

Untargeted Metabolomics in Fermented Food Systems

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Abstract

Fermented foods are chemically complex systems in which substrate composition, microbial community dynamics, and physicochemical conditions interact to generate thousands of metabolites across diverse chemical classes. Conventional targeted analytical approaches quantify predefined compounds with high precision but operate within a restricted chemical space, systematically excluding emergent features central to product identity, safety, and sensory character. Untargeted metabolomics addresses this limitation by capturing global chemical fingerprints of fermented matrices, enabling discovery-driven investigation across a broad fraction of the metabolome. This review examines the application of untargeted metabolomics across key research areas in fermented food science, including fermentation monitoring, microbial interactions, flavour development, process optimisation, post-fermentation stability, and safety assessment. Across these domains, untargeted approaches reveal system-level metabolic relationships beyond the reach of targeted analyses, while also presenting interpretive challenges. A central limitation is the annotation bottleneck: despite high feature detection rates, only a small fraction of signals are structurally identified, constraining mechanistic interpretation and cross-study comparability. Additional challenges in data processing, statistical validation, and interlaboratory reproducibility further limit data interpretation. Addressing these constraints through improved spectral libraries, standardised workflows, and integration with complementary omics is essential for advancing untargeted metabolomics towards robust knowledge generation in fermented food systems.

Keywords: untargeted metabolomics; fermented foods; metabolite annotation; microbial interactions; flavour development; food safety



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1. Introduction

Fermented foods represent one of the most chemically complex categories of food matrices that analytical science is required to characterise. The chemical composition of a fermented product is neither fixed at the moment of inoculation nor does it converge towards a stable endpoint; rather, it reflects the evolving output of a system in which substrate composition, microbial community succession, inter-species interactions, and physicochemical conditions interact across timescales ranging from hours to months. Microbial communities and physicochemical conditions generate thousands of metabolites spanning diverse chemical classes and contributing to product quality, safety, and sensory properties. Importantly, a substantial fraction of these metabolites arise from microbial co-metabolism, emerging from competitive and cooperative interactions within complex communities; as such, their formation is highly sensitive to initial conditions and cannot be

reliably inferred from the metabolic capabilities of individual species. The chemical landscape that ultimately defines fermented food quality, safety, and functionality is therefore best understood as an emergent, system-level property rather than a predictable sum of isolated biochemical processes. Although targeted analytical approaches remain indispensable for quantitative analysis and regulatory applications, they face important limitations when applied to highly dynamic and chemically diverse fermentation systems.

Targeted methods—whether based on reference standards, selected ion monitoring, or validated multi-residue panels—quantify predefined analytes with high precision but operate under a closed-world assumption: only compounds that are already known and expected can be detected. In fermented food matrices, where the chemical space encompasses a vast diversity of yeast-derived secondary metabolites—including esters, higher alcohols, organic acids and other transformation products generated during fermentation—often spanning a wide dynamic concentration range [1], targeted strategies may overlook unexpected or previously uncharacterised metabolites [2,3], as they are inherently restricted to metabolites present at relatively high concentrations and fail to capture the broader chemical diversity of the system [4]. Consequently, they may not fully capture that key quality markers may correspond to previously undescribed oxidation products, that safety signals may emerge as subtle metabolic perturbations well before regulated contaminants reach critical thresholds, or that aroma profiles may be governed by a limited number of precursor–product relationships spanning distinct chemical classes. The consequence is not merely incomplete characterisation, but a structurally biased representation of fermentation chemistry—one that reflects the constraints of assay design rather than the underlying metabolic reality of the system. Implemented primarily through high-resolution liquid chromatography–mass spectrometry and, for volatile fractions, gas chromatography–mass spectrometry, untargeted approaches detect tens of thousands of metabolic features per sample, spanning polar and non-polar primary metabolites, secondary metabolites, and transformation products [5,6]. The resulting datasets capture global chemical fingerprints of fermented matrices at specific time points and, in longitudinal designs, enable reconstruction of fermentation trajectories at the system level. Importantly, this fingerprinting strategy is inherently hypothesis-generating rather than hypothesis-confirming: instead of validating predefined targets, it allows the identification of features that differentiate conditions, evolve over time, or correlate with process variables and quality attributes. Several recent reviews have explored metabolomics and multi-omics applications in fermented foods, including Asian fermented products such as kimchi, soy sauce, douchi, and other traditional fermented foods [7–9]. However, critical discussions regarding metabolite annotation, biological interpretation, reproducibility, quality control, and methodological standardisation remain comparatively limited.

When applied to fermented food systems, this discovery-driven paradigm has revealed metabolites arising from microbial interactions that cannot be predicted from monoculture studies, enabled early detection of contamination through system-level perturbation patterns, and uncovered precursor–product relationships linking specific metabolic classes to sensory outcomes [10,11]. Collectively, these findings underscore the ability of untargeted metabolomics to capture emergent chemical behaviour that extends beyond the scope of targeted analytical frameworks. Nevertheless, untargeted metabolomics should be viewed as complementary rather than alternative to targeted approaches, as biological interpretation, quantitative validation, and regulatory decision-making frequently require confirmatory targeted or semi-targeted analyses.

The field has now matured to the point where the limitations of untargeted metabolomics are increasingly recognised. While analytical platforms have improved substantially over the last decade, data interpretation and metabolite identification re-

main major challenges limiting the full exploitation of untargeted metabolomics. Current analytical platforms generate feature-rich datasets with high within-run reproducibility; however, only a small fraction of detected features can be confidently annotated at the structural level. This challenge may be particularly pronounced in fermented foods, where microbial metabolites, biotransformation products, Maillard-derived compounds, and matrix-specific metabolites remain underrepresented in current spectral databases and reference libraries [12]. In addition, the reproducibility of feature-level findings across laboratories remains limited, and the link between statistically discriminant features and biologically meaningful mechanisms is often unclear [13]. As a consequence, multivariate models built on large sets of unannotated features can discriminate fermented products with high accuracy, yet provide limited insight into the underlying chemistry driving these differences. Furthermore, current workflows may fail to detect biologically relevant low-abundance compounds, highlighting that both unidentified features and undetected metabolites contribute to the incomplete characterisation of fermented food metabolomes.

These interpretive constraints are not incidental but reflect fundamental limitations, including incomplete spectral reference databases, insufficient standardisation and quality control practices, and analytical workflows prioritising metabolome coverage over structured biological interpretation in complex food matrices.

This review examines the application of untargeted metabolomics across key areas of fermented food research, including the monitoring of dynamic fermentation processes, the characterisation of microbial metabolism and inter-species interactions, the elucidation of flavour and aroma development, the optimisation of process conditions, the assessment of post-fermentation stability, and the detection of safety-relevant chemical changes. For each area, the capacity of untargeted approaches to reveal insights beyond the scope of targeted analyses is evaluated alongside the interpretive limitations that constrain the translation of findings into actionable knowledge. Particular attention is given to challenges associated with metabolite annotation, biological interpretation, reproducibility, quality control, and the translation of untargeted metabolomics findings into robust and actionable knowledge. The review concludes with an assessment of the methodological and conceptual challenges that currently limit the field, together with practical recommendations for experimental design, quality control, metabolite annotation, and data reporting. Rather than providing a catalogue of applications, this work aims to define the current capabilities and limitations of untargeted metabolomics as an analytical framework for understanding fermented food systems.

