



Microbial function in food fermentations: Current research and future directions

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ABSTRACT

Complex microorganisms are the drivers of fermentation ecosystem and play a pivotal role in determining product yield and quality. Although significant efforts have been devoted to characterizing microbial composition and diversity, these cannot fully elucidate microbial functions that are critical to the fermentation process and product quality. There is an urgent need to shift research focus toward microbial function, particularly those closely associated with food flavor, texture, quality, and nutrition. Currently, function prediction based on amplicon sequencing and omics techniques remain the predominant strategies for investigating microbial function in food fermentations. However, these methods generate population-averaged data, masking functional heterogeneity, rare taxa, and key contributors within low-abundance populations. Emerging technologies, stable-isotope probing and single-cell sequencing offer promising alternatives. This review provides an overview of methods for investigating microbial function in food fermentations, spanning conventional to emerging techniques, while critically assessing their respective advantages and limitations. Additionally, we summarize recent research in microbial functions in fermented foods. By providing methodological insights and future perspectives, this review aims to guide targeted research on microbial function in complex fermentation systems.

1. Introduction

Fermented foods and beverages have played a pivotal role in human history and cultural development, arising from the dynamic interactions between diverse microorganisms and plant or animal substrates. These processes yield a variety of products such as cheese, liquor, beer, wine, and sausage (Fig. 1). These fermentation products not only hold substantial economic importance but also confer numerous health benefits (Leech et al., 2020; Mukherjee et al., 2024). However, most traditional fermentations are spontaneous, which often lead to the fluctuation of product quality and yields, posing considerable challenges to food security and economic loss (Galimberti et al., 2021; Wu et al., 2021). The underlying cause of these fluctuations lies in the complex microbial communities driving spontaneous fermentation, where limited knowledge of microbial functional traits and difficulties in community management hinder process control (Wei et al., 2024a, b). In these fermentation systems, microorganisms from diverse sources—including

starter cultures, raw materials, air, water, equipment, and manufacturing environment—converge to form dynamic, multi-species communities that colonize the fermentation matrix (Louw et al., 2023). Throughout the fermentation stages, these microorganisms perform coordinated metabolic functions to finish the conversion of raw materials into flavor compounds, including enzyme production, the hydrolysis of macromolecules (e.g., carbohydrates and proteins), and subsequent biochemical conversion of sugars and amino acids into desirable flavor metabolites (Wei et al., 2023). Given this complexity, effective regulation and precise management of fermentation microbiota are critical to ensuring consistent production of high-quality fermented foods. However, a major challenge is that the functional roles of many microorganisms in spontaneous fermentations remain poorly characterized. Without a deeper understanding of microbial functionality, targeted process optimization and quality control in food fermentation will remain elusive.

With the development of high-throughput sequencing, we have

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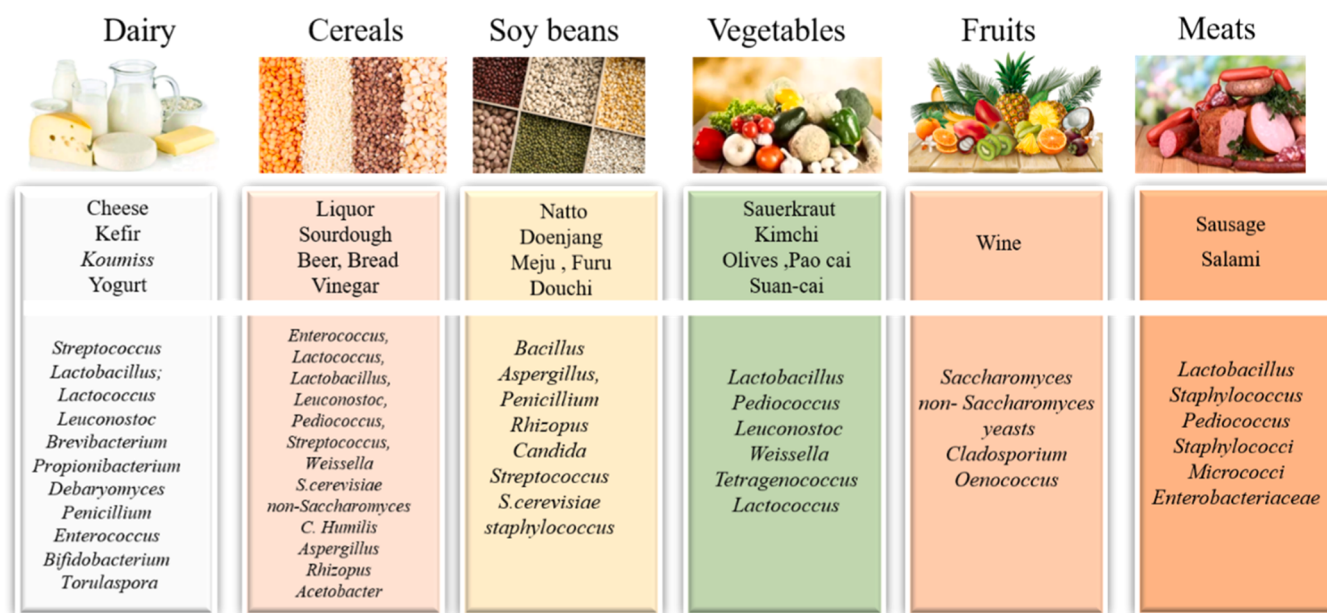


Fig. 1. Representative fermented foods, along with their primary substrates and dominant microbial consortia.

gained unprecedented insights into the microbial structure and diversity within diverse fermentation ecosystems. These foundational studies have not only facilitated the identification of bacterial, fungal, and other microbial communities in fermented foods but have also revealed critical correlations between microbial composition and specific food production processes and parameters (Du et al., 2023; Leech et al., 2020; Louw et al., 2023). Nevertheless, the understanding of the exact functional contributions of individual microorganisms to the yield and quality of fermented products remains incomplete.

Current methodologies for analyzing microbial functionality in fermented foods can be classified into three categories. The first approach involves functional prediction based on marker genes from high-throughput sequencing data, primarily utilizing tools such as PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Yang et al., 2023b). However, these predictions are limited by the existing genomic databases, which are heavily skewed toward microorganisms relevant to human health and biotechnological applications (Sun et al., 2020). The second approach encompasses single-omics techniques, including metagenomics, metatranscriptomics, metaproteomics, and metabolomics (Feng et al., 2020; Leech et al., 2020; Wei et al., 2023; Yang et al., 2023a). While these methods are widely applied, they each provide insights from a partial perspective—focusing exclusively on DNA (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics), or metabolites (metabolomics)—thereby failing to deliver a holistic understanding of microbial function. The third and most advanced approach is multi-omics integration, which synergizes various omics technologies to systematically explore microbial species, genes, proteins, and metabolites (Kang et al., 2022). Despite its potential, omics techniques generate population-averaged data, masking functional heterogeneity, rare taxa, and key contributors within low-abundance populations (Blattman et al., 2020; Hatzepichler et al., 2020). Moreover, it remains inadequate for precisely delineating the roles of individual microbes and tracing the metabolic pathways from nutrient substrates to flavor compounds. This limitation significantly hinders our ability to decipher the true metabolic contributions of microorganisms during fermentation. Given these challenges, there is an urgent need for more refined methodologies capable of elucidating microbial functions with greater precision and establishing definitive connections between microbial activity and the biosynthesis of flavor compounds in fermented foods.

Such advancements would substantially enhance our ability to optimize fermentation processes and improve product quality.

Stable isotope probing (SIP) has emerged as a powerful tool for linking microbial taxa to metabolic functions without requiring prior knowledge of their underlying enzymatic or genetic mechanisms (Caro et al., 2023; Vyshenska et al., 2023). By tracking the incorporation of stable isotopes into biomolecules, SIP allows researchers to identify active microbial participants in specific biochemical processes, thereby advancing our understanding of microbiota-driven biogeochemical cycling. Single-cell sequencing provides a good resolution by enabling genomic, transcriptomic, proteomic, and epigenomic profiling at the individual cell level. This technique circumvents the limitations of bulk omics approaches, revealing functional heterogeneity within microbial communities and clarifying the precise roles of individual strains in complex ecosystems (Vandereyken et al., 2023). To date, SIP and single-cell sequencing have been instrumental in dissecting microbiota functions in diverse environments, including the gut (Chijiwa et al., 2020), soil (Li et al., 2024b), and marine systems (Arandia-Gorostidi et al., 2023). However, their application in food fermentation ecosystems remains underexplored. Given the intricate interplay of microorganisms in food fermentations—where metabolic activities play a decisive role in the product quality, flavor development, and process efficiency—these techniques hold significant untapped potential. Integrating SIP and single-cell sequencing into fermentation research could unravel the specific contributions of key microbial players, elucidating their roles in substrate utilization, metabolite production, and flavor compound formation. Bridging this knowledge gap is not only critical for advancing fundamental microbial ecology but also for optimizing industrial fermentation practices.

In this review, we systematically examine and critically assess the methodologies used to decipher the functional roles of complex microbiota within ecosystems. We synthesize key insights derived from conventional approaches and explore the emerging potential of stable isotope probing and single-cell sequencing in the food fermentations. Furthermore, we discuss current challenges and future perspectives in microbiota functional analysis, identifying critical gaps and directions for subsequent research.

2. Conventional methods for analyzing microbial community functions in food fermentations

Current approaches for investigating microbial functions in fermentation ecosystems can be categorized into three main groups: (1) functional prediction based on marker gene sequencing, (2) single-omics techniques, and (3) multi-omics integration strategies. Below, we critically examine these methodologies, highlighting their applications, strengths, and limitations in the context of food fermentation research.

2.1. Functional prediction via high-throughput marker gene sequencing

High-throughput amplicon sequencing of conserved marker genes (e.g., 16S rRNA for bacteria, ITS/18S rRNA for fungi) enables phylogenetic characterization of microbial communities but provides only indirect insights into functional potential (De Filippis et al., 2017; Sun et al., 2020). To bridge this gap, bioinformatic tools such as PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) leverage taxonomic profiles to infer metabolic capabilities by referencing annotated genomes in databases. PICRUSt's algorithms predict the presence of functional genes of microbial taxa without fully sequenced genomes by mapping their marker genes to homologous taxa with known sequenced genomes (Langille et al., 2013). Different from PICRUSt1 that rely heavily on the Greengenes database, PICRUSt2 significantly expanded its databases, genomic coverage and compatibility with diverse OTU clustering methods, enhancing its utility in fermentation studies (Douglas et al., 2020). This tool has been applied to analyze microbial functions in traditional fermentations, including *Baijiu* (Yang et al., 2023b), *Yucha* (Zhang et al., 2016), *Fu brick tea* (Li et al., 2019), *Fermented Vegetables* (Peng et al., 2018), and *Traditional Indian Food Idli* (Mandhanja et al., 2019).

However, predictive accuracy by PICRUSt is constrained by database completeness, which remains skewed toward medically or industrially relevant microbes. Therefore, limited representation of fermentation-associated genomes may lead to erroneous functional annotations, particularly for understudied or uncultured taxa (Sun et al., 2020).

2.2. Single-omics approaches for microbial function elucidation

Single-omics techniques—metagenomics, metatranscriptomics, metaproteomics, and metabolomics—provide targeted insights into microbial functions by analyzing genes, transcripts, proteins, or metabolites, respectively.

2.2.1. Metagenomics

Metagenomics has emerged as a powerful tool for comprehensive microbial community analysis, enabling the non-targeted and unbiased profiling of entire genomic content within complex ecosystems. Unlike traditional approaches, metagenomics sequences genomic DNA directly after fragmentation and library construction, bypassing PCR amplification steps that may introduce biases (Yang et al., 2021b; Zhang et al., 2019). By providing both taxonomic resolution at the species level and detailed gene information, metagenomics offers unique insights into the functional potential of microbiota in fermentation systems. This capability is particularly valuable for establishing connections between microbial species and their metabolic pathways, thereby facilitating the prediction of community functional profiles. A key strength of metagenomics lies in its ability to reconstruct microbial genomes *de novo* through advanced binning techniques, enabling the identification of novel species and unculturable microorganisms along with their metabolic traits (Du et al., 2023). This feature has revolutionized our understanding of microbial diversity in food fermentation processes. However, several important limitations must be acknowledged. First, the functional information derived from metagenomic data reflects potential rather than actual microbial activities, as it is based solely on gene presence without considering expression levels. Second, the

technique cannot differentiate between viable and non-viable cells, a significant drawback in fermentation systems (long-term fermentation processes) where DNA from lysed cells may persist and distort community profiles. Technical challenges also exist in data analysis and interpretation. The massive datasets generated by metagenomic sequencing require substantial computational resources and sophisticated bioinformatics pipelines. Furthermore, the inherent complexity of fermentation microbiomes often leads to difficulties in accurately assigning functional genes to their host organisms, with low-abundance species frequently being underrepresented in the analysis (Alneberg et al., 2014; Nielsen et al., 2014; Sangwan et al., 2016; Yao et al., 2024). These limitations highlight the need for developing optimized metagenomic approaches specifically designed for fermentation ecosystems, incorporating improved DNA extraction protocols, advanced bioinformatics tools, and potentially complementary techniques like metatranscriptomics to validate functional predictions.

2.2.2. Metatranscriptomics

Metatranscriptomics enables the functional characterization of active microbial communities by quantifying gene expression through messenger RNA (mRNA) extraction. Unlike metagenomics, which provides a static snapshot of microbial genetic potential, metatranscriptomics reveals which genes are transcriptionally active and to what extent under specific environmental conditions (Bashiardes et al., 2016). This approach is particularly valuable in food fermentation research, where dynamic microbial community drive metabolic processes that influence flavor, texture, and product quality. By identifying actively transcribed genes, researchers can pinpoint microbial species contributing to fermentation and elucidate their functional roles in real time (Bashiardes et al., 2016; Ma et al., 2019). Fermentation environments often impose significant stress on microbial communities, such as high salinity in soy sauce production (Devanathi and Gkatzionis, 2019), low pH in vinegar production (Lu et al., 2018), and high lactic acid in *Baijiu* production (Deng et al., 2020; Wang et al., 2021). Metatranscriptomic analysis helps uncover stress-responsive genes, shedding light on microbial adaptation mechanisms and identifying novel enzymes with potential industrial applications.

Despite its advantages, metatranscriptomics faces several technical challenges. The extraction of high-quality mRNA from fermentation matrices is challenging due to various contaminants such as high levels of polysaccharides, proteins, and strong colored biomass. Additionally, the short half-life of mRNA limits the detection of transient gene expression changes, and the presence of transcripts does not always correlate with protein activity. These limitations constrain the broader application of metatranscriptomics in food fermentation studies, necessitating complementary approaches such as metaproteomics for a more comprehensive understanding of microbial functionality.

2.2.3. Metaproteomics

Metaproteomics provides a direct assessment of microbial functionality by identifying and quantifying proteins within complex microbial communities. This approach offers insights into post-translational modifications, enzyme activities, and cellular responses to environmental perturbations, making it a powerful tool for studying food fermentation ecosystems. Early metaproteomic methods relied on two-dimensional gel electrophoresis followed by mass spectrometry (MS), a labor-intensive and low-throughput process. Advances in liquid chromatography (LC) coupled with high-resolution MS have revolutionized the field, enabling large-scale, high-throughput protein analysis (Yang et al., 2020).

In food fermentations, metaproteomics has been instrumental in linking microbial enzymes to metabolic pathways responsible for flavor development, substrate utilization, and stress adaptation (Wang et al., 2020; L. Yang et al., 2023). However, several challenges remain. First, protein identification depends on accurate genomic databases; errors in nucleotide sequences can propagate into incorrect protein annotations,

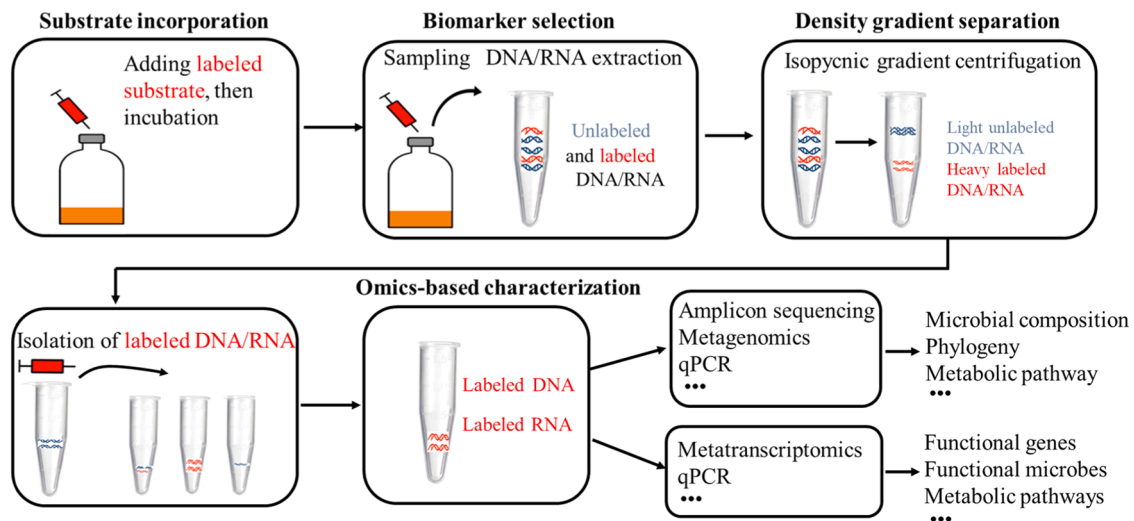


Fig. 2. The procedure of metagenomic and metatranscriptomics stable-isotope probing.

affecting peptide identification and taxonomic classification (Duarte et al., 2024). Second, the presence of material-derived proteins can obscure microbial protein detection, complicating data interpretation (Cardenas et al., 2014). To overcome these limitations, future efforts should focus on improving sequencing accuracy, expanding microbial protein databases, and developing specialized databases tailored to fermented food microbiomes. Additionally, refining protein extraction and separation techniques will enhance the sensitivity and reliability of metaproteomic analyses. By integrating metatranscriptomics and metaproteomics, researchers can achieve a more holistic understanding of microbial contributions to fermentation processes, paving the way for optimized fermentation strategies and novel biotechnological applications.

2.2.4. Metabolomics

A biological system lies a dynamic interplay between genes, transcripts, proteins, and metabolites, with metabolites serving as the direct mediators of biochemical activity (Dunn et al., 2011; Goodacre et al., 2004). Unlike genomics and proteomics, which predict potential cellular functions, metabolomics captures the real-time biochemical state of an organism, offering a snapshot of physiological and metabolic responses to environmental stimuli. This approach detects, identifies, and quantifies low-molecular-weight metabolites (<1500 Da), providing critical insights into metabolic fluxes that underlie fermentation processes (Liu and Locasale, 2017). Metabolomic include two complementary strategies: Untargeted metabolomics and targeted metabolomics (Johanningsmeier et al., 2016). Advanced chromatographic separation coupled with mass spectrometry (MS) and multivariate statistical analysis allows for high-resolution metabolic mapping. In food fermentation research, this technique has been instrumental in identifying key aroma and flavor compounds that define product quality, discriminating taste markers and bioactive metabolites in fermented foods, and linking microbial metabolic activity to sensory attributes, streamlining the discovery of novel flavor-producing strains (Yang et al., 2021b). Despite its strengths, metabolomics faces challenges in metabolite annotation due to the vast chemical diversity and limited reference databases. Future efforts should prioritize the development of comprehensive, fermentation-specific metabolite libraries to enhance detection accuracy and functional interpretation.

2.2.5. Multi-omics integration

While single-omics approaches (metagenomics, metatranscriptomics, metaproteomics, and metabolomics) each provide valuable insights into microbial function at distinct molecular levels, they fail to

capture the full complexity of microbial ecosystems. To bridge this gap, integrated multi-omics strategies have emerged, enabling a more holistic understanding of microbial interactions and metabolic networks in fermented foods. For example, combined genomics, metatranscriptomics, and metabolomics revealed microbial communities and their metabolic function during *Baijiu* fermentation (Yuan et al., 2023; Song et al., 2017; Wei et al., 2023). Metaproteomics and metabolomics uncovered metabolic differentiation shaping microbial ecology in *Daqu* fermentation (Yang et al., 2023b). Genome-scale metabolic modeling integrated with transcriptomics elucidated microbial interactions behind flavor development in Cheese production (Melkonian et al., 2023). Metabolomics and transcriptomics delineated functional stability in microbial coexistence in kefir fermentation (Blasche et al., 2021).

Despite its potential, multi-omics integration faces key challenges: (1) Bulk omics data represent the average of a bulk population, masking microbial heterogeneity and obscuring strain-level functional variations. (2) Functional redundancy exists among microorganisms and shared metabolic pathways among microbes complicate precise attribution of specific functions. (3) The functions of rare taxa and key contributors within low-abundance populations are difficult to elucidate.

3. Stable-isotope probing

Stable isotope probing (SIP) has emerged as a transformative technique for elucidating the metabolic roles of microorganisms within their native environments, circumventing the need for prior knowledge of their genetic or biochemical pathways. By introducing heavy stable isotopes (e.g., ^{13}C , ^{15}N , ^{18}O) into specific substrates, SIP enables the tracking of isotopic incorporation into microbial biomolecules, thereby linking metabolic activity to phylogenetic identity in a culture-independent manner (Vyshenska et al., 2023). This approach is particularly powerful when combined with omics techniques, allowing researchers to resolve the functional contributions of uncultivated taxa within intricate microbial consortia.

Taking metagenomic and metatranscriptomics stable-isotope probing as examples, a typical SIP-omics experiment involves four key steps, as depicted in Fig. 2. (1) **Substrate incorporation**: A labeled compound (e.g., ^{13}C -glucose) is introduced into a microbial community, where active populations assimilate the isotope into biomolecules such as DNA, RNA, or phospholipid fatty acids (PLFAs). (2) **Biomarker selection**: Depending on the research question, different biomarkers (DNA, RNA, proteins, or whole cells) are targeted for analysis. For nucleic acid-based SIP, DNA and RNA are commonly used due to their compatibility with

sequencing technologies. (3) **Density gradient separation:** Isotopically labeled ("heavy") DNA or RNA is separated from unlabeled ("light") nucleic acids via isopycnic centrifugation using media such as cesium chloride (CsCl) for DNA or cesium trifluoroacetate (CsTFA) for RNA. (4) **Omics-based characterization:** High-throughput sequencing of gradient fractions reveals the taxonomic and functional profiles of active microorganisms, providing insights into their metabolic roles (Barnett et al., 2023; Nieto et al., 2024; Barnett and Buckley 2023).

Recent advancements in SIP integrated with omics technologies expand our understanding of microbial activity in diverse ecosystems. For instance, DNA-SIP coupled with metagenomics has uncovered key players in carbon cycling, including previously overlooked degraders of complex organic matter (Zheng et al., 2024; Barnett et al., 2023). RNA-SIP with ^{13}C -labelled substrates (acetate, formate, and methanol) identified active denitrifiers involved in methane oxidation, shedding light on cryptic nitrogen-cycling pathways (Nwoba et al., 2024). SIP-guided metagenomic binning enabled the cultivation of elusive phenanthrene degraders in contaminated soils, demonstrating the potential for targeted microbial isolation (Li et al., 2024b).

Despite its success in environmental microbiology, SIP applications in fermentation systems remain underexplored. Several challenges hinder its broader adoption: (1) **Substrate complexity and microbial competition.** Fermented foods contain diverse carbon and nitrogen sources, making it difficult to trace specific metabolic fluxes. Additionally, microbial competition may lead to uneven isotope distribution, skewing SIP results (Jin et al., 2024). To address this, researchers can use well-defined and food-relevant labeled substrates (e.g., ^{13}C -sugars or ^{15}N -amino acids) and optimize incubation times to capture key metabolic turnover events. (2) **Biomarker recovery in dense matrices.** Food matrices (e.g., cheese, kimchi) often contain inhibitory compounds (e.g., fats, salts, acids) that interfere with nucleic acid extraction and gradient separation (Carbonne et al., 2022). These can be solved by pre-treatment steps (e.g., enzymatic digestion of proteins/fats) and optimized gradient protocols (e.g., adjusted CsCl densities) to improve biomarker recovery. (3) **Low biomass and high host DNA background.** Many fermented foods have low microbial biomass, and host-derived DNA (e.g., from plant/animal ingredients) can dominate

sequencing data (Singh et al., 2014). This can be solved by selective labeling strategies (e.g., brief pulse-chase experiments) and host DNA depletion methods (e.g., methylation-based enrichment) to enhance microbial signal detection. (4) **Regulatory acceptability.** Using isotopes in a food-processing is often prohibited. Therefore, SIP experiments must be conducted in defined laboratory-scale model systems that simulate the food matrix. The insights gained can then be validated using non-labeled and conventional methods.

While SIP is a powerful technique, its application in complex matrices (like fermentation systems) requires careful experimental design to avoid common pitfalls and ensure robust interpretation of results. (1) **Mitigating isotope cross-feeding and dilution:** A major challenge is isotope cross-feeding, where primary consumers of the labeled substrate excrete metabolites (e.g., organic acids, amino acids) that are subsequently assimilated by secondary microorganisms. This can misidentify secondary feeders as primary degraders. Additionally, the presence of large unlabeled endogenous pools of carbon or nitrogen can dilute the isotope label, requiring longer incubation times to achieve sufficient enrichment, which in turn increases the risk of cross-feeding. To address these issues, pulse-chase experiments are essential. A short "pulse" of labeled substrate is followed by a longer "chase" with unlabeled substrate, allowing researchers to trace the flow of isotopes through the microbial food web and distinguish primary from secondary consumers. Furthermore, using highly enriched substrates (>98 atom % ^{13}C) and optimizing incubation time to be just long enough to detect label incorporation are critical steps (Vyshenska et al., 2023; Zheng et al., 2024). (2) **Choice of biomarker:** The selection of nucleic acid biomarker involves a trade-off between stability and temporal resolution. DNA-SIP provides a stable record of activity but missing rapid metabolic shifts. In contrast, RNA-SIP reflects real-time metabolic activity due to the high turnover rate of RNA, offering superior temporal resolution to capture dynamic processes in fermentation. However, RNA is more labile and technically challenging to work with. The choice should align with the research question: DNA-SIP for identifying actively growing populations over days, and RNA-SIP for mapping rapid metabolic responses over time (Nwoba et al., 2024). (3) **Quantitative interpretation and controls:** Identifying taxa in the "heavy" fraction is

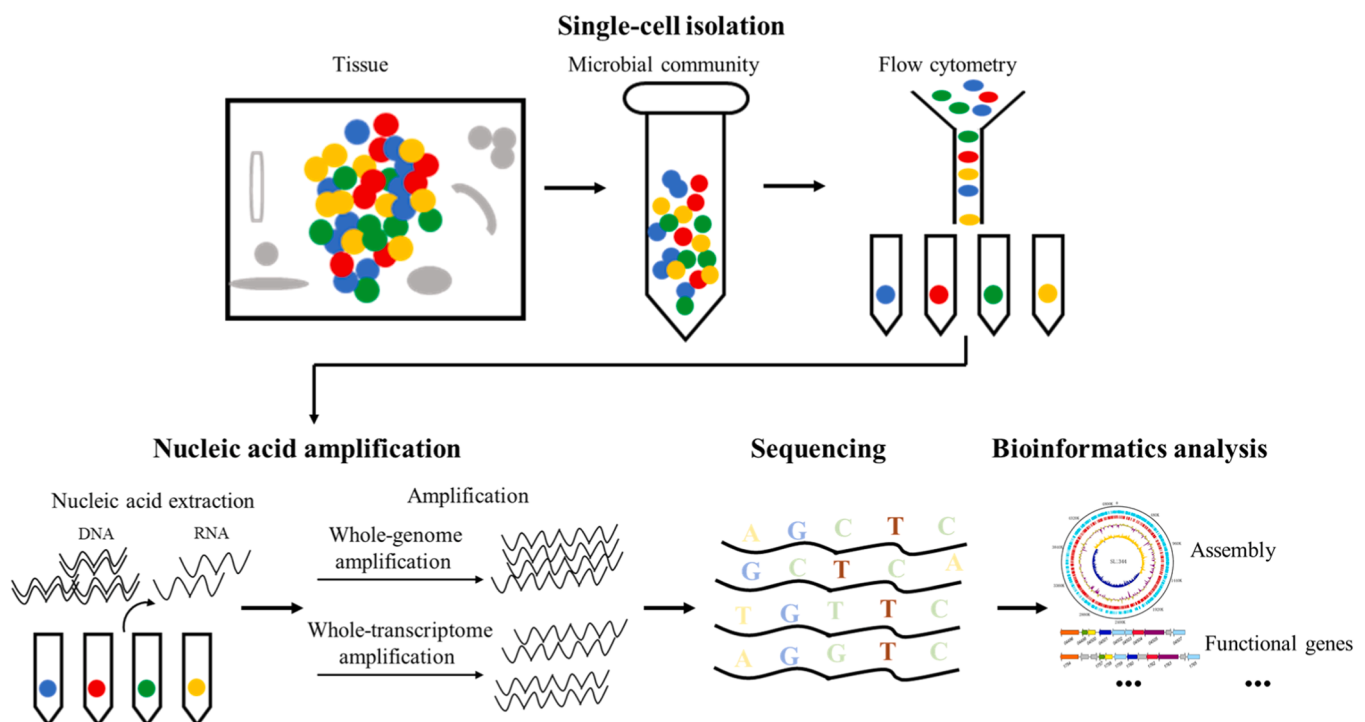


Fig. 3. The procedure of single-cell metagenomics and metatranscriptomics.

merely insufficient, therefore, quantitative interpretation is needed. The atom % excess of the heavy isotope in each gradient fraction must be measured (e.g., by isotope-ratio mass spectrometry) to confirm successful label incorporation and to establish a detection threshold (e.g., ^{13}C DNA must be heavier than a defined atom % ^{13}C value) above which taxa are considered truly labeled. This prevents false positives from background noise. Crucially, inclusion of appropriate controls is non-negotiable. Killed controls (autoclaved or chemically fixed samples) confirm that label incorporation is due to biological activity and not abiotic absorption. Parallel incubations with unlabeled substrate are essential to account for any buoyant density shifts caused by the substrate or matrix itself, not the isotope (Zheng et al., 2024; Barnett et al., 2023; Li et al., 2024b).

Following the resolution of challenges associated with complex food matrices and experimental pitfalls in fermented foods, SIP technique can be applied to guide precision regulation of fermentation processes. For example, SIP technique can be used for the development of novel yogurt starter culture for enhanced flavor. Traditional starter underproduce the desirable butter-like aroma compound diacetyl. For that, a lab-scale model fermentation can be established using milk medium amended with ^{13}C -labeled citrate (a direct precursor to diacetyl) (Melkonian et al., 2023). The heavy nucleic acids from active citrate-utilizers are separated via density-gradient centrifugation and subjected to metagenomic sequencing. Consequently, a specific strain is chosen as the primary consumer of citrate and the putative key contributor to diacetyl synthesis. Then, the strain is isolated and its high diacetyl-producing phenotype is validated in pure culture. Finally, the strain is rationally combined with traditional starters (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) into a defined starter. Defined starter is validated at pilot scale using non-labeled milk, where metabolomic profiling and sensory analysis confirm a significantly superior flavor profile compared to the original culture. The specific SIP-based identification and isolation of functional microorganisms translate microbial ecology insights into industrial application, embodying the core principle of precision fermentation.

4. Single-cell sequencing technology

Bulk sequencing provides population-averaged data that often overlook functional heterogeneity among individual cells. In addition, the functional activity of rare taxa and key contributors within low-abundance populations are often obscured (Hatzenpichler et al., 2020). To overcome these limitations, single-cell sequencing (SCS) has emerged as a powerful tool, enabling high-resolution analysis of genomic, transcriptomic, and metabolic activities at the individual cell level. Recognized as Nature Methods "Method of the Year" in 2013 and again in 2019 for single-cell multimodal omics, SCS has revolutionized our understanding of cellular heterogeneity, microbial interactions, and functional adaptations (Vandereyken et al., 2023). By resolving genetic and phenotypic variations among seemingly identical cells, SCS provides insights into uncultivated microorganisms, rare subpopulations, and dynamic regulatory networks that are otherwise obscured in bulk analyses (Wu et al., 2024b; Zhang et al., 2025).

The single-cell sequencing experiment includes four key steps, as depicted in Fig. 3. (1) **Single-cell isolation.** Techniques include limited dilution, micromanipulation, laser capture microdissection, raman tweezers, vortex and phase separation, fluorescence-activated cell sorting (FACS), flow cytometry, and microfluidics. Among these, microfluidic technology has rapidly developed to an ideal isolation method due to its lower cost and higher throughput, offering significant advantages when combined with single cell sequencing in reducing the noise and demonstrating promising applications (Shang et al., 2024). (2) **Nucleic acid amplification.** Due to the picogram-scale DNA/RNA content in single cells, whole-genome amplification (WGA) or whole-transcriptome amplification (WTA) is essential. Amplification methods include PCR-based and non-PCR-based methods. PCR-based

methods contain degenerative oligonucleotide PCR (DOP-PCR), ligation-mediated PCR (LM-PCR) and primer extension preamplification (PEP), emulsion paired isolation and concatenation PCR (epic PCR). Non-PCR-based methods contain multiple displacement amplification (MDA), primase-based whole-genome amplification (WGA), and multiple annealing and looping-based amplification cycles (MLBACs) (Yasen et al., 2020). (3) **Sequencing.** High-throughput platforms (e.g., Illumina, PacBio) are applied to generate genomic or transcriptomic data. (4) **Bioinformatics analysis.** Computational tools are used to assemble genomes, annotate genes, and reconstruct metabolic pathways (Lan et al., 2023).

SCS has been instrumental in deciphering microbial functions in complex ecosystems. For example, Chijiwa et al. (Chijiwa et al., 2020) applied single-cell genomics to identify metabolic responders to inulin at the species level in gut microbiota without relying on reference genomes, obtaining 346 single-amplified genomes and revealing novel functional roles of uncultured bacteria. The rumen microbiome, a complex ecosystem with limited pangenome information and transcriptional data. Jia et al. (Jia et al., 2024) combined droplet-based single-cell RNA sequencing with pangenome analysis, uncovering functional heterogeneity of among 2534 microbial species, among which 172 core active species were grouped into 12 functional clusters. Most notably, they discovered for the first time the function of *Basfia succiniciproducens* in carbohydrate metabolic, redefining the classic knowledge of carbohydrate metabolism. Moreover, single-cell sequencing has facilitated the identification of new single cell genome (Yu et al., 2017), microbe-microbe interactions (Nakayama et al., 2019), and novel viruses and their host interactions (Jarett et al., 2020).

Despite its success in gut and environmental microbiomes, SCS remains underutilized in fermentation ecosystems. However, in a study examining the bacterial community of koumiss, Yao et al. (Yao et al., 2017) employed a single cell amplification technique. They serially diluted each sample until it contained approximately 100 cells and then proceeded with sequencing. This approach allowed them to detect three low-abundance taxa—*L. otakiensis*, *Streptococcus macedonicus*, and *Ruminococcus torques*—that carry putative genes related to lactose metabolism and the synthesis of typical flavor compounds. This finding suggests that many microorganisms, despite their low abundance, play significant roles in fermentation ecosystems and have not yet been discovered. Thus, single-cell sequencing holds considerable promise for uncovering microbial functions in food fermentations. However, unlike the gut microbiomes, which has well-established microbial genomic databases, fermented foods lack high-quality reference genomes for many indigenous microbes. This can be addressed by combining SCS with short-read (Illumina) and long-read (PacBio, Nanopore) sequencing to improve genome assembly and leveraging metagenomic datasets to reconstruct near-complete genomes before SCS analysis. In addition, due to the high-fat/high-protein matrices of many fermented foods (e.g., cheese, fermented meat), standardized protocols establishment is prerequisite in each fermentation matrices. During food fermentation, microbial functions fluctuate, making it difficult to capture transient metabolic states. Therefore, time-resolved SCS can be used to sampling at multiple fermentation stages to track functional dynamics. Promisingly, single-cell stable isotope probing is a promising method to identify active microorganisms in substrate utilization and flavor compound formation at the single-cell level (Alcolombri et al., 2022). By addressing these challenges, SCS could be used in fermentation microbiomes, paving the way for microbial functionality analysis and precision fermentation.

5. Research progress of microbial community function in food fermentations

Currently, a variety of methods have been extensively employed to analyze microbial functions, aiming to comprehend and manage the microbiota in a wide array of fermented foods.

5.1. Cheese

Cheese represents a complex ecosystem of microbial fermentation, comprising dynamic communities derived from defined starter cultures, indigenous milk microbiota, adjunct cultures, and making environment. These microbial consortia serve as biological catalysts that orchestrate the biochemical transformations responsible for cheese's organoleptic and textural properties. Numerous studies have employed multi-omics approaches to investigate the structure-function relationships between microbial metabolism and critical cheese quality parameters, including flavor profile development, rheological characteristics, nutritional value, and product safety (Melkonian et al., 2023). Initially, complete genome sequences of thousands of strains, primarily lactic acid bacteria, were obtained to analyze gene function and evolution in Cheese production. Subsequently, omics methods have been utilized to reveal the in situ microbial community function. For instance, metagenomic analyses have uncovered putative functional genes and their associated metabolic pathways in the microbiota of three types of cheese rinds (bloomy, natural and washed), highlighting the potential contributions of less common species, such as *Pseudoalteromonas haloplanktis* and *Psychrobacter immobilis* (Wolfe et al., 2014). Metagenomics analysis has also predicted the metabolic capacity of the Mexican cotija cheese microbiota related to the branched chain amino acids metabolism, which is essential for the production of a broad range of flavor compounds and free fatty acids. Additionally, this work identified genes associated with bacteriocin production and immunity, which affect the growth dynamics of individual species within the community (Escobar-Zepeda et al., 2016).

Metatranscriptomic provides information on the transcriptional regulation of genes under specific environmental conditions during a particular period. For example, a study found that temperature is a key parameter that accelerates cheese ripening. Further, metatranscriptomic analysis indicated that increasing the ripening temperature enhances the cheese maturation rate by promoting gene expressions related to proteolysis, lipolysis, and amino acid/lipid catabolism. This leads to a higher concentration of free amino acids and fatty acids, thereby enhancing the potential flavor characteristics (De Filippis et al., 2016). High-quality RNA extraction from cheese samples is fundamental for metatranscriptomic analysis. A previous study demonstrated that the RNA can be extracted from various cheese types without prior cell separation from the cheese matrix. Moreover, they found that the efficiency of RNA extraction varies significantly between cheese types, with the integrity of extracted RNAs from pressed cooked cheeses being lower than those from other types of cheeses (Carbonne et al., 2022).

Multi-omics integration methods are used to reveal deeper mechanistic insights into cheese fermentation and flavor formation driven by complex multi-species communities, complementing the missing information obtained by single-omics analysis. For example, metagenomic and metatranscriptomic analyses were used to investigate how different ripening conditions (warm and cold) influenced the functional diversity of Swiss-type cheeses (Duru et al., 2018). In this study, the authors reconstructed four different bacterial genomes (*Lactococcus lactis*, *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii* subsp. *shermanii* strain JS) and showed that these species harbor pathways for flavor formation, such as the production of methanethiol, free fatty acids, acetoin, diacetyl, acetate, ethanol, and propionate. Meanwhile, *L. lactis* dominated (~80–90 %) within the microbial community. The metatranscriptomic data revealed that 651 genes were differentially expressed between warm and cold ripening conditions, with genes responsible for flavor compound production being downregulated in cold ripening condition. In contrast, proteases, peptidases, dipeptide transporters, amino acid permeases, amino acid catabolism, fatty acid β -oxidation, and biosynthesis genes were upregulated in warm ripening condition, revealing that warm ripening temperature promote the development of flavor compounds.

Competitive and cooperative microbial interactions play a crucial

role in the flavor formation during cheese fermentation. Recently, multi-omics approaches have been extensively employed to elucidate these microbial interactions. For example, Melkonian et al. (Melkonian et al., 2023) investigated the impact of microbial interactions on cheese flavor characteristics over the course of a year-long Cheddar cheese production process using genomics, genome-scale metabolic modeling, metatranscriptomics, and metabolomics. They constructed a microbial community comprising a commercial starter culture along with *Streptococcus thermophilus* and *Lactococcus* strains. By strain dropout strategy, they discovered the significant role of *S. thermophilus* in promoting *Lactococcus* growth and influencing flavor compound formation. Notably, the proteolytic activity secreted by *S. thermophilus* supplies the *Lactococcus* community with a necessary nitrogen source, enhancing de novo nucleotide biosynthesis. Furthermore, competitive metabolic interactions occur within the *Lactococcus* community, where *L. cremoris* and *L. lactis* strains compete for available citrate, resulting in the accumulation of key metabolites such as diacetyl and acetoin, which ultimately affect the final cheese flavor. Significantly, the study identified the importance of strain interactions in cheesemaking: different *L. lactis* strains influence the activity of *S. thermophilus* in distinct ways. This research underscores the critical role of strain-specific metabolic interactions among microorganisms in shaping the flavor profile of cheese, providing evidence of microbial functional diversity within complex food microbial ecosystems.

5.2. Kefir

Kefir, famous for its high nutritional value and health benefits, is traditionally produced by inoculating milk with a kefir grain – a protein and exopolysaccharide matrix harboring a variety of microorganisms. This mixture, primarily consisting of lactic acid bacteria, acetic acid bacteria, yeasts, and other microorganisms, is responsible for the fermentation process. The rich microbiota is intimately linked to the enhancement of kefir's sensory characteristics and health-promoting functions due to their metabolic activities (Cheng et al., 2024). Unveiling the microbial community and their conserved features is essential for managing and controlling the fermentation process. A study investigated the heterogeneity of milk kefir microbial populations globally using metagenomics and pan-metagenome analysis. The researchers collected 64 kefir grains from 25 countries for metagenomics-based identification and characterization of microorganisms. They first identified the core features of microbial communities across all kefir samples, discovering a separation into four distinct communities, each represented by *Lactobacillus helveticus*, *Lactobacillus kefirifaciens* subsp. *kefirifaciens*, *Lactococcus lactis* subsp. *Lactis*, and *L. cremoris* subsp. *Cremoris*. Further functional analysis revealed that 222 metabolic pathways were shared among different communities, suggesting that despite different species composition, various types of kefir maintain similar characteristics to produce consistent flavor quality in the final products (Walsh et al., 2023).

Metabolic interactions among diverse species form the foundation for the long-term coexistence of microbial communities and the consistent product quality. Recently, multi-omics methods have been employed to reveal the mechanisms behind the stable coexistence of communities in Kefir fermentation. For example, Blasche et al. used metagenomics, metabolomics, transcriptomics, and large-scale mapping of inter-species interactions to elucidate the spatiotemporal orchestration and metabolite dynamics of prokaryotes and yeasts that maintain community stability in a model Kefir ecosystem. Shotgun metagenomic sequencing revealed that the core community consisted of *Lactobacillus* species (mainly *L. kefirifaciens* and *L. kefir*), *Leuconostoc* species (usually *L. mesenteroides*), *Lactococcus lactis*, and *Acetobacter* species. Bacterial communities displayed different compositions in the kefir grain and milk fractions: they remained stable in grains, while undergoing significant changes in the fermented milk during fermentation. The study identified six stages based on microbial growth trends: stage I

dominated by *L. kefirifaciens*, stages II and III marked by rapid growth of *L. lactis* and *L. mesenteroides*, respectively, stage IV dominated by *L. kefir*, stage V characterized by stagnation of most species, and stage VI marked by the growth of *Acetobacter fabarum* and *L. kefir*. Further analysis revealed the microbial sequential colonization mechanism mediated by extensive metabolic changes: early-colonizing species provide available metabolites (mainly amino acids and lactate) for the subsequent species (Blasche et al., 2021).

5.3. Chinese liquor

Baijiu (Chinese liquor), one of the world's oldest distilled spirits, is produced through the spontaneous fermentation of grains such as sorghum, wheat, rice, corn, using *Daqu* as starters and pit mud as the fermentation vessel. During fermentation, abundant microorganisms from various sources – including the natural environment, *Daqu*, tools, and the ground – converge to form a complex multispecies community to convert raw materials to various flavor compounds (Jin et al., 2017). Unraveling the microbial mechanisms of action in the fermentation process is the foundation of achieving quality control and enhancing the flavor profile of *Baijiu*.

Currently, metatranscriptomics stands as the primary method for uncovering the core functional microorganisms and their metabolic activities that contribute to the formation of flavor compounds during *Baijiu* fermentation. For example, during Jiang-flavor *Baijiu* fermentation, metatranscriptomics analysis demonstrated that core yeasts (*Pichia*, *Schizosaccharomyces*, *Saccharomyces*, and *Zygosaccharomyces*) are involved in high levels of ethanol production in the initial stage, while *Lactobacillus* plays a role in the production of lactic acid and acetic acid in the subsequent stage (Song et al., 2017). In the case of Strong-flavor *Baijiu* fermentation, metatranscriptomics analysis demonstrated that both dominant taxa (*Limosilactobacillus fermentum*, *Kazachstania africana*, *Saccharomyces cerevisiae*, and *Pichia kudriavzevii*) and keystone taxa (*Thermoascus aurantiacus*, *Weissella confusa*, and *Aspergillus amstelodami*) are implicated in ethyl acetate biosynthesis through the high expression of O-acetyltransferase and ethanol acetyltransferase, as well as in the production of higher alcohols (isoamyl alcohol, phenethyl alcohol, and isobutanol) production via the Ehrlich pathway and anabolic pathway (Yuan et al., 2023). Imbalance in the content of flavor compounds can lead to inferior quality products. Employing a metatranscriptomics approach, Wei et al. (Wei et al., 2024b) discovered that *Saccharomyces cerevisiae* produces the highest levels of higher alcohols during Jiang-flavor *Baijiu* fermentation, and a higher abundance of *Saccharomyces* leads to higher levels of higher alcohols in the first fermentation cycle. Therefore, controlling the abundance of *Saccharomyces* is a potential strategy for reducing higher alcohol production and improving flavor quality in the first cycle.

Daqu, the starter for *Baijiu* production, provides the microorganisms, flavor compounds, enzymes, and raw materials for *Baijiu* fermentation. It is produced through spontaneous fermentation with multi-species community (Wu et al., 2023). *Daqu* is made from wheat and includes a two-step process: one month of fermentation in a fermentation room and three months of drying in a storage room to mature. Metaproteomics and metatranscriptomics are widely used to explore microbial function in *Daqu* production. Metaproteomics analysis has revealed that *Daqu* contributes 80 % of carbohydrate hydrolases for *Baijiu* fermentation, with alpha-amylase and glucoamylase from *Aspergillus*, *Rhizomucor*, and *Rhizopus* being responsible for starch hydrolysis and ethanol production (Wang et al., 2020). Yang et al. (Yang et al., 2023b) identified 1030 proteins in *Daqu* by metaproteomics and protein annotation indicated that microbial metabolic pathways are predominantly enriched in 14 pathways. Amino acid metabolism, regulated by *Neurospora crassa*, *Aspergillus nidulans*, *Bacillus subtilis*, and *Oceanobacillus iheyensis* leads to the microecological differentiation of yellow, white, and black *Daqu*. Temperature is a critical driver in shaping the corresponding microbial communities in *Daqu* production. *Daqu* can be classified into three types

based on the maximum temperature: high-temperature *Daqu* (> 60 °C), medium-temperature *Daqu* (50 – 60 °C), and low-temperature *Daqu* (40 – 50 °C). Yi et al. (Yi et al., 2019) elucidated the effect of temperature on active microbial communities and functional enzymes through metatranscriptomics. They found that *Aspergillus* and *Penicillium* are the most active fungi at 70 °C, and 230 carbohydrate-active enzymes are identified as potential saccharifying enzymes. Carbohydrate and energy pathways associated with saccharification and fermentation exhibit lower expression levels at 70 °C compared to those at 62 °C; however, some enzymes related to aromatic compound degradation are only detected at 70 °C. Huang et al. (Huang et al., 2017) investigated the function of active microbial communities in Nong-flavor *Daqu* production using metatranscriptomics. They discovered that fungal communities are much more diverse, and the enzymes produced by fungi are more abundant than those produced by bacterial communities. Fungal communities secrete thermostable carbohydrate-active enzymes, including endo-1,3-β-D-glucanase, endo-1,5-α-L-arabinanase, and endo-β-1,3-1,4-glucanase, which are involved in glycolysis, ethanol metabolism, pyruvate metabolism, and citrate cycle.

Pit mud, a special fermentation vessel for Strong-flavor *Baijiu* production, is intimately linked to the liquor quality by virtue of its enrichment of a variety of anaerobic microbes that generate numerous flavor compounds. Pit mud is used repeatedly and matures over time through the domestication of liquor-brewing microbes during fermentation with only mature pit mud being suitable for brewing high-quality liquor. Therefore, the quality of pit mud is directly related to its mature time. Zheng et al. (Zheng et al., 2015) utilized high-throughput sequencing of 16S rDNA and iTRAQ-based proteomic to investigate the microbial diversity and the expression of aroma-forming functional proteins in 30- and 300-year pit muds. They discovered that *Clostridium* and *Lactobacillus* were the predominant microbes in both samples, but the 300-year-old pit mud had a higher presence of aroma-forming functional microorganisms such as methanogens and *Clostridium*. Proteomic analyses identified 63 proteins associated with aroma formation, 59 of which were highly expressed in the 300-year-old pit mud. These proteins play a role in methanogenesis and the formation of caproic acid and butyric acid during liquor fermentation. The findings suggest that older pit mud contributes to the production of higher quality Chinese liquor. However, the natural maturation period of over 20 years is a critical constraint on high-quality liquor production, and the scarcity of naturally mature pit mud impedes the growth and development of the baijiu industry. Therefore, the production of artificial pit mud (APM) to increase high-quality liquor output is a viable solution, and efforts in this direction have been made. APM is typically produced by inoculating a starter culture into a mixture of fresh soil, natural mature pit mud, wheat, and soybean meal, followed by incubation under anaerobic conditions for 30 to 60 days. Liu et al. (Liu et al., 2020) compared the prokaryotic taxonomic and functional dynamics from the first to fourth batches fermented in APM using metagenomic, metaproteomic, and metabolomic analyses. Metagenomic analysis revealed the presence of 36 prokaryotic classes and 195 genera across all samples, with *Bacilli* and *Clostridia* consistently dominating, and the relative abundance of *Bacilli* decreasing from the first to the fourth batch fermentation. Metaproteomic analysis identified 213 prokaryotic proteins from 70 genera, with *Lactobacillus* (18.31 %), *Clostridium* (9.86 %), and *Sporomusa* (5.16 %) being the main sources of these proteins. Moreover, 47 differential proteins were found between the first and fourth batches, with 16 and 31 proteins enriched and depleted in the fourth batch, respectively. These differential proteins, primarily associated with *Lactobacillus*, were involved in metabolic processes such as ADP metabolism, purine ribonucleoside diphosphate metabolism, and purine nucleoside diphosphate metabolism. These results aid in the optimization of APM cultivation technique and the improvement of liquor quality.

5.4. Fermented tea

Pu'er tea, a popular traditional Chinese tea, is produced through a natural solid-state fermentation process using sun-dried green tea leaves as the raw material. It is renowned for its health benefits on human body, including hypolipidemic, antioxidative, and toxicity suppressing. The combination of metagenomics and metaproteomics has unveiled the microbial communities and enzymatic profiles of Pu'er tea over a 21-day fermentation period. Metagenomics analysis revealed that *Proteobacteria* and *Aspergillus* were the dominant bacteria and fungi, respectively. Subsequent metaproteomics analysis identified 335 proteins, categorized into 28 Biological Processes and 35 Molecular Function categories. The majority of bacterial proteins were affiliated with *Proteobacteria*, while the majority of fungal proteins were attributed to *Aspergillus*. Microbial extracellular enzymes were primarily involved in the degradation of xylan, pectin, cellulose, and arabinosyl-xylan-polysaccharides of the plant cell wall – which facilitate the maceration and soft-rotting of tea leaves. Concurrently, catalase, catalase-peroxidase, and peroxiredoxin were found to catalyze the oxidation of catechins (Zhao et al., 2015).

Moreover, a comprehensive review of the fermentation mechanisms of Pu'er tea throughout the entire process has been revealed by an integrated meta-omics approach combining amplicon sequencing, metaproteomics, and metabolomics (Zhao et al., 2019). Initially, the bacterial genus *Proteobacteria* showed a decrease, while *Firmicutes* showed an increase during fermentation. Fungal genus *Rasamsonia*, *Thermomyces*, and *Aspergillus* were dominant at the intermediate stage, with *Aspergillus* being dominant at other stages of fermentation. Metaproteomics analysis revealed that the majority of identified proteins were classified as catalytic activity and binding based on the GO annotation, with biological processes primarily being cellular process and metabolic process. The most common metabolic pathways were identified as glycolysis/gluconeogenesis, ribosome, oxidative phosphorylation, and the citrate cycle based on the KEGG pathways annotation. Carbohydrate-active enzymes (CAZymes), responsible for the assembly and breakdown of complex carbohydrates, including oligosaccharides, polysaccharides and glycoconjugates to nucleic acids, proteins, lipids, polyphenols, and other natural compounds, play an important role in fermented foods (Andre et al., 2014). During Pu'er tea fermentation, a substantial number of proteins were annotated as CAZymes. These enzymes are hypothesized to degrade plant and fungal polysaccharides, such as cellulose, xylan, xyloglucan, pectin, starch, lignin, galactomannan, and chitin. On the one hand, these CAZymes break down polymers into monomers and oligomers for microbial growth; on the other hand, the degradation of plant polysaccharides can destroy tea leaves cells, softening tea leaves. Finally, metabolomics analysis uncovered that the content of phenolic compounds, including gallates, decreased while gallic acid and ellagic acid increased after fermentation. The shift may be attributed to several factors: phenolic glycosides being hydrolyzed by glycoside hydrolases, gallates being hydrolyzed by tannase to produce gallic acid, and phenolic compounds being oxidized, modified, or degraded by various enzymes including catechol O-methyltransferase, phenol 2-monooxygenase, salicylaldehyde dehydrