC (benzaldehyde, cyanide, isocyanate) identified in corn silage.	Chen et al. (2025)
Sorghum silage (without or with *Lactiplantibacillus plantarum* inoculation)	Phenolic compounds, AA, small peptides, and other classes of compounds.	LC–ESI-MS/MS	Untargeted	Sorghum silage contains 10 bioactive metabolites, including polyphenols and cinnamates.	Kharazian et al. (2024)
Different feed and feed raw materials	Mycotoxins and their metabolites	LC-MS/MS	Targeted	Deoxynivalenol-3-glucoside and zearalenone-4-sulfate frequently detected in feed.	Streit et al. (2013)
Corn silage and high-moisture corn	Mycotoxins and other chemical classes	UHPLC-HRMS (Orbitrap)	Targeted / Untargeted	Corn silage rich in polyamines, peptides; high-moisture corn in flavonoids, mycotoxins.	Lapris et al. (2025)
Feed additives and poly-herbal formulations	Polyphenols, terpenoids, alkaloids, sulfur compounds	UHPLC-HRMS (Orbitrap)	Untargeted	Polyherbal additives characterized by terpenoids, stilbenes, and phenolic subclasses	Rocchetti et al. (2024)

formation, and downstream milk composition. Including these environmental dimensions reinforces the potential of F3-metabolomics as a tool to support both productivity and sustainability in the dairy sector.

Thus, feed metabolomics can support a molecular-level feed formulation and generates hypotheses for verification in biofluids and dairy products. This is particularly valuable under climate uncertainty, where dynamic metabolite profiling can guide forage selection, harvest timing, conservation, and functional feed additive use (Wróbel et al., 2025). A comprehensive overview of recent studies on metabolomics applied to feed matrices, dietary strategies, and forage conservation is reported in Table 1, highlighting current feed metabolomics applications in dairy science. These studies show how metabolomics characterizes feedstuffs, particularly forages and silages, and reveals how specific compounds can influence animal and product traits, laying the foundation for integrating feed chemistry into a broader systems-level framework. A consistent theme across several studies is the characterization of phenolic compounds and flavonoids, known for their bioactivity in the rumen and antioxidant properties. Verhulst et al. (2024) demonstrated that red clover isoflavones, such as biochanin A and formononetin, can serve as marker compounds to differentiate species; however, they used targeted LC-MS/MS requiring a-priori knowledge of the main compounds and not considering pedoclimatic effects on phenolic distribution. In the same way, Amrit et al. (2023) reported elevated polyphenol content and antioxidant activity in pasture grasses grown in winter and spring, using a targeted LC-MS/MS approach. Kharazian et al. (2024) identified ten phenolic compounds with potential biofunctional roles, including apigenin, caffeic acid, and gallic acid, in sorghum silage, expanding the discussion around plant secondary metabolites in ruminant nutrition. No information about the confidence level in phenolics annotation was reported. Beyond polyphenols, several studies explored VOC as indicators of feed quality and sensory potential. Yuan et al. (2022) found that ketones increased during alfalfa degradation, offering a potential VOC-based marker of silage deterioration. Liu et al. (2024) identified terpenoids, esters, and heterocyclic compounds in alfalfa silage, with compounds such as methyl and ethyl benzoate contributing to aroma. Chen et al. (2025) highlighted benzaldehyde, cyanide, and isocyanate as characteristic VOC in corn silage, associated with bitter almond flavor and potential feed aversion. These studies underscore the value of VOC profiling both for quality control and for understanding how feed-derived aroma compounds may influence milk sensory attributes. However, the previously mentioned works were based on a triple-quadrupole MS-system, thus limiting our understanding on the comprehensive VOC profiles of the studied feedstuffs. A third key axis in feed metabolomics is mycotoxin surveillance. Streit et al. (2013) provided a comprehensive screening of regulated and emerging mycotoxins, detecting over 130 compounds across diverse feed matrices, with deoxynivalenol-3-glucoside and zearalenone-4-sulfate being most prevalent. Similarly, Gallo et al. (2021) provided a comprehensive screening of 500 fungal metabolites in 64 corn silages, using a LC-MS/MS approach, demonstrating a major abundance of emerging *Fusarium*-produced mycotoxins, such as siccanol, moniliformin, equisetin, epiequisetin and bikaverin. More recently, Lapris et al. (2025) showed how UHPLC-HRMS (based on Orbitrap-MS metabolomics) can differentiate corn silage from high-moisture corn, not only by mycotoxin profiles but also by bioactive peptides and phenolic acids. These multi-residue workflows highlight how metabolomics can serve feed safety assessments far beyond traditional targeted testing. Interestingly, several studies extend feed metabolomics to functional modulation of fermentation via inoculants or feed additives. Su et al. (2023) showed that ensiling corn silage with *Lactiplantibacillus plantarum* altered key metabolite concentrations, including 3-phenyllactic acid and pyridoxine, suggesting microbial transformation of substrates. The authors used a combination of different MS-platforms, such as SPME-GC-MS, GC-TOF-MS, and LC-Orbitrap-MS, thus providing comprehensive information about the effect of microbial inoculant. Xia et al. (2023) reported enhanced accumulation of ferulic acid, apigenin, and organic acids in ryegrass silage inoculated with *L. rhamnosus*, reinforcing the value of bio-inoculants to steer the fermentative metabolome. Similarly, Zhang et al. (2025) by using HRMS coupled with robust data processing and data analysis steps showed that *Lycium barbarum* by-products enriched alfalfa silage in flavonoid biosynthesis pathways, adding potential functional value. The impact of carbohydrate supplementation on the silage metabolome was addressed by Wang et al. (2020), who found by using UHPLC-TOF-MS that fructose and pectin enhanced small peptide concentrations while reducing triterpene glycosides. These findings underscore the importance of fermentable sugar profiles in modulating microbial activity and downstream metabolic features. Lastly, Rocchetti et al. (2024) applied untargeted UHPLC-HRMS to polyherbal feed additives, identifying and semi-quantifying terpenoids, alkaloids, and sulfur compounds as key discriminants. This study provides a glimpse into the complexity of functional additives, and the power of untargeted metabolomics based on UHPLC-HRMS to profile several classes of bioactive compounds. While feed metabolomics studies provide valuable insights into the chemical complexity of feedstuffs, many still lack longitudinal sampling and integration with downstream biofluid or product data. Replication levels and environmental metadata are often insufficient to capture temporal or seasonal variability. Future works should combine feed compositional profiling with parallel measurements in animals and products, enabling causal inference within the F3-metabolmics framework.

4.2. *Fluid metabolomics: metabolic fingerprinting of biofluids as a functional bridge between feed and food*

Within the F3-metabolomics continuum, fluid metabolomics is the metabolic interface translating dietary and environmental inputs into physiological outcomes. Defined here for the first time in animal science, it refers to the omics-based profiling of biofluids such as saliva, ruminal fluid, blood, urine, milk, and feces. These matrices act as dynamic sentinels of microbial and host metabolism, bridging upstream nutritional inputs with downstream product attributes. Offering a robust alternative to invasive tissue sampling, biofluid analysis enables longitudinal monitoring across production cycles (Goldansaz et al., 2017) and supports precision livestock tools for early disease detection, nutritional diagnostics, and performance prediction. Our literature survey indicates that, despite the growing number of metabolomics-based studies in livestock science, the integration of biofluids remains fragmented, with most investigations limited to single matrices or isolated time points. An equally critical issue is the representativeness of biofluid samples: the physiological state of the animal, diurnal variation, feeding regime, and even the sampling method can profoundly influence the measured metabolome (Bando et al., 2010; Goldansaz et al., 2017). Without harmonized protocols that define when, how, and under

what conditions biofluids are collected, the reproducibility and comparability of results across studies are severely compromised. By formalizing fluid metabolomics as a distinct conceptual layer, this review positions biofluid metabolomics as the metabolic backbone of systems-level approaches in dairy science, while also emphasizing the need for standardized, reproducible sampling strategies to ensure biological relevance. Each biofluid targets specific metabolic domains. Rumen fluid reveals fermentation dynamics through VFAs, ammonia, lactate, and microbial metabolites, informing feed digestibility and fermentative balance (Nur Atikah et al., 2018; Oyebade et al., 2024). Blood plasma or serum reflects host metabolic adaptations to diet, health, and physiological demands, enabling quantification of diverse metabolite classes (Wang and Kadarmideen, 2019; Zhao et al., 2024). Urine contains waste and detoxification metabolites (e.g., urea, hippurate, creatinine), indicating nitrogen efficiency, gut microbial activity, and systemic stress (Boudra et al., 2022). Milk bridges maternal metabolism and product quality, its composition shaped by diet, physiological stage, and metabolic health (Zhu et al., 2021; Suh, 2022), with targeted/untargeted profiling revealing biomarkers such as sugars, fatty acids, oligosaccharides, and minor bioactives. Less explored matrices like saliva and feces offer non-invasive access to oral and hindgut microbial activity, host-microbiota co-metabolism, and overall health, though their systematic use in dairy science remains limited (Saraphol et al., 2025; Vallejo-Mateo et al., 2025). When integrated with feed metabolomics, fluid metabolomics can trace dietary compounds in biofluids, e.g., polyphenols or terpenes from forage tracked in rumen fluid and plasma to assess bioavailability, transformation, and systemic impact (Bešlo et al., 2022). Elevated plasma NEFAs and ketone bodies indicate early negative energy balance and ketosis risk (Lisuzzo et al., 2023), urinary creatinine-to-hippurate ratios relate to protein catabolism and microbial metabolism (Prahl et al., 2022), and milk fatty acid shifts (saturated/unsaturated ratios) reflect lipid mobilization or rumen fermentation (Kupczyński et al., 2024). These examples highlight fluid metabolomics' predictive power when diet, biofluid, and product layers are captured simultaneously, enabling early detection of metabolic disorders and real-time adjustment of feeding strategies to optimize performance and protect animal health. An integrated overview of recent studies applying metabolomics to biofluids under various dietary and management conditions is reported in Table 2, illustrating the central role of fluid metabolomics in decoding the biochemical responses that link feed to food. Particularly, it offers a powerful lens through which dietary strategies, physiological status, and metabolic outputs in dairy ruminants can be simultaneously explored. As shown in Table 2, studies targeting various fluids, mainly plasma, milk, rumen, urine, feces, and serum, highlight the remarkable capacity of metabolomics to capture functional and systemic responses to feeding regimes, environmental pressures, and animal health status. Several studies exemplify the ability of plasma metabolomics to detect early metabolic imbalances. Previous works (Huang et al., 2023; Huang et al., 2024) reported significant metabolic shifts in tryptophan, alanine, glutamate, and citrate cycle pathways, associated respectively with ketosis onset (working on plasma and milk) and subclinical hyperketonemia (working on plasma only). These metabolic alterations align with changes in purine/pyrimidine metabolism, reinforcing the role of energy and nitrogen-related pathways as sensitive markers of health and productivity. Similarly, Toral et al. (2023), although working only in untargeted UHPLC-HRMS mode, highlighted nitrogen metabolism as central to feed efficiency in dairy sheep, with both plasma and milk metabolomes indicating AA turnover as a key driver. However, further research should clarify milk production-level effects and validate findings across different diets. The combination of targeted and untargeted approaches enables detailed resolution of functional pathways. For instance, Shi et al. (2022) applied targeted lipidomics and reported disruptions in choline and glycerophospholipid metabolism in goats exposed to aflatoxin B1, while Kenéz et al. (2018) demonstrated that preweaning nutrition altered the phospholipidome of Holstein heifers. In both cases, metabolomics allowed early detection of metabolic disruptions even in the absence of overt clinical signs.

Beyond blood-derived matrices, rumen fluid analysis captures direct diet-microbiota-host interactions. Studies by Mu et al. (2019) and Li et al. (2022) demonstrated how dietary interventions (e.g., N-carbamylglutamate supplementation or high production status) significantly alter rumen metabolic profiles, modulating pathways related to arginine, lipid biosynthesis, and terpenoid metabolism. Particularly, untargeted metabolomics (although not quantitative) was very powerful in providing affected metabolic pathways. Saleem et al. (2012) further reported accumulation of potentially harmful biogenic amines (e.g., N-nitrosodimethylamine and dimethylamine) under high grain diets, highlighting the diagnostic potential of rumen metabolomics for dietary-induced dysbiosis. These authors carried out quantitative DFI-MS/MS metabolomics by using commercially available kit to determine concentrations of amino acids, sugars, acylcarnitines, sphingolipids, and glycerophospholipids in the rumen samples. The utilization of these commercial kits represents a key advancement to perform both untargeted and targeted quantitative omics studies in dairy science. Interestingly, multi-fluid studies provide deeper insight into systemic nutrient dynamics. For instance, Sun et al. (2015) characterized metabolic signatures across milk, rumen, serum, and urine, revealing AA metabolism as the most discriminant marker between high- and low-quality forages. Similarly, Oyebade et al. (2024) linked direct-fed microbials to beneficial shifts in both ruminal and plasma metabolites, supporting the potential for synbiotic interventions to influence host metabolism. A growing body of work also emphasizes heat stress, methane emissions, and milk production traits as targets of fluid metabolomics. Jorge-Smeding et al. (2024), using targeted quantitative metabolomics, showed that short-term heat stress led to more pronounced metabolic shifts in milk than plasma, underscoring the mammary gland's sensitivity to environmental stress. Additionally, previous works (Yanibada et al., 2020; Yanibada et al., 2021) exploiting targeted and untargeted metabolomics approaches, outlined that enteric methane emissions correlate with discriminant blood and milk metabolites, some of microbial origin, suggesting that metabolomics can help bridge rumen microbiome function with systemic physiology and emissions control. Urine and feces, though less frequently explored, offer promising, non-invasive matrices. Boudra et al. (2022) validated spot urine collection for robust N-level discrimination, while Saraphol et al. (2025) identified fecal metabolite pathways activated by nutrient deficiencies in thin cows. Ghaffari et al. (2025) demonstrated that AA and bile acid metabolism in feces shifts significantly across lactation stages, reflecting microbial turnover and lipid mobilization. Interestingly, this is one of the first studies to perform a detailed longitudinal analysis of the fecal metabolome in dairy cows, covering late lactation, dry-off, calving, and mid-lactation using a targeted metabolomics approach. Milk remains a focal biofluid for understanding the end-point of feed conversion and physiological adaptation. Multiple studies (e.g., Xi et al., 2017; Xu et al., 2022; Marina

Table 2
Recent applications of fluid metabolomic approaches for the chemical characterization of different animal biofluids.

Biofluids	Analytical platform	Omics study type	Key findings	References
Plasma; Milk	LC-MS/MS	Untargeted	Tryptophan and nucleotide metabolism linked to ketosis onset and milk yield.	Huang et al. (2024)
Plasma; Milk	UHPLC-QTOF-MS	Untargeted	AA and nitrogen metabolism strongly influence feed efficiency in dairy sheep.	Toral et al. (2023)
Plasma	UPLC QTOF-MS; UPLC-QTRAP-MS	Untargeted metabolomics; Targeted lipidomics	Distinct plasma metabolome identifies subclinical hyperketonemia in goats.	Huang et al. (2023)
Plasma; Rumen fluid	CIL/LC-MS	Untargeted	Microbial supplementation increases health-promoting rumen and plasma metabolites.	Oyebade et al. (2024)
Plasma	UHPLC-ESI-QTOF-MS	Untargeted	AFB1 exposure alters choline, lipid, and AA metabolism in goat plasma.	Shi et al. (2022)
Plasma	FIA-MS/MS; LC-MS/MS	Targeted	Phospholipid shifts reflect nutritional interventions in Holstein heifers.	Kenéz et al. (2018)
Plasma	UHPLC-TOF-MS	Untargeted	Transition alters lipid, sugar, and nucleotide metabolism in plasma.	Luo et al. (2019)
Plasma; Milk	UHPLC-HRMS	Targeted	Heat stress affects milk metabolome more than plasma, especially AA metabolism.	Jorge-Smeding et al. (2024)
Plasma	NMR; LC-MS; LC-MS/MS	Untargeted / Targeted	Plasma metabolome mirrors enteric methane variation; microbial metabolites involved.	Yanibada et al. (2020)
Plasma	UPLC-QTOF-MS	Untargeted	Identification of differentially expressed metabolites as a function of heat stress.	Li et al. (2025)
Blood; Serum	UHPLC-HRMS	Untargeted	Lactation-stage biomarkers in blood/serum associated with milk traits.	Fu et al. (2025)
Blood	GC-TOF-MS	Untargeted	Metritis linked to plasma markers of lipolysis, stress, and immune dysfunction.	Casaro et al. (2023)
Urine	HILIC-MS/MS	Targeted	Spot vs. total urine sampling equally valid; detects dietary nitrogen level effects.	Boudra et al. (2022)
Serum	LC-MS/MS	Targeted	BCAA and Acylcarnitines in serum associate with overconditioning in cows.	Ghaffari et al. (2019)
Rumen fluid	^{1}H NMR; GC-MS; FIA-MS/MS	Targeted	High cereal intake elevates ruminal biogenic amines and nitrosamine precursors.	Saleem et al. (2012)
Rumen fluid	LC-MS	Untargeted	High-yield cows show elevated unsaturated lipid biosynthesis in rumen fluid.	Mu et al. (2019)
Rumen fluid	UHPLC-HRMS	Untargeted	NCG supplementation alters rumen metabolites related to nitrogen and AA metabolism.	Li et al. (2022)
Rumen fluid	^{1}H NMR	Targeted	Milk replacer frequency does not affect rumen metabolome in calves.	Zened et al. (2024)
Rumen fluid; Urine; Feces	^{1}H NMR	Untargeted	Biofluids reflect diet-induced shifts; HCD alters microbial and host metabolism.	Kim et al. (2023)
Rumen fluid; Urine; Feces; Milk; Serum	^{1}H NMR	Untargeted	Core metabolite patterns distinguish fluid types; acetate, lactate, hippurate among top.	Kim et al. (2021)
Rumen fluid; Milk	^{1}H NMR	Targeted	Rumen and milk profiles show disease-related metabolites and milk quality markers.	Eom et al. (2020)
Rumen fluid; Serum	GC-TOF-MS	Untargeted / Targeted	High milk protein yield linked to AA and VFA changes in rumen and serum.	Xue et al. (2020)
Feces	UHPLC-QTOF-MS	Untargeted	Thin cows show fecal pathway shifts linked to nutrient deficiency responses.	Saraphol et al. (2025)
Feces	FIA-MS/MS; LC-MS/MS	Targeted	Fecal AA drop and bile acid rise reflect dry period vs. lactation metabolism.	Ghaffari et al. (2025)
Saliva	UHPLC-QTOF-MS	Untargeted	Saliva modulates rumen microbiota growth; individual-specific responses observed.	Palma-Hidalgo et al. (2023)
Milk	UPLC-Q-TOF MS^{E}	Untargeted	Mastitis stages reflected in milk metabolome; key AA and energy metabolites altered.	Xi et al. (2017)
Milk	UHPLC-HRMS; HILIC-MS	Untargeted	Milk features linked to glucose, lipid, AA, and hormonal metabolism affect yield.	Marina et al. (2024)
Milk	UHPLC-HRMS	Untargeted	Pyrimidine metabolites in milk indicate high-starch corn diet effects.	Rocchetti et al. (2022a)
Milk	NMR; LC-QTOF-MS	Untargeted	Milk metabolome reflects methane emission levels; lower emissions correlated with a healthier profile.	Yanibada et al. (2021)
Milk	^{1}H NMR	Targeted	Dimethyl sulfone and hippurate in milk linked to pasture-based systems.	Rojas-Gómez et al. (2025)
Milk; Serum	UHPLC-MS	Untargeted	Serum and milk profiles reveal effects of BCAA supplementation.	Xu et al. (2022)
Milk	UHPLC-QTOF-MS	Untargeted	Milk lipid and polyphenol profiles discriminate corn silage vs. hay-based diets.	Rocchetti et al. (2020)

(*continued on next page*)

Table 2 (*continued*)

Biofluids	Analytical platform	Omics study type	Key findings	References
Milk	UHPLC-HRMS	Untargeted	Mycotoxin-contaminated corn silage alters milk sphingolipid and redox metabolites.	Rocchetti et al. (2021b)
Milk	UHPLC-HRMS	Untargeted	Hippurate, enterolactone, equol in milk mark corn silage-based systems.	Rocchetti et al. (2022a)
Milk	UHPLC-HRMS	Untargeted /Targeted	Mycotoxin biomarkers in milk reflect *Fusarium* contamination levels in feed.	Rocchetti et al. (2021b)
Milk	UHPLC-HRMS	Untargeted / Targeted	Milk pyrimidines reflect dietary carbohydrate sources and odd-chain fatty acid synthesis.	Giagnoni et al. (2025)
Milk	GC-MS;^{1}H NMR	Untargeted	Maize partially replaced by legumes alters milk metabolome modestly.	Lanza et al. (2021)
Milk	LC-MS/MS	Untargeted	Grazing increases sulfur-rich and AA-derived milk metabolites.	Ashokhan et al. (2021)
Milk	UHPLC-ion mobility-HRMS	Untargeted	First application of ion mobility-HRMS to discriminate raw milk from individual cows across three production supply chains, revealing AA and lipids as the key discriminant metabolites.	Riboni et al. (2025)
Milk; Rumen fluid	UHPLC-HRMS	Untargeted	Rumen acetate and milk phospholipids linked via Fibrobacter activity.	Wang et al. (2024a)
Milk; Rumen fluid	^{1}H NMR	Untargeted / Targeted	Pasture diets elevate milk hippurate; TMR increases rumen VFAs.	O'Callaghan et al. (2018)
Milk; Rumen fluid	HPLC-QTOF-MS	Untargeted	Green feeding modulates milk and rumen metabolites, especially lipids and AA.	Neglia et al. (2023)
Milk; Rumen fluid; Serum; Urine	GC-TOF-MS	Untargeted	Multi-fluid analysis reveals AA metabolism distinguishes forage quality.	Sun et al. (2015)

et al., 2024) demonstrated how milk metabolomics can trace disease (mastitis), efficiency (feed conversion), or metabolic responses to specific feeding strategies. Additional studies (Rocchetti et al., 2020; Lanza et al., 2021; Rocchetti et al., 2021a; Rocchetti et al., 2022a; Giagnoni et al., 2025) further reinforce that milk is highly responsive to dietary composition, mycotoxin exposure, and forage type, then making it an ideal readout for tracing feed-to-food biochemical continuity. A bottleneck of milk metabolomics studies is often represented by their untargeted nature; therefore, we suggest performing more combined targeted and untargeted studies to validate some interesting candidate biomarkers and metabolomic routes. Although saliva can be collected easily and repeatedly, it remains underexplored in livestock science compared to classical biofluids such as blood, milk, urine, and rumen fluid (Tapio et al., 2016). It contains endogenous metabolites, microbial products, and enzymes reflecting both systemic status and oral health, and in ruminants plays a key role in rumen buffering and fermentation through substantial bicarbonate and phosphate input (Matthews et al., 2018). Salivary metabolomic profiling can thus provide indirect yet valuable insights into ruminal homeostasis, dietary adaptation, or early signs of metabolic stress, as demonstrated by Palma-Hidalgo et al. (2023) in goat saliva-rumen microbiome interactions. In parallel, exhaled breath analysis ("exhalomics") is emerging as an ultra-minimally invasive tool for detecting volatile metabolic signatures related to energy metabolism, microbial fermentation, and immune responses (Islam et al., 2024; Islam et al., 2023). Recent advances (e.g., SESI-HRMS) allow identification of VOCs from systemic or microbial origins without invasive rumen fluid collection methods such as rumenocentesis (Barrientos-Blanco et al., 2025). Together, saliva and exhaled breath represent promising frontiers in fluid metabolomics for precision livestock farming, provided that future efforts focus on method standardization, biomarker validation, and integration into multi-fluid omics frameworks. Taken together, the research studies here included in fluid metabolomics area demonstrate strong potential for linking diet-induced metabolic shifts to animal physiology and production traits. However, analytical approaches vary widely, and cross-study comparability remains limited. In many cases, biofluid sampling is not synchronized with feed or product collection, limiting the capacity to track metabolic fluxes. Harmonized protocols and integrated multi-matrix sampling designs will be essential to fully exploit fluid metabolomics as the mechanistic bridge in F3-metabolomics scheme.

4.3. Food metabolomics: tracing the chemical fingerprint of dairy products back to feed and farming systems

Within the F3-metabolomics framework, dairy food metabolomics is the final analytical layer, using advanced metabolomics to profile dairy products (such as raw milk for cheese manufacturing, cheese, yogurt, butter, whey) and capture the cumulative effects of feed, physiology, and environment. It translates dietary and metabolic inputs into compositional traits, enabling mechanistic insights into product variability, functionality, and authenticity. Both targeted (e.g., CLA, SCFA, antioxidant peptides) and untargeted (LC-HRMS, ^{1}H NMR) metabolomic approaches are applied (Stilo et al., 2021; Rocchetti et al., 2023; Stobiecka et al., 2022; Badawy et al., 2023; Rocchetti and O'O'Callaghan, 2021; Zhu et al., 2021). These detect compounds influencing nutritional and sensory quality, serving as biomarkers for feeding regime, origin, or seasonality. Cheese ripening studies track lipid, peptide, AA, and volatile changes to optimize flavor and texture (Piras et al., 2013; Le Boucher et al., 2015; Rocchetti et al., 2021b), while NMR fingerprinting supports PDO traceability (Rocchetti and O'O'Callaghan, 2021). Integrating food- with fluid-metabolomics reveals carry-over effects where metabolic shifts (e.g., oxidative stress, energy balance) alter milk composition, thus potentially affecting quality (Tufarelli et al., 2023) or technological properties (Kyriakaki et al., 2023; Smet et al., 2010). Positioned as a biochemical link between production and product, dairy food metabolomics validates feeding and management impacts, strengthens traceability, and identifies authenticity and sustainability markers. A comprehensive overview of metabolomics applications to dairy product characterization across diverse

feeding systems and production contexts, what we define as dairy Food metabolomics within the F3-metabolomics framework, is reported in Table 3. A core objective of dairy food metabolomics is the identification of metabolite patterns that encode traceability, authenticity, and nutritional quality. Several studies have addressed this goal within the context of European PDO cheeses. In Parmigiano Reggiano PDO cheese, previous works (Becchi et al., 2023; Becchi et al., 2024) revealed that both geographical origin (mountain *vs* lowland) and feeding system (permanent meadow *vs* conventional) significantly affect the metabolomic fingerprint of ripened cheese, with fatty acids, phospholipids, terpenoids and PUFA emerging as key discriminants. Similarly, Cavallini et al. (2023) confirmed the ability of ^{1}H NMR to resolve 31 informative metabolites capable of distinguishing mountain Parmigiano Reggiano. Comparable findings emerged in Grana Padano PDO cheese studies. Maestrello et al. (2024) linked lipid unsaturation patterns to seasonal feed changes; Rocchetti et al. (2018) highlighted plant-derived compounds, lipids, and oligopeptides as robust markers separating PDO from non-PDO products. These results support the notion that the chemical profile of dairy products retains imprints of both feeding regimen and terroir, aligning with traditional cheesemaking values. While it is true that many examples rely on GC or LC separations coupled with MS techniques, the distinctive value of metabolomics emerges from systems-level interpretation rather than the analytical platform alone. Untargeted metabolomics allows the detection of low-abundance, diet-derived metabolites that would be overlooked by targeted approaches, the construction of biomarker panels instead of single-analyte readouts, and the mapping of metabolites into functional pathways that can inform predictive models. For instance, volatile terpenes from pasture can be simultaneously profiled in feed, plasma, and milk, revealing not only presence but also carry-over and metabolic transformation, insights unattainable by conventional chromatographic methods alone. Therefore, F3-metabolomics leverages the combination of high-resolution measurements and cross-matrix integration to provide a mechanistic understanding beyond classical chemistry. Beyond Italian cheeses, metabolomic studies in traditional fermented products, such as Iranian yoghurt (Bakhshayesh et al., 2024) and Greek Graviera (Ralli et al., 2023), have detected esters, aldehydes, AA, and CLA/ω3-PUFA as key markers of compositional quality, with links to milk traits and feeding system. These confirm the functional sensitivity of microbial-driven fermentations to input quality. The VOC have become prominent readouts of feed-induced sensory shifts. In Mozzarella di Bufala Campana, Sacchi et al. (2020) and Salzano et al. (2020) showed how sorghum forage or PDO status impact esters, ketones, and specific VOC (e.g., talopyranose), directly influencing flavor and olfactory perception. Montasio cheese (Aprea et al., 2016) and uncooked pressed cheeses (Manzocchi et al., 2021; Cardin et al., 2024) similarly demonstrated that VOC profiles respond to cow diet, with herbage type and botanical richness influencing both flavor precursors and microbial enzyme activities relevant for ripening. Butter and cheddar cheese were also shown to reflect feeding systems. Other published papers (O'O'Callaghan et al., 2016; O'Callaghan et al., 2017) linked pasture-based systems to increases in β-pinene, toluene, and beneficial FA profiles. These metabolites not only enhance flavor quality but are also potential biomarkers of grazing systems, supporting product labeling and consumer transparency. Kefalograviera cheese (Tzora et al., 2022) stands out for its comprehensive quantification of over 120 metabolites, including proteolytic products, AA, lipids, vitamins, and

Table 3
Recent applications of food metabolomic approaches for the chemical characterization of dairy products when considering the different feeding strategies and milk sources.

Dairy products	Analytical platform	Omics study type	Key findings	**References**
Parmigiano Reggiano PDO cheese	UHPLC-HRMS	Untargeted	Mountain vs lowland farm origin influences cheese metabolome; key markers are FAs and glycerophospholipids.	Becchi et al. (2024)
Parmigiano Reggiano PDO cheese	UHPLC-HRMS	Untargeted	Permanent meadows feeding enhances terpenoids and PUFA in Parmigiano Reggiano.	Becchi et al. (2023)
Grana Padano PDO cheese	UHPLC-QTOF-MS	Untargeted	Lipids, oligopeptides, and plant-derived metabolites distinguish PDO from non-PDO Grana Padano.	Rocchetti et al. (2018)
Iranian traditional yoghurt	GC-MS	Untargeted	Metabolites in yogurt correlate with FA composition; esters and aldehydes most representative.	Bakhshayesh et al. (2024)
Grana Padano PDO cheese	^{1}H NMR	Untargeted	Seasonal feeding affects FA unsaturation in Grana Padano lipid profile.	Maestrello et al. (2024)
Parmigiano Reggiano PDO cheese	^{1}H NMR	Untargeted	Mountain Parmigiano Reggiano shows distinct metabolic profile; 31 discriminant compounds identified.	Cavallini et al. (2023)
Butter	GC-MS	Untargeted	Five VOC, including toluene and β-pinene, discriminate pasture-derived butters.	O'Callaghan et al. (2016)
Cheddar cheese	GC-MS	Untargeted	Pasture feeding improves FA profile of Cheddar cheese over TMR.	O'Callaghan et al. (2017)
Graviera PDO Cheese	^{1}H NMR	Untargeted	Production area affects CLA, ω3-PUFA, and polar metabolites in Graviera PDO.	Ralli et al. (2023)
Montasio PDO Cheese	SPME-GC-MS	Targeted	Ripening and diet modulate Montasio cheese aroma-related VOC.	Aprea et al. (2016)
Mozzarella di Bufala Campana PDO cheese	SPME-GC-MS	Targeted	Fresh forage alters mozzarella VOC, reducing creamy and fruity notes.	Sacchi et al. (2020)
Uncooked pressed cheese	HS-SPME-GC-MS	Untargeted / Targeted	Feeding system (pasture *vs* indoors) affects cheese VOC; 8 key volatiles vary with forage use.	Manzocchi et al. (2021)
Caciotta Cheese	HS-GC-MS	Untargeted / Targeted	Mountain Caciotta cheese shows high alcohols and ketones, low terpenes; farm origin impacts flavor precursors.	Cardin et al. (2024)
Kefalograviera Cheese	UHPLC-ESI-QTOF-MS	Targeted	Dietary flaxseed and lupin influence cheese profile; over 120 quantified metabolites including proteolysis products.	Tzora et al. (2022)
Mozzarella di Bufala Campana PDO cheese	GC-MS	Untargeted	PDO vs non-PDO mozzarella discriminated by sugar and FA derivatives; talopyranose and ribitol among markers.	Salzano et al. (2020)

nucleotides. These changes responded to sheep diets supplemented with flaxseed and lupins, suggesting the potential of food metabolomics to monitor nutritional modulation of dairy products. Food metabolomic applications in dairy systems reveal clear connections between feeding strategies, metabolic markers, and product characteristics. Yet, many studies focus on discrimination rather than mechanistic understanding, and only a minority combine product analysis with upstream feed and biofluid profiling. Embedding food metabolomics within a multi-matrix framework will enhance the predictive capacity for product quality, authenticity, and functionality.

5. Data integration across the feed-fluid-food axis

The central aim of the F3-metabolomics paradigm is to integrate feed metabolomics, fluid metabolomics, and food metabolomics within a single analytical continuum. The strength of this framework does not lie in a simple serial arrangement of analyses, but in its ability to connect complementary metabolomic datasets from feeds, animal biofluids, and dairy products in order to trace the metabolic fate of nutrients, bioactive compounds, and contaminants across the entire production chain. Importantly, this section concerns the integration of metabolomics data exclusively, and not a broader multi-omics approach.

For example, polyphenol-rich forages can be tracked from silage (feed metabolomics) through rumen metabolites and plasma profiles (fluid metabolomics) to cheese composition (food metabolomics). Similarly, reduced-protein diets influence ruminal ammonia and volatile fatty acids, with downstream effects on urinary nitrogen excretion and milk protein content. By connecting these metabolomic layers, F3-metabolomics enables hypothesis-driven investigations into the mechanisms linking nutrition, animal physiology, and product quality, providing a conceptual scaffold for future predictive models even before aggregated multi-matrix datasets are fully available. While the experimental validation of this framework remains ongoing, several targeted examples illustrate its

Table 4
Recommended best practices for implementing the F3-metabolomics framework in dairy research.

Step	Best operating practices
1. Experimental design	- Clearly define research objectives and hypotheses, including mechanistic questions linking feed, biofluid, and product outcomes. - Select representative feed, biofluid, and dairy product samples covering the intended production system (e.g., sampling corn silage, plasma, and milk for studying nutrient carry-over or feed-related biomarkers). - Plan longitudinal or time-course sampling to capture dynamic changes. - Include appropriate biological replicates and controls to ensure statistical robustness. - Record detailed metadata: diet composition, animal physiological status, environmental factors, and processing conditions to facilitate reproducibility and cross-study comparisons. - Explicitly consider potential limitations in metabolite coverage and matrix completeness, acknowledging that no single matrix or platform provides full system coverage.
2. Sampling and preservation	- Use standardized sampling protocols for each matrix (feed, biofluids, dairy products). - Minimize pre-analytical variation by controlling time of collection, animal physiological status, and environmental conditions. - Ensure biological representativeness by selecting sampling windows that reflect the physiological state of interest (e.g., lactation stage, health status) and considering pooled replicates to reduce individual variability. - Define fixed collection windows (relative to feeding, milking, or time of day) to improve repeatability across trials and laboratories. - Apply immediate preservation techniques (e.g., snap-freezing, use of preservatives) to prevent metabolite degradation. - Store samples under validated conditions (-80 °C or liquid nitrogen) until analysis.
3. Analytical platforms	- Select targeted or untargeted metabolomics approaches based on study goals, acknowledging limitations in coverage and annotation for each platform. - Combine complementary platforms (e.g., LC-MS, GC-MS NMR) to increase metabolome coverage. - Include internal standards and pooled quality control samples to monitor analytical performance and ensure data reliability. - Validate methods for sensitivity, reproducibility, and matrix effects for targeted determinations. - Recognize that analytical data are a partial view; integration across platforms and matrices is essential for deriving biologically meaningful insights.
4. Data processing and integration	- Use standardized preprocessing pipelines (normalization, transformation, scaling) to reduce analytical bias. - Use open-source tools where possible (e.g., XCMS, MetaboAnalyst, MS-DIAL). - Apply unsupervised (e.g., HCA, PCA) and supervised multivariate statistical analyses (PLS-DA, OPLS-DA) for pattern recognition and biomarker discovery. - Combine univariate and multivariate approaches; test robustness with cross-validation tools. - Explore advanced computational approaches, including machine learning algorithms (e.g., random forest, support vector machines, neural networks) to improve predictive modeling, feature selection, and multi-omic integration. - Explicitly document assumptions and known limitations of each data set; integration should aim at identifying causal or mechanistic links, not just statistical correlations. - Ensure reproducibility by documenting all processing steps and sharing scripts/code when possible.
5. Interpretation and reporting	- Contextualize metabolomic findings within the feed-fluid-food continuum. - Report both biological relevance and technical limitations of the study. - Highlight potential applications for precision feeding, product quality enhancement, and sustainability. - In targeted studies, validate candidate biomarkers in independent cohorts, increasing translational value for farm application. - Follow community guidelines for metabolomics data reporting and annotation standard levels. - Critically discuss what each omics layer contributes and how integration enhances understanding of production drivers, ensuring that F3-metabolomics is presented as a framework for insight rather than merely a descriptive tool.

potential. Feed metabolomic profiling of polyphenol-rich silages can guide precision feeding strategies (Lapris et al., 2025), optimizing nitrogen utilization and antioxidant intake. These upstream dietary modifications are then reflected in fluid metabolomics readouts, such as plasma and milk metabolite shifts, enabling early detection of metabolic imbalances or nutrient deficiencies (Huang et al., 2024; Zhao et al., 2025). Downstream, food metabolomics analysis traces dietary metabolites into dairy products, serving as authenticity markers and indicators of functional quality (Suh, 2022). Moreover, linking feed nitrogen content to urinary nitrogen excretion provides a direct avenue to evaluate environmental impact, highlighting F3-metabolomics' utility in sustainability assessment. Collectively, these examples illustrate how this approach integrates metabolomics data across multiple matrices to inform decision-making at nutritional, production, and product levels.

Tools developed for multi-omics integration (Herráiz-Gil et al., 2023), such as MB-PLS and DIABLO for supervised analysis (Singh et al., 2019; Mishra and Liland, 2023), or similarity network fusion, clustering, and multi-omic factor analysis for unsupervised exploration (García-Pérez et al., 2024), may further support future F3-metabolomics studies. When applied to metabolomics-only datasets, these tools can reveal covarying features or latent biochemical modules across feed, fluid, and food matrices. Pathway enrichment tools such as KEGG, HMDB, BMDB, and LMDB can contextualize metabolites within biological networks, enabling visualization of fluxes across compartments. Machine learning methods (random forests, SVMs, deep neural networks) are increasingly used to predict performance, health, or quality traits, though they require rigorous validation, robust feature selection, and interpretability to avoid overfitting (García-Pérez et al., 2024). A recent review by Freire et al. (2025) summarized ML and deep learning applications in dairy science, noting that spectroscopy remains the most widely used technology in combination with ML tools. While most applications focus on milk, interest is now expanding to the broader dairy sector.

Our literature searching further underscores the promise of F3-metabolomics integration, considering, for example, the influence of feed-derived compounds such as volatile terpenes on the sensory properties of milk and dairy products (Kilcawley et al., 2018), the metabolomic profiling of different cow biofluids as a function of feeding system (Kim et al., 2021), or the impact of feed additives on metabolic phenotypes (Yanibada et al., 2021). Despite encouraging potential, the field still faces challenges. Heterogeneity in sampling strategies, analytical platforms, and metabolite annotation levels often impedes full integration. Moreover, temporal asynchrony, where feed, fluid, and food samples are collected at different stages, complicates the longitudinal mapping of metabolic transformations.

To address these issues, future research should emphasize standardized sampling protocols, harmonized metadata collection, and longitudinal designs capable of capturing dynamic interactions across the feed-fluid-food continuum. To facilitate reproducibility and cross-study comparability, we propose a set of best-practice guidelines summarizing key methodological considerations for each stage of the F3-metabolomics workflow (Table 4). These recommendations aim to provide operational guidance that can be readily adopted and adapted by different research groups. The F3-metabolomics framework encourages a holistic, system-level view of dairy production based entirely on metabolomics. By integrating feed, physiological, and product metabolite profiles within a unified analytical approach, this paradigm supports the identification of causative biomarkers, the optimization of precision feeding strategies, and enhanced traceability and sustainability throughout the dairy value chain.

6. Applications in precision livestock farming and sustainability

Integrating F3-metabolomics into PLF can enhance efficiency, traceability, and sustainability in dairy by linking feed composition, animal metabolism, and product quality. In precision feeding, metabolomic biomarkers of rumen efficiency, nitrogen use, and amino acid metabolism (Rocchetti et al., 2022b; Malheiros et al., 2023; Giagnoni et al., 2025) allow ration adjustments to cut methane and nitrogen losses (Zhang et al., 2020). Additionally, real-time data integration into farm software could guide dynamic dietary strategies. Food metabolomics also supports authentication and quality control schemes, distinguishing PDO cheeses from imitations and, when coupled with blockchain, enabling transparent supply chains (Castellini et al., 2022a; Castellini et al., 2022b). In food safety, metabolites from mycotoxins, drug residues, or spoilage organisms (Hew et al., 2024) offer early warnings across the feed-milk chain. For example, derivatives of zearalenone, fumonisins, and aflatoxins can be identified in both feed and milk, enabling upstream-downstream surveillance strategies. Moreover, from a circular bioeconomy view, metabolomics can assess alternative feeds-by-products, insect meals, polyherbal blends (Rocchetti et al., 2023; Gao et al., 2024; Wang et al., 2024b; Rossi et al., 2025), ensuring they sustain animal health and product quality. Embedding F3-metabolomics in PLF aligns dairy production with the triple bottom line: economic viability, environmental stewardship, and social trust.

7. Challenges and future perspectives of the F3-metabolomics paradigm

Despite the rapid adoption of metabolomics in dairy science, several limitations persist across all F3-metabolomics layers. Within single matrices, whether feed, rumen fluid, or milk, metabolite coverage remains incomplete, with many detected features unannotated or lacking functional characterization. Differences in analytical platforms (LC-MS, GC-MS NMR) and variable data-processing pipelines further hinder cross-study comparability. Additionally, most studies remain descriptive, rarely integrating pathway information or causal inference to link diet, metabolism, and product traits. Recognizing these limitations is essential: it clarifies that F3-metabolomics is proposed not as a comprehensive solution but as a structured framework to progressively integrate partial, complementary insights across the feed-fluid-food continuum, ultimately enabling systems-level understanding despite individual methodological gaps. While the integration of feed-, fluid-, and food-metabolomics represents a promising conceptual advance toward a comprehensive feed-to-food understanding in dairy systems, several methodological and interpretative challenges remain. We acknowledge that the true benefits of F3-metabolomics will only be fully realized once integrated experiments are conducted across

diverse sample sets and analytical platforms. This review does not claim to present completed F3-metabolomics studies but aims to define the framework, synthesize current literature, and provide a roadmap for future investigations. By highlighting gaps, such as limited longitudinal sampling, inconsistent annotation levels, and incomplete cross-matrix integration, we emphasize that F3-metabolomics serves as a conceptual scaffold to guide research towards coordinated, multi-matrix, and multi-omic experiments. Future studies should prioritize synchronized sampling of feed, biofluids, and dairy products, combined with standardized workflows and high-resolution platforms, to enable mechanistic interpretation and predictive modeling. In this sense, F3-metabolomics represents a forward-looking framework for dairy science, offering a vision for integrated precision feeding, product traceability, and sustainable production rather than immediate experimental proof. Current feed metabolomics studies, though increasingly sophisticated, are often limited to single feed matrices and rarely extend their insights beyond descriptive profiling. This restricts their utility in tracing feed-derived metabolites across the metabolic continuum. Moreover, the lack of standardization in sample preparation, data processing, and compound annotation hampers comparability across studies and laboratories; therefore, this review also aims to provide detailed operational guidelines to support the adoption of best practices in dairy science (Table 4). The extensive use of untargeted approaches (46 out 70 of the reviewed studies reported in Table 1, 2, and 3) frequently results in low annotation rates, leaving many detected features unidentified or functionally uncharacterized. Critically, the carry-over potential of key bioactive compounds, such as isoflavones, mycotoxins, or terpenoids, from feed to animal to product is seldom explored in a quantitative or mechanistic manner (Aprea et al., 2016; Sacchi et al., 2020; Tzora et al., 2022). These gaps underscore the need for truly integrative experimental designs where feed, biofluids, and final products are studied in parallel, enabling the reconstruction of metabolic linkages and causal inference, the core ambition of the F3-metabolomics framework. Similarly, the expanding body of fluid metabolomic research offers a window into the dynamic metabolic status of dairy animals but is frequently confined to isolated biofluids (27 out 41 of the reviewed studies reported in Table 2), limiting its capacity to capture systemic responses. Despite the availability of powerful tools, from LC-MS/MS to NMR, the lack of harmonized platforms and quantitative consistency across studies complicates cross-comparison and biomarker validation. Many metabolites of interest are of microbial origin, yet metagenomic data are often missing, preventing the elucidation of host-microbiota interactions. Moreover, while correlations between fluid metabolites and performance traits are increasingly reported (Xue et al., 2020; Shi et al., 2022), few studies confirm whether these biomarkers play functional or causal roles in metabolic regulation or production efficiency. Therefore, fluid metabolomics must embrace more holistic designs that include multiple biofluids, longitudinal sampling, and integration with both upstream feed data and downstream product analysis, reinforcing its role as the central metabolic conduit of the F3-metabolomics axis. At the terminal end of this continuum, food metabolomics provides the final readout of how feeding strategies and physiological processes translate into the chemical and functional quality of dairy products. Yet, despite its potential, most food metabolomic studies remain disconnected from upstream layers. Without synchronized sampling or common analytical pipelines, it becomes difficult to model causality or interpret the impact of diet or metabolism on specific product attributes. Many studies focus heavily on lipidomic profile or VOC (O'Callaghan et al., 2016; Aprea et al., 2016; Sacchi et al., 2020; Manzocchi et al., 2021), while other compound classes such as microbial metabolites, peptides, or minor phytochemicals remain underexplored. Additionally, the heterogeneity in product types, ripening stages, and production contexts, especially within PDO systems, calls for greater methodological rigor and harmonization. Just to provide a highly relevant example, UHPLC-ion mobility-HRMS was only recently applied by Riboni et al. (2025) for the assessment of raw milk traceability. In particular, the authors achieved high-confidence identification of 153 discriminant features for tracing Grana Padano PDO milk samples at farm level, mainly belonging to amino acids, glycerolipids, and glycerophospholipids. This represents the first study in which ion mobility was coupled to HRMS for milk metabolomics profiling, providing an extra dimension of ion separation and, crucially, the collision cross section (CCS) as an orthogonal identification parameter. The inclusion of CCS values markedly enhances annotation confidence, which is especially critical in dairy science for the high-resolution quantification of structurally similar lipid species that are otherwise challenging to differentiate. To position dairy food metabolomics as a powerful downstream biosensor of the feed-animal-product chain, future works should adopt paired sampling strategies, longitudinal approaches, high-resolution platforms, and multi-omic fusion, enabling the tracing of molecular signals from pasture to plate. Ultimately, the challenges faced by each individual omics layer are not isolated, but interconnected. Their resolution lies in the co-design of studies that integrate these dimensions in a coordinated, system-wide manner. The F3-metabolomics paradigm thus invites a methodological shift: from compartmentalized omic investigations toward truly transversal metabolomics, capable of capturing the biochemical continuity that links soil, feed, animal metabolism, and dairy products.

8. Conclusions

The F3-metabolomics framework, integrating Feed-, Fluid-, and Food-metabolomics, offers a structured, systems-level approach to track the biochemical continuum from feed to food in dairy production. By positioning fluid metabolomics as the central link, it enables researchers to connect dietary inputs with metabolic pathways and final product outcomes, supporting biomarker identification, metabolic flux interpretation, and predictive modeling for precision livestock farming. Potential applications include optimizing nutrient supply, enhancing feed efficiency, reducing environmental emissions, authenticating product origin, and safeguarding animal health and welfare. Its full potential depends on critical advances: standardizing sampling protocols, improving annotation confidence, and harmonizing inter-omic data to ensure reproducibility; exploring alternative matrices such as saliva or exhaled breath for minimally invasive monitoring; and strengthening connections between omics outputs and on-farm decision tools to translate molecular insights into practice. With the growing availability of multi-omic databases and more integrated analytical pipelines, F3-metabolomics is well positioned to guide a new era of traceable, sustainable dairy production, bridging research and practice, and aligning productivity with environmental and ethical commitments.

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Gabriele Rocchetti: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Antonio Gallo:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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