2. Conceptual and Analytical Framework

The distinction between targeted and untargeted metabolomics reflects not only a technical difference but a fundamental divergence in analytical epistemology. Targeted approaches quantify predefined metabolites using selected or multiple reaction monitoring on triple-quadrupole instruments, providing high sensitivity, precision, and absolute quantification for compounds with established identity and fragmentation behavior [14,15]. While this hypothesis-confirming framework is well suited to quantitative analysis, validation studies, and regulatory applications, its coverage is inherently restricted to predefined compounds. This limitation may reduce its ability to capture the full chemical complexity of highly dynamic systems such as fermented food matrices, where the metabolome extends beyond existing analytical panels. Fermentation does not generate a static metabolite inventory; rather, the combined and sequential activities of diverse microbial consortia—shaped by substrate depletion, pH evolution, temperature, and inter-species interactions—drive the continuous formation and transformation of a chemically diverse metabolome spanning organic acids, esters, higher alcohols, biotransformed polyphenols, bioactive peptides, and

structurally uncharacterised secondary metabolites [2,3]. In this context, targeted panels provide information on predefined metabolites but may overlook unexpected metabolites and transformation products that contribute to product identity, batch-to-batch variability, and functional properties.

Untargeted metabolomics partially addresses this limitation by acquiring data across a broad fraction of the ionisable or volatile chemical space, enabling post-acquisition compound annotation and facilitating the generation of new hypotheses from complex datasets. However, biological interpretation and validation frequently require complementary targeted or semi-targeted approaches [9]. Importantly, untargeted and targeted metabolomics should be regarded as complementary rather than competing analytical strategies. In practice, untargeted approaches are primarily used for discovery, systems-level characterisation, and hypothesis generation, whereas targeted workflows remain essential for quantitative confirmation, validation, and regulatory applications. Figure 1 provides an overview of the untargeted metabolomics workflow in fermented food systems, from sample collection and data acquisition to biological interpretation and industrial applications, while also highlighting current analytical challenges and future opportunities for predictive fermentation management.

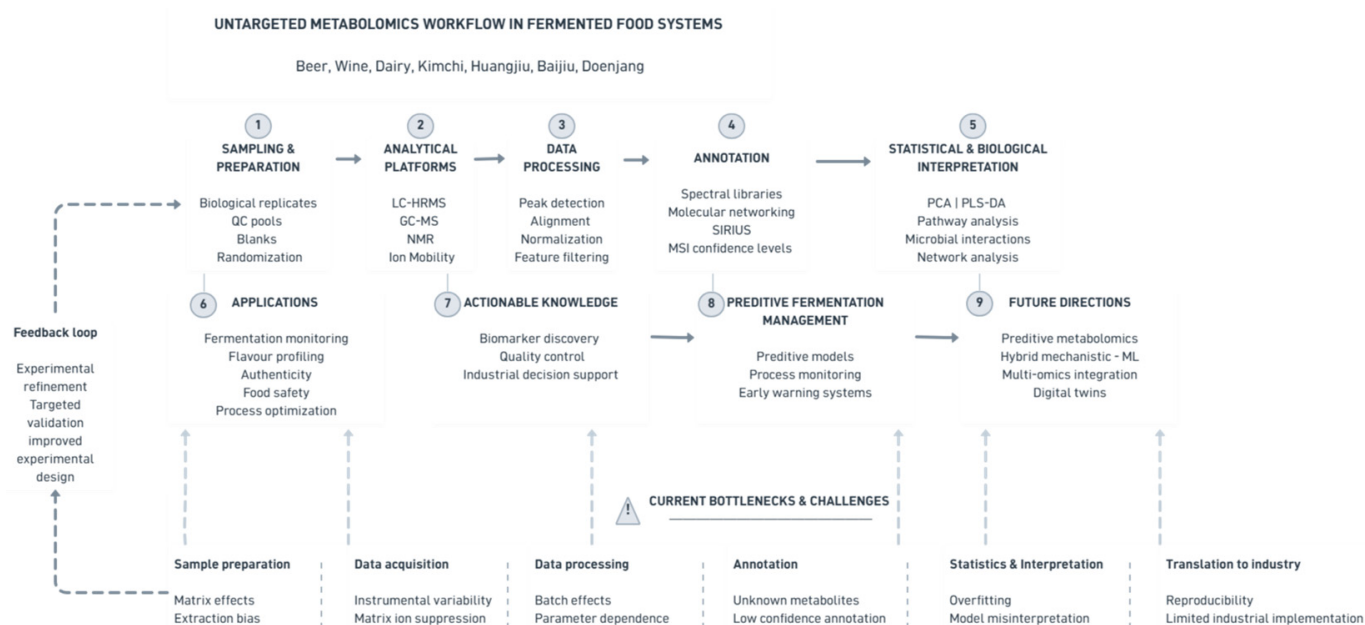


Figure 1. General workflow, applications, current challenges, and future directions of untargeted metabolomics in fermented food systems. Solid arrows indicate the primary analytical workflow, whereas dashed arrows represent iterative feedback processes and the influence of critical limitations across different stages of the metabolomics pipeline.

The implementation of untargeted metabolomics in fermented matrices introduces analytical challenges that are more pronounced than in simpler biological systems. High concentrations of ethanol, organic acids, sugars, and polyphenols generate strong matrix effects; in electrospray ionisation, co-eluting compounds can suppress or enhance analyte signals, thereby reducing both feature detectability and the accuracy of relative quantification [16]. As a result, sample preparation must balance a fundamental trade-off between reducing matrix complexity and preserving metabolome coverage. This limitation is particularly evident in volatile profiling of fermented beverages, where comparative evaluations of extraction techniques—including dynamic headspace, solid-phase microextraction (SPME), solid-phase extraction, liquid–liquid microextraction, and stir-bar sorptive extraction—have shown that each method captures only a distinct subset of volatile com-

pounds. The overlap in annotated features between methods is typically limited, with SPME and solid-phase extraction emerging as complementary rather than redundant approaches [17]. Consequently, no single extraction protocol can comprehensively recover the metabolome of fermented foods, and multi-method strategies are increasingly required for robust characterisation. The optimal sample preparation strategy is also strongly matrix-dependent, as fermented beverages, dairy products, soybean-based fermentations, meat products, and cereal fermentations differ substantially in ethanol, fat, salt, protein, and polyphenol contents, which directly influence extraction efficiency, matrix effects, and metabolite recovery.

Beyond analytical considerations, batch-to-batch variability represents an additional challenge intrinsic to fermented systems. Microbial community stochasticity and substrate heterogeneity introduce substantial inter-sample variation. In addition, distinguishing biological variability from technical batch effects remains a major challenge in large-scale fermentation studies, particularly when samples are collected across different production batches, seasons, or fermentation stages. These sources of variability further complicate the interpretation of untargeted metabolomics data, reinforcing the need for carefully structured workflows that integrate both analytical and biological sources of uncertainty. Strategies such as experimental randomisation, batch-aware statistical modelling, and appropriate quality control procedures are therefore essential to minimise confounding effects and improve the reliability of biological interpretations.

At the instrumental level, LC-HRMS, GC-MS, and NMR provide complementary rather than interchangeable views of the fermentation metabolome. LC-HRMS—particularly using quadrupole-Orbitrap or QTOF instruments in data-independent acquisition (DIA) modes—offers the broadest coverage for non-volatile and semi-volatile compounds across a wide polarity range, with sub-5 ppm mass accuracy supporting molecular formula assignment and concurrent MS/MS acquisition for all detected features [5,13]. Multi-column strategies (e.g., reversed-phase and HILIC in both ionisation modes) can collectively detect thousands of structurally distinct features per sample, although the proportion annotated at high confidence remains limited by current spectral database coverage, particularly for microbial metabolites, fermentation-derived transformation products, and matrix-specific compounds that remain underrepresented in available reference libraries. GC-MS retains a distinct advantage for volatile compound profiling: its spectral reproducibility and the availability of extensive electron ionisation libraries enable higher-confidence annotation of aroma-active compounds than is typically achievable in LC-MS/MS [11]. The use of two-dimensional GC coupled to TOFMS further increases peak capacity, resolving co-eluting esters, alcohols, and terpenoids in complex matrices such as wine and beer. NMR occupies a complementary niche: although less sensitive, it is inherently quantitative, non-destructive, and structurally informative for isomer discrimination, making it particularly valuable for fermentation monitoring and food authentication [18,19]. A comparative overview of the main analytical platforms used in untargeted metabolomics of fermented foods, including their capabilities, limitations, and typical applications, is provided in Table 1.

Across all platforms, compound annotation remains a major bottleneck. This limitation is particularly relevant in fermented foods, where extensive microbial metabolism and biotransformation generate compounds that are frequently absent from conventional metabolomics databases. Although annotation rates are often low in untargeted LC-MS datasets, the true proportion of confidently annotated features in fermented food systems remains uncertain and depends strongly on matrix complexity, analytical platform, and database coverage.

Table 1. Analytical platforms used in untargeted metabolomics of fermented foods.

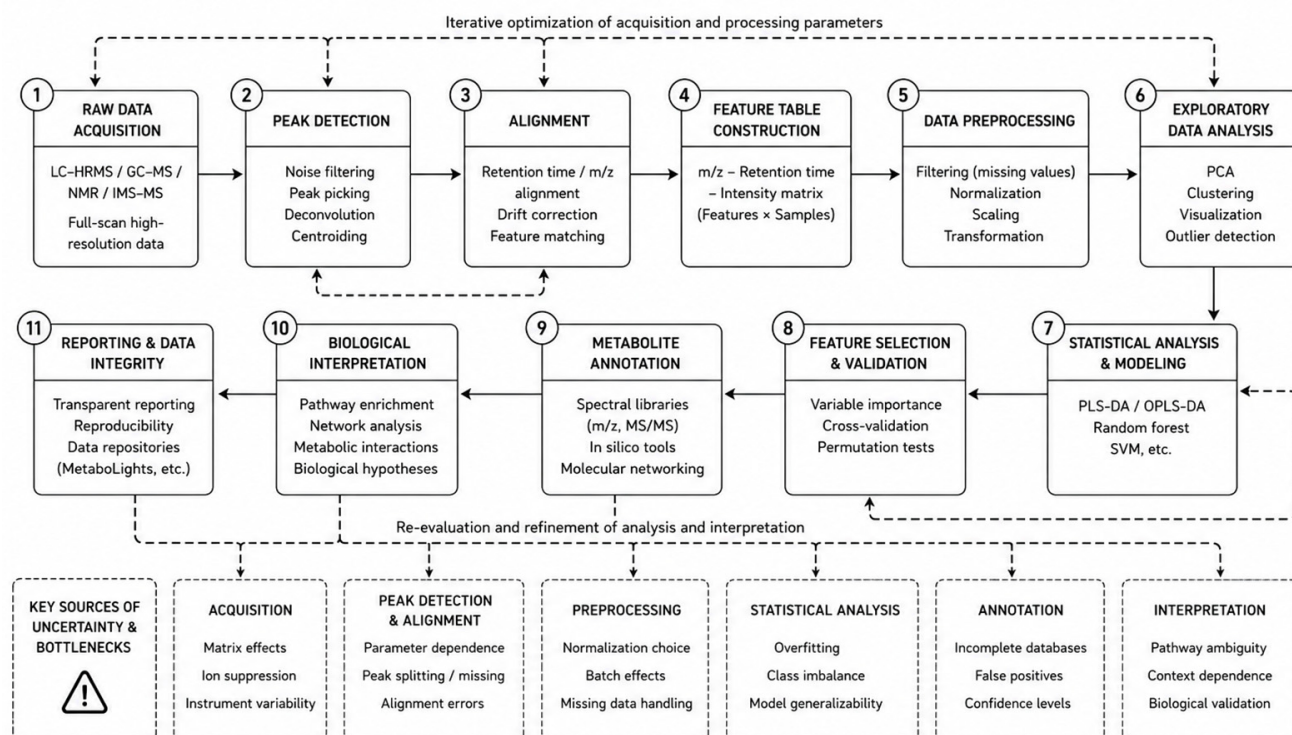
Platform	Analysed Fraction	Key Advantages	Main Limitations	References
LC-HRMS (Liquid Chromatography–High-Resolution Mass Spectrometry)	Broad coverage of non-volatile and semi-volatile compounds across a wide polarity range	Sub-5 ppm mass accuracy supporting molecular formula assignment and concurrent MS/MS acquisition for all detected features in DIA modes; multi-column strategies (reversed-phase and HILIC, both ionisation modes) can detect thousands of structurally distinct features per sample	High-confidence annotation remains limited by spectral database coverage; low annotation rates are common for fermentation-derived metabolites and transformation products; many signals arise from adducts, in-source fragments, and informatic artefacts rather than novel metabolites	[20–26]
GC-MS (Gas Chromatography–Mass Spectrometry)	Volatile fractions (volatile compound profiling in fermented matrices)	Spectral reproducibility with extensive electron ionisation libraries enables higher-confidence annotation of aroma-active compounds than is typically achievable in LC-MS/MS; two-dimensional GC coupled to TOFMS increases peak capacity, resolving co-eluting esters, alcohols, and terpenoids in complex matrices (e.g., wine and beer)	Inapplicability to thermolabile compounds; risk of degradation artefacts in biological samples containing heat-sensitive metabolites; lack of coverage of polar non-volatile metabolites; requirement for derivatization; sample preparation and extraction strategies are highly dependent on analyte volatility and matrix composition, often requiring selective extraction or derivatisation procedures; feature sets dependent on the extraction method used	[27–30]
NMR (Nuclear Magnetic Resonance Spectroscopy)	Quantitative, non-destructive metabolomic profiling with structural information supporting isomer discrimination; particularly valuable for fermentation monitoring and food authentication	Inherently quantitative, non-destructive, and structurally informative for isomer discrimination	Lower sensitivity than MS-based platforms	[31,32]
PTR-ToF-MS (Proton Transfer Reaction–Time-of-Flight Mass Spectrometry)	Real-time monitoring of the global VOC fingerprint during fermentation	Captures non-linear evolution of VOC fingerprints in real time during active fermentation	Limited structural information, restricted compound identification capability without complementary techniques, and challenges in resolving isomeric compounds	[33]

Abbreviations: DIA = data-independent acquisition; GC-MS = Gas Chromatography–Mass Spectrometry; HILIC = Hydrophilic Interaction Liquid Chromatography; HRMS = High-Resolution Mass Spectrometry; LC-MS/MS = Liquid Chromatography–Tandem Mass Spectrometry; LC-HRMS = Liquid Chromatography–High-Resolution Mass Spectrometry; ML = machine learning; MS/MS = tandem mass spectrometry; NMR = Nuclear Magnetic Resonance Spectroscopy; PTR-ToF-MS = Proton Transfer Reaction–Time-of-Flight Mass Spectrometry; QTOF = Quadrupole Time-of-Flight; TCA = tricarboxylic acid; TOFMS = time-of-flight mass spectrometry; UPLC-HRMS = Ultra-Performance Liquid Chromatography–High-Resolution Mass Spectrometry; VOC = volatile organic compound.

Recent advances—including knowledge-guided multi-layer network approaches, in silico MS/MS spectral prediction tools such as SIRIUS and CANOPUS, machine learning-assisted annotation strategies, and ion mobility-derived collision cross-section measurements—are beginning to extend annotation into previously inaccessible regions of chemical space. Nevertheless, these approaches remain complementary rather than definitive solutions, and their performance in highly complex fermented food matrices

3. Data Processing and Interpretation

DATA PROCESSING AND INTERPRETATION WORKFLOW IN UNTARGETED METABOLOMICS OF FERMENTED FOOD SYSTEMS



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Feature detection—the extraction of chromatographic peaks from raw LC-MS data—represents an early and influential source of variability. Algorithm parameters governing peak width, intensity thresholds, and noise filtering must be carefully adapted to the dataset, as suboptimal parameterisation can inflate noise-derived signals or reduce sensitivity towards low-abundance metabolites [34]. In fermented food matrices, this challenge is particularly pronounced: co-elution of structurally related compounds (e.g., isomeric short-chain fatty acids or amino acid conjugates arising from proteolysis) can complicate peak deconvolution, while baseline instability driven by high-abundance endogenous components may introduce artefactual signal features that are difficult to distinguish from genuine metabolites [2]. Cross-sample alignment, which seeks to match features across injection runs despite retention time and m/z drift, is likewise sensitive to matrix-driven variability. In systems where composition changes substantially across fermentation stages or production batches, achieving consistent alignment remains a non-trivial challenge rather than a fully resolved step in the workflow [34,35].

Once a feature table has been constructed, normalization is required to reduce analytical variability that does not reflect underlying biological differences. The choice of normalization strategy is therefore consequential, as different approaches perform variably depending on dataset structure, the proportion of differentially abundant features, and the relative contribution of biological and technical variance [36,37]. Quality control (QC)-based correction methods—in which a pooled sample is repeatedly injected throughout the analytical sequence to model and correct signal drift—are widely recommended for large LC-MS datasets [38]. However, in time-resolved fermentation experiments, where samples collected at successive stages differ systematically in composition, QC-based correction may attenuate genuine biological variation if the QC sample does not adequately capture the compositional range of the study. This limitation may be particularly relevant in longitudinal fermentation studies, where substantial compositional changes occur throughout the process and a single pooled QC sample may not fully represent all fermentation stages [20]. Batch effects—systematic variability arising from instrument drift, reagent differences, or variation in extraction efficiency—can influence entire metabolite classes and are particularly challenging in multi-batch fermentation studies spanning different production runs or laboratories. If not properly accounted for, batch effects can lead to artificial sample groupings in multivariate analyses, potentially obscuring the underlying biological structure of the data [39].

The gap between a detected, normalised feature and a structurally annotated metabolite represents a major limitation in the interpretive power of untargeted metabolomics. Even under best-practice conditions, only a limited fraction of detected molecular features in untargeted datasets receive confident structural annotations [40]. This reflects both the incomplete coverage of reference spectral databases and the substantial contribution of adducts, in-source fragments, and isotope peaks to the overall feature count [41]. In fermented food matrices, this challenge is further compounded by the presence of bio-transformation products—such as glycoside hydrolysis products, esterified phenolic acids, and strain-specific bioactive peptides—that are often absent from general metabolomics databases derived primarily from biological fluids and model organisms [42]. Although values close to 10% are frequently reported in the metabolomics literature, the true annotation rate in fermented food metabolomics remains uncertain and is likely to depend on matrix complexity, analytical platform, and database coverage. This issue is increasingly recognised in fermented foods, where microbial metabolites, fermentation-derived transformation products, and matrix-specific compounds remain poorly represented in existing databases.

The Metabolomics Standards Initiative (MSI) confidence levels provide a widely adopted framework for communicating annotation certainty. Confirmed structures (Level 1) require comparison with authentic reference standards analysed under identical conditions, whereas putatively annotated compounds (Level 2) rely on spectral library matching or *in silico* fragmentation predictions. Tentative annotations (Level 3) are based on compound class inference from mass and fragmentation patterns alone [38]. In practice, most annotated features in food fermentation studies fall within Levels 2 and 3, reflecting the inherent limitations of current annotation strategies.

However, the distinction between these levels is not always clearly maintained in the literature, which can lead to an overinterpretation of chemical information that may not be fully supported by the available evidence [38]. Recent advances in *in silico* annotation tools, including platforms such as SIRIUS and CANOPUS and workflows such as COSMIC, are beginning to extend structural annotation into chemical space not covered by existing libraries [43]. While these approaches represent important progress, uncertainty remains at lower confidence levels, and independent validation using reference standards continues to be required for unambiguous structural confirmation.

Multivariate statistical analysis plays a central role in extracting biological meaning from high-dimensional metabolomics datasets. Unsupervised approaches such as principal component analysis (PCA) provide unbiased overviews of variance structure and are particularly useful for identifying analytical outliers and assessing the internal consistency of sample groupings prior to hypothesis testing [44,45]. Supervised methods, notably partial least squares discriminant analysis (PLS-DA) and its orthogonal variant (OPLS-DA), are widely applied in fermented food studies to identify features that contribute to differences between fermentation conditions, starter cultures, or product classes [44]. However, the supervised nature of these models introduces a recognised risk of overfitting, especially in datasets where the number of features greatly exceeds the number of samples, as is common in fermented food metabolomics. Permutation testing and cross-validation are commonly used safeguards, although their application and reporting are not always consistent, and insufficient filtering of non-informative features prior to model construction may lead to inflated variable importance (VIP) scores and reduced reproducibility of candidate biomarkers [45]. Consequently, discrimination performance alone should not be interpreted as evidence of biological relevance, particularly when candidate biomarkers lack structural annotation or independent validation.

Pathway enrichment analysis extends interpretation by mapping annotated metabolites onto known metabolic pathways, but it inherits the uncertainties of upstream processing steps. Annotation limitations, incomplete pathway databases, and bias toward well-characterised pathways mean that enrichment results are more appropriately interpreted as hypothesis-generating rather than confirmatory [46]. Increasingly, robust interpretive frameworks integrate metabolomics with complementary omics approaches, such as metagenomics or metaproteomics, using information on microbial functional potential to contextualise metabolite dynamics and constrain the range of biologically plausible interpretations [42].

Collectively, data processing and interpretation should be regarded not as downstream steps, but as integral components of the analytical workflow that strongly influence the conclusions of a fermented food metabolomics study. The reproducibility of such studies depends not only on the quality of raw data, but also on transparent reporting of processing parameters, normalisation strategies, and annotation confidence levels. Best-practice guidelines published in high-impact venues have begun to formalise these requirements, although their adoption in applied food science remains variable [12,38]. Future progress will depend not only on improved analytical performance, but also on greater standard-

isation of data processing workflows, broader adoption of reporting guidelines, and the development of fermented-food-specific annotation resources.

4. Applications in Fermented Food Systems

4.1. Monitoring of Fermentation Processes

Fermentation unfolds as a succession of metabolic phases driven by shifting microbial populations, substrate availability, and accumulating end-products. These phases are not discrete transitions but continuous chemical trajectories, and their characterisation requires a monitoring strategy capable of capturing the metabolome as a whole rather than a predefined subset of analytes. Untargeted metabolomics enables fermentation to be described as a trajectory through chemical space: at each time point, the global metabolic fingerprint reflects not only product accumulation, but also intermediate dynamics—including precursor depletion, transient metabolite pools, and pathway-level flux shifts—that are not readily captured by targeted approaches, a principle that also underpins the successful discrimination of beer styles based solely on metabolomic fingerprints [47] and the resolution of product-specific characteristics such as style and barrel-aging history [48]. A time-course UPLC-HRMS study of probiotic-fermented milk illustrated this temporal structure, with substantial divergence in metabolomic profiles during the first 36 h followed by stabilisation during ripening, and time-point-specific metabolites associated with TCA cycle activity, glutamate metabolism, and fatty acid pathways [20]. Complementary flavour-focused studies have further shown that fermentation may exhibit discrete “critical time nodes”, at which coordinated metabolic transitions coincide with major sensory-relevant changes, including increased odor-active values linked to branched-chain amino acid biosynthesis pathways [49]. This trajectory is consistent with an initial phase of active catabolic remodelling followed by metabolic consolidation, a pattern that would be difficult to resolve using endpoint measurements alone. Volatile fingerprinting of lager beer across five brewing stages provides a complementary example, showing a transition from aldehyde- and furan-dominated profiles in wort to a predominance of aliphatic esters and higher alcohols following fermentation [27]. This shift reflects the transition from thermally driven Maillard chemistry to yeast-mediated biosynthesis. Importantly, the timing and breadth of these changes are not easily reconstructed from targeted monitoring strategies focused on final-product compounds, underscoring the value of untargeted approaches for capturing the full temporal and chemical complexity of fermentation processes.

Comparable observations have been reported in traditional Asian fermented beverages. Metabolomic profiling of Huangjiu fermentation revealed pronounced temporal shifts in amino acid metabolism, organic acid production, and aroma precursor formation throughout the brewing process, enabling discrimination of fermentation stages beyond conventional physicochemical indicators [50]. These findings reinforce the concept that fermentation can be described as a continuous metabolic trajectory and demonstrate that the applicability of untargeted metabolomics extends across culturally and technologically distinct fermentation systems.

The ability to resolve fermentation stages through metabolic transitions and to associate each stage with a chemically coherent signature represents a key strength of untargeted metabolomics relative to targeted approaches. In wine, HRMS-based profiling of single-strain and mixed *Saccharomyces*/non-*Saccharomyces* fermentations has shown that the non-volatile metabolome encodes not only species-level differences but also intraspecific variation within *Saccharomyces cerevisiae*, including hundreds of strain-specific metabolites linked to differential nitrogen metabolism [21,22]. Such resolution is difficult to achieve with targeted flavour panels, which typically capture end-point outcomes without fully reflecting the underlying metabolic processes. Real-time PTR-ToF-MS monitoring

of wine fermentation further illustrates this point, revealing non-linear evolution of the global VOC fingerprint. Notably, simultaneous inoculation of *Metschnikowia pulcherrima* with a *Saccharomyces*/*Torulaspora* pair produced a volatile profile distinct from that of any pairwise combination [33]. This deviation from additivity reflects competitive and cooperative microbial interactions that modulate flux through ester and higher alcohol biosynthesis, highlighting patterns that are not readily predictable from monoculture-based targeted analyses.

A comparable level of mechanistic insight is observed in dairy fermentation systems. NMR-based metabolomic profiling of yoghurt production at high temporal resolution has shown that formate dynamics differ between starter culture combinations, reflecting the complementary metabolic roles of *Streptococcus thermophilus*, which produces formate from pyruvate, and *Lactobacillus* species, which require it for growth [31]. The resulting formate trajectory provides a sensitive indicator of process state and microbial interaction, capturing aspects of metabolic interdependence that are not typically resolved by conventional analytical approaches. Such observations illustrate how untargeted metabolomics can reveal functionally relevant metabolic relationships that underpin fermentation performance and product characteristics.

The detection of process deviations—defined as departures from an expected metabolic trajectory—extends the application of untargeted metabolomics into the domain of quality control, although practical challenges remain. In Chinese liquor fermentation, metabolome-wide profiling across four consecutive seasons showed that inter-season metabolic variation exceeded intra-process variability, driven by temperature-dependent shifts in microbial community composition that propagated into altered product profiles despite controlled fermentation conditions [51]. A targeted monitoring system calibrated on a single season would likely identify such cross-seasonal batches as anomalous, but without providing direct insight into the underlying cause. In contrast, global metabolic fingerprints capture both the magnitude and chemical nature of these deviations, supporting more informed interpretation of process variability. Similarly, in wine, GC-MS-based metabolomics demonstrated that structurally related triazole fungicides produced distinct metabolic perturbation patterns, differentially affecting amino acid, carbohydrate, and fatty acid pathways [28]. These findings suggest that contaminant-driven deviations can generate sufficiently specific metabolic signatures to enable discrimination between residue types.

Despite this potential, important limitations remain. Deviations are typically observed as displacements in multivariate ordination space, but translating these patterns into actionable process interventions depends on the structural annotation of the underlying features. Although advances in spectral databases, computational tools, and molecular networking have improved annotation capabilities, a substantial proportion of metabolomic signals detected in fermented food systems remain only partially characterised. Consequently, the transition from anomaly detection to mechanistic process diagnosis continues to represent a key challenge. Future progress may depend not only on generating larger metabolomic datasets, but also on improving annotation confidence and integrating metabolomic information into predictive process-control frameworks capable of supporting industrial decision-making.

4.2. Microbial Metabolism and Interactions

Untargeted metabolomics enables the characterisation of emergent metabolic behaviour arising from microbial interactions rather than isolated organism activity, a distinction that is analytically foundational. The metabolome of a fermented food is not simply an aggregate of the outputs of individual taxa, but rather a chemical record of substrate competition, sequential transformation of shared intermediates, and the metabolic repro-

gramming that organisms undergo in response to their neighbours. These community-level dynamics generate chemical complexity that is not readily predictable from monoculture-based targeted assays, as many relevant metabolites arise specifically from interaction-dependent processes. In Baijiu solid-state fermentation, combined metatranscriptomics and metabolomics showed that increases in community modularity—defined as the organisation of taxa into functionally distinct sub-communities—were associated with enhanced volatile flavour production that could not be attributed to any single genus [52]. The metabolic output therefore reflected properties of the microbial network as a whole rather than those of its individual components, a relationship which could only be observed at the level of the global metabolome.

A key mechanism linking microbial interactions to metabolome complexity is cross-feeding, whereby metabolic intermediates are transferred between species, altering flux through downstream biosynthetic pathways. In kefir, a landmark multi-omics study combining metabolomics, transcriptomics, and interaction mapping demonstrated that early colonisers of the milk matrix released amino acids and lactate that enabled subsequent community members to establish and, in some cases, dominate the fermentation [53]. Stable coexistence thus emerged not solely from niche differentiation but from metabolite-mediated facilitation, representing a system-level behaviour that is not readily captured in monoculture studies. Similar community-level dynamics have been observed during kimchi fermentation, where longitudinal metabolomic analyses revealed that shifts in microbial composition were closely associated with coordinated changes in amino acid, organic acid, and carbohydrate metabolism, highlighting the strong coupling between microbial succession and metabolome evolution in complex fermented ecosystems [54]. A similar principle is evident in wine fermentation. uHRMS profiling of single and sequential fermentations with *Lachancea thermotolerans*, *Starmerella bacillaris*, *Metschnikowia pulcherrima*, and *S. cerevisiae* showed that mixed fermentations produced metabolites absent from monoculture conditions, with the non-volatile metabolome resolving both species-level and strain-level differences linked to nitrogen metabolism [21].

A direct consequence of this community-level interdependence is the formation of metabolites that are structurally or biosynthetically unexpected, and therefore difficult to anticipate from the known biochemistry of individual species. For example, untargeted metabolomics of *Hanseniaspora uvarum* co-fermentation with *S. cerevisiae* in Cabernet Sauvignon wine showed reduced glycolytic and TCA cycle intermediates alongside altered polyphenolic and indole derivative profiles relative to monoculture conditions [55]. These patterns likely reflect shifts in metabolic flux driven by resource competition and interaction-dependent regulation, although their precise biochemical interpretation remains incomplete. This highlights a central challenge of the approach: while untargeted metabolomics can sensitively detect interaction-driven chemical changes, translating these features into structurally annotated and mechanistically interpretable metabolites remains constrained by limited spectral database coverage. In complex fermented matrices, annotation rates remain low, meaning that the most chemically informative region of the metabolome—the interaction-dependent fraction—is often the least well characterised. Integrative multi-omics strategies, linking metabolomic features to genomic biosynthetic potential, offer a promising route to address this gap, although their routine application still faces significant analytical and bioinformatic challenges [7,56].

4.3. Development of Flavour and Aroma Compounds

Flavour in fermented foods is the cumulative sensory expression of a metabolic system rather than the sum of a finite set of aroma-active compounds. The sensory character of products such as wine, ripened cheese, or fermented condiments reflects the superposition

of substrate-derived precursors, microbially generated intermediates, and the secondary reactions between them—a chemical space that is sufficiently broad that no hypothesis-driven targeted assay can comprehensively survey it in advance, reflecting the intrinsic chemical diversity and multicomponent nature of food aroma systems [57].

The limitation of targeted flavour chemistry is therefore not only one of analytical coverage, but also of scope. By constraining measurement to a predefined compound inventory, targeted approaches may overlook contributions that were not anticipated, whereas in fermented matrices, some of the most relevant aroma determinants arise outside established analytical frameworks. Untargeted metabolomics enables the linking of chemical complexity to sensory perception by capturing coordinated metabolic patterns rather than isolated compounds. Wine flavouromics studies have shown that untargeted volatile profiling explains a larger fraction of sensory variance between wine styles than targeted quantification of key odorants [29], consistent with the idea that perceptual signals are distributed across the metabolome rather than concentrated in a small number of compounds [2].

A central analytical advantage of untargeted metabolomics in flavour research lies in its capacity to resolve precursor–product relationships that remain obscured in targeted volatile analyses. Evidence from traditional Asian fermented beverages further supports the value of untargeted metabolomics for flavour research. In strong-aroma Baijiu, comprehensive metabolomic profiling enabled the discrimination of products according to geographical origin and production characteristics, revealing regional flavour signatures associated with complex volatile metabolite patterns that would be difficult to capture using conventional targeted approaches [58]. These findings reinforce the concept that flavour emerges from coordinated metabolomic networks rather than from a limited number of characteristic aroma compounds. Volatile aroma compounds represent metabolic endpoints, and their abundance is governed by upstream transformations in non-volatile pools of amino acids, fatty acids, glycoconjugates, and peptides. Targeted volatile panels capture these endpoints but do not fully account for the precursor context that determines their formation. Integrating volatilome profiling with LC-MS-based polar metabolomics allows this causal chain to be reconstructed. In fermented sausage, combined analysis of volatile and non-volatile fractions revealed correlations between 17 characteristic volatiles and 20 differential non-volatile metabolites, with divergence in lipid-derived compounds linked to microbial treatment [23]. While the volatile profile confirmed distinct aroma signatures, the integrated metabolomic approach provided mechanistic insight by identifying the lipid substrate pools whose differential degradation drove ester and aldehyde formation. Similarly, in fermented brown goat milk, shifts in triglyceride composition reduced precursor availability for lipid oxidation and attenuated off-odour development [59]. In Camembert, untargeted metabolomics simultaneously tracked volatile discriminants alongside lipolysis and proteolysis dynamics in the non-volatile fraction, providing a coherent account of how matrix biochemistry drives aroma evolution during ripening [60].

A further contribution of untargeted metabolomics to flavour research is its ability to reveal aroma-relevant compounds that are not typically included in targeted panels. Sensomics approaches that characterise the flavour-active metabolome without prior compound selection have identified compounds in fermented dairy products that fall outside conventional frameworks, including sub-threshold species, atypical lactones, and sulphur-containing metabolites whose individual concentrations may be low but whose combined contribution influences perceived complexity [61]. These findings refine the mechanistic understanding of flavour formation but also highlight a key limitation: in complex fermented matrices, only a small fraction of detected features can be confidently annotated, leaving many potentially relevant compounds without structural identity or sensory at-

tribution [62]. While correlations between unidentified features and sensory attributes can be established, their interpretation remains constrained. Bridging this gap will require advances in spectral library coverage, closer integration of sensory analysis within metabolomics workflows, and multi-omics strategies capable of linking unknown features to biosynthetic pathways and microbial function [56].

4.4. Process Optimisation and Control

Process conditions in fermentation do not modulate individual metabolites in isolation; rather, they reshape the overall metabolic state of a coupled biological–chemical system. Modest changes in temperature, substrate composition, or inoculum can shift a fermentation along qualitatively different trajectories in chemical space, even when conventional process indicators—such as pH, titratable acidity, or viable cell counts—appear comparable across conditions. This divergence is analytically important, as quality attributes including sensory character, textural stability, and product typicality emerge from the global metabolome rather than from a limited set of routinely monitored variables. Untargeted metabolomics enables the identification of these system-wide responses, supporting a transition from parameter-based to data-driven optimisation strategies. In kimchi fermentation across 4–15 °C, LC-MS profiling detected approximately 5000 metabolites, with more than 3000 features increasing at 10–15 °C relative to 4 °C, alongside marked divergence in microbial community composition and central carbon metabolism within a similar pH range [24]. This example illustrates how temperature functions as a high-dimensional control input, simultaneously influencing community succession and metabolic flux in ways that are not readily captured by targeted assays.

The practical value of this perspective lies in the use of global metabolic fingerprints as intermediate phenotypes—state descriptors that more closely reflect sensory and quality outcomes than conventional process variables. In yogurt fermented across five temperatures (30–45 °C), untargeted metabolomics identified distinct sets of differential metabolites across pairwise comparisons, spanning lipid-like molecules, organic acids, and organoheterocyclic compounds that collectively contribute to mouthfeel and flavour [63]. While targeted panels of acids or aroma compounds can confirm that temperature influences fermentation, the global fingerprint provides insight into which region of metabolic space a given condition occupies and how this relates to sensory attributes. A similar pattern is observed in *Streptococcus thermophilus* milk fermentation, where metabolic profiling at 37 °C and 42 °C revealed distinct volatile fingerprints despite overlapping bulk fermentation metrics, reflecting differential routing through branched-chain amino acid catabolism and glycolysis [64,65]. Substrate composition exerts comparable effects: fermentation of soybean residues by *Aspergillus oryzae* or *Bacillus subtilis* following different pre-treatment temperatures produced non-additive shifts in organic acid, amino acid, and isoflavone profiles, indicating that microbial metabolic strategies depend strongly on substrate history [66]. Across these systems, the relationship between process inputs and metabolic outputs is often non-linear and cannot be fully inferred from knowledge of individual variables in isolation, as further demonstrated in large-scale beer metabolomics studies integrating chemical profiling, sensory evaluation and machine learning, where global metabolic fingerprints enabled accurate prediction of flavour attributes and consumer appreciation across diverse products [67].

This capacity to resolve system-level responses to process inputs supports the development of data-driven optimisation frameworks, in which experimental designs systematically explore process space, map conditions onto metabolomic fingerprints, and identify regions associated with desired product properties. An example is provided by NMR-based optimisation of malolactic fermentation in sea buckthorn juice, where

profiling of six *Lactiplantibacillus plantarum* strains across different pH values and fermentation times enabled the identification of condition-dependent metabolic trajectories and informed process recommendations aligned with specific functional objectives [32]. Nevertheless, important limitations remain. Many discriminating features detected in such studies are not structurally annotated, meaning that metabolomic differences can be identified without fully resolving the underlying biochemical mechanisms. In addition, correlations between fingerprints and sensory outcomes may be statistically robust but not always readily transferable into generalisable process control strategies, particularly when fingerprints are sensitive to batch effects and matrix variability [2]. Used critically, untargeted metabolomics extends process understanding beyond single-analyte specifications; however, translating fingerprint-level discrimination into mechanistically grounded and actionable control remains an ongoing challenge.

4.5. Shelf-Life and Post-Fermentation Changes

Fermentation termination does not correspond to chemical stasis; rather, storage initiates a new phase of metabolic evolution characterised by progressive transformation of the product matrix. Residual enzymatic activity, spontaneous chemical reactions, and low-level microbial metabolism continue to reshape the metabolome, typically shifting the system from biosynthetic to degradative processes. These changes occur across multiple pathways in parallel, including proteolysis, lipolysis, and oxidation, whose products interact to define the final sensory and nutritional properties of the product. Untargeted metabolomics enables the monitoring of these evolving metabolic landscapes during storage, providing insight beyond predefined quality markers. In fermented milk co-inoculated with *Bifidobacterium animalis* and *Lactocaseibacillus paracasei*, LC-MS profiling over 21 days of cold storage identified 21 discriminatory metabolites and showed that purine and pyrimidine metabolism—rather than the lactic acid pathways typically monitored—dominated post-fermentation biochemical changes [68]. A comparable time-series analysis in *Lactobacillus casei* Zhang milk demonstrated that 15 volatile metabolites were sufficient to distinguish six discrete fermentation and storage stages, with the largest transition occurring at fermentation termination and subsequent evolution proceeding through distinct chemical states [69]. These observations indicate that storage is not simply a linear decline from a fermentation endpoint, but a structured sequence of metabolic transformations.

The ability of untargeted metabolomics to resolve degradation pathways becomes particularly evident in systems where targeted analyses provide only a partial view. In Mongolian cheese, LC-MS profiling identified 51 differential metabolites across five chemical classes during storage, with pathway analysis implicating β -alanine metabolism and glycine/serine/threonine catabolism as key contributors to quality deterioration [25]. These coordinated metabolic shifts were associated with the accumulation of bitter peptides, short-chain fatty acids, and odour-active compounds linked to sensory decline. Because these processes span multiple chemical classes, they are difficult to capture using targeted assays focused on a limited set of markers. A similar complexity is observed in red wine. Untargeted LC-MS fingerprinting of wines stored under different oxygen regimes showed that metabolomic divergence was governed not only by aldehyde accumulation, but by the differential transformation of flavanol-derived intermediates. Oxidative conditions favoured the formation of ethyl-linked tannin–anthocyanin adducts and pyranoanthocyanins, whereas anoxic conditions promoted direct-linked adducts and sulfonated tannins associated with reductive off-odours [70]. These polyphenol transformation networks are not readily accessible through targeted measurements of free anthocyanins or standard staling indices, yet they play a central role in defining the oxidative trajectory and sensory evolution of wine.

Despite its ability to capture system-level chemical evolution during storage, untargeted metabolomics in shelf-life research remains subject to important interpretive limitations. Time-course studies typically yield large numbers of differential features, but only a fraction can be structurally annotated and linked to specific degradation pathways, particularly for complex intermediates arising from proteolysis, lipid oxidation, and polyphenol transformation [71,72]. An additional challenge lies in distinguishing quality-relevant metabolic changes from physicochemical alterations that occur independently of sensory deterioration. Reviews of metabolomics in dairy systems have noted that candidate stability markers identified in one product context often show limited transferability across matrices or storage conditions, reflecting the strong product specificity of post-fermentation chemistry [73]. Addressing these challenges will require integrating untargeted metabolomic data with mechanistic models of degradation processes specific to each product class, as well as expanding spectral databases to better represent the reaction networks that drive storage instability in fermented foods.

4.6. Safety and Quality Markers

Conventional safety monitoring of fermented foods operates within a predefined analytical scope, in which only compounds included in the assay design can be reliably detected. Targeted chromatographic and immunoassay panels quantify known hazards—such as regulated mycotoxins, biogenic amines, and bacterial toxins—with high sensitivity and throughput, but they are inherently limited in their ability to detect compounds that fall outside this predefined space [74]. This limitation is structural rather than purely analytical. Fermented food systems generate a wide range of compounds through microbial metabolism, substrate transformation, and spontaneous chemical reactions, including modified toxin forms, biosynthetic precursors, and matrix-specific degradation products that are not routinely monitored. As a result, emerging or context-dependent hazards linked to specific combinations of microbiota, substrate, and process conditions may remain undetected. Untargeted metabolomics provides a complementary strategy by enabling broader chemical surveillance and facilitating the detection of unexpected compounds and early indicators of safety or quality change.

The value of this approach is particularly evident in cases where early metabolic signals precede the accumulation of regulated hazards. In aflatoxigenic *Aspergillus* populations, untargeted metabolomics combined with molecular networking identified biosynthetic precursors and structurally related analogues as early warning biomarkers capable of predicting aflatoxin contamination risk 35–47 days before toxin levels became quantifiable, with a machine-learning model achieving 97.8% classification accuracy across field isolates [26]. A targeted assay focused solely on regulated aflatoxins would likely not have detected these early-stage indicators. Importantly, such findings should be interpreted as evidence of screening potential rather than regulatory readiness. Classification accuracy in a discovery study does not by itself establish suitability for release decisions, routine surveillance, or formal risk assessment. Before implementation, putative early-warning biomarkers require independent validation across matrices, production batches, laboratories, and analytical platforms, followed by conversion into targeted confirmatory assays with defined performance characteristics.

Similarly, in wine, GC-MS profiling revealed that pesticide residues in grape pulp induced broad metabolic perturbations affecting carbohydrates, amino acids, and short-chain fatty acid derivatives—compound classes closely linked to sensory properties [28]. These findings demonstrate that untargeted metabolomics captures not only the presence of contaminants but also their wider metabolic impact. In addition, hypothesis-free screening enables the detection of structurally related co-contaminants, including mod-

ified toxin forms and emerging analogues whose toxicological significance is still being established [75,76].

A key limitation of this approach lies in the gap between detection and actionable interpretation. Untargeted analyses typically generate large numbers of discriminating features, many of which remain unannotated and therefore cannot be readily incorporated into risk assessment frameworks [77]. This constraint is particularly relevant in food safety applications, where unidentified features must ultimately be structurally characterised before they can support regulatory or quality decisions. Bridging this gap requires the development of validated workflows for feature prioritisation and structural elucidation, as well as the integration of untargeted screening with targeted confirmatory assays. Expanding food-relevant spectral reference libraries and systematically annotating the chemical space associated with fermented food matrices will be essential for enabling untargeted metabolomics to function not only as a discovery tool but also as a practical component of early warning systems.

Representative applications of untargeted metabolomics across different fermented food matrices are summarised in Table S1 (Supplementary Materials). The examples provided are not intended to be exhaustive, but rather to illustrate the diversity of study designs, analytical approaches, and biological insights currently reported in the field.

5. Discussion: Limitations and Recommendations

Data acquisition is no longer the main bottleneck in untargeted metabolomics; instead, the main challenges lie in data interpretation and metabolite identification. Current LC-MS platforms routinely detect tens of thousands of features from fermented food matrices, but the transition from raw signal to biological understanding is still limited by several factors, including annotation, analytical reproducibility, statistical validity, and the overall interpretive framework. These are not minor technical issues, but key points where unsupported conclusions can enter the literature. Among these constraints, annotation is the key limitation.

A large fraction of detected features remain unannotated, which limits biological interpretation. In fermented food systems, this problem is particularly evident: fermentation produces chemically diverse, matrix-specific metabolites—including modified phenolics, unusual organic acids, and microbially derived intermediates—that are poorly represented in spectral libraries developed mainly for clinical metabolomics. This limitation is also evident in detailed profiling studies of complex food matrices, where hundreds of volatile metabolites can be detected while only a subset can be confidently identified, with a significant proportion remaining unknown or newly reported [30]. Importantly, part of the problem comes from the data itself. As shown by Sindelar and Patti, many unannotated LC-MS signals are not new metabolites but artefacts such as adducts, in-source fragments, isotopologues, and contaminants [41]. The implications are clear: features that cannot be structurally assigned cannot be mechanistically interpreted, independently validated, or translated into process knowledge. For this reason, annotation confidence should be reported using MSI levels, clearly distinguishing confirmed identities (Level 1), spectral matches (Level 2), putative classes (Level 3), and unknowns (Level 4) [38]. Reporting “identified metabolites” without indicating confidence level can be misleading, especially when these metabolites are used to support biological conclusions. Improving annotation in fermented food systems will require both better spectral libraries and the use of combined strategies, including *in silico* tools such as SIRIUS/COSMIC [43], ion-mobility data [78], and network-based approaches that relate unknown features to known compounds [79].

These annotation issues are made worse by the statistical structure of untargeted datasets. Such datasets are typically high-dimensional, with more features than samples,

creating conditions where statistical artefacts can easily arise. Supervised methods, especially PLS-DA, can produce apparently strong models even from noise if validation is not properly applied [45]. Uninformative features can distort model structure and inflate VIP scores, increasing the risk of false positives being carried into interpretation. This risk is particularly relevant in fermented food studies, where sample sizes are often limited. To address this, dimensionality reduction and univariate filtering should be treated as essential steps in the workflow and applied before supervised modelling. At the same time, redundancy from adducts and isotopologues should be resolved during data processing, as keeping these features increases dimensionality without adding useful information and reduces model stability [80]. Experimental design is equally important: biological replication, balanced group sizes, and the inclusion of blanks and controls determine whether the statistical model is valid.

Beyond statistical issues, reproducibility remains a major limitation. Variability in untargeted metabolomics is not random but arises from identifiable sources such as column ageing, ionisation differences, and instrument drift. These effects introduce non-biological variation that can be difficult to separate from real metabolic differences, especially in fermentation studies that span multiple time points or batches. A survey by the mQACC consortium showed that while most laboratories use pooled QC samples and internal standards, only 36% include technical replicates, and reporting of QC procedures is often incomplete [81,82]. Without technical replication, it is not possible to separate biological from analytical variation, which makes reported effect sizes unreliable. Pooled QC samples remain the most effective way to monitor and correct signal drift, and QC-based filtering is a robust way to select stable features [83]. For fermented food matrices, matrix-matched QC samples are particularly important, as ion suppression varies across products and fermentation stages and is not well captured by generic references.

At a broader level, these issues also affect reproducibility between laboratories. Inter-laboratory variability in untargeted metabolomics is often underestimated. Studies have shown that feature overlap between laboratories analysing the same samples can be as low as ~25%, even when the overall data structure appears similar [84]. This highlights an important point: similar PCA patterns do not mean that the same compounds are being detected. In fermented food research, where independent validation is still limited, this means that reported discriminant metabolites should be treated as method-dependent until confirmed by other approaches. Improving reproducibility will require better data sharing, including raw data and full metadata (instrument settings, extraction methods, chromatographic conditions, and QC results), and the adoption of standard reporting guidelines such as those proposed by mQACC.

Ultimately, these limitations come together at the level of biological interpretation. Even when features are correctly annotated and statistically validated, linking metabolomic patterns to fermentation mechanisms requires that metabolites be connected to the reactions that produce them and to the microbial or chemical processes involved. Pathway analysis can help, but in fermented systems—where many compounds come from microbial metabolism, Maillard reactions, or matrix-specific transformations—standard pathway databases often provide incomplete coverage. As a result, pathway results should be treated as hypothesis-generating rather than definitive. A more reliable approach is to integrate metabolomics with process-level data, such as microbial composition, substrate chemistry, pH, temperature, and sampling time. While this does not fully solve the annotation problem, it helps link metabolomic patterns to measurable biological processes, making interpretation clearer and more transferable across systems.

Looking forward, the major challenge in fermented food metabolomics may no longer be the generation of analytical data, but rather the transformation of increasingly complex

datasets into reproducible, interpretable, and actionable knowledge. While recent advances in machine learning, digital twins, and multi-omics integration offer exciting opportunities, their successful implementation will depend on overcoming persistent limitations in annotation confidence, model robustness, and interlaboratory reproducibility. In this context, hybrid approaches combining mechanistic knowledge of fermentation processes with data-driven models may represent a particularly promising direction, enabling metabolomics to evolve from a primarily descriptive tool into a predictive framework capable of supporting process control and industrial decision-making.

6. Conclusions

Untargeted metabolomics has changed how fermented food systems can be studied by capturing global metabolic fingerprints rather than predefined compounds. Across the studies reviewed, it has revealed system-level chemical patterns underlying flavour development, microbial interactions, fermentation dynamics, post-fermentation stability, and safety-related changes. Importantly, several findings discussed here—including metabolites emerging from microbial co-cultures, early perturbation signals preceding regulated contaminant thresholds, and degradation pathways not captured by targeted assays—would probably have remained inaccessible without untargeted approaches.

However, the main limitation of the field remains interpretive rather than analytical. Many discriminant features are still structurally uncharacterised, cross-laboratory reproducibility is limited, and reporting practices remain insufficiently standardised, making it difficult to compare and integrate findings across studies. Progress will therefore depend on improved food-relevant spectral libraries, stronger annotation and validation workflows, robust quality-control practices, and closer integration with microbiome, process, and sensory data. If these challenges are addressed, untargeted metabolomics can move beyond descriptive fingerprinting towards mechanistic understanding, predictive fermentation management, and more reliable decision-making in fermented food quality, safety, and innovation.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fermentation12070311/s1>, Table S1: Applications of untargeted metabolomics across key research areas in fermented food systems: study design, principal findings, and analytical platforms. References [85,86] are cited in the supplementary materials.

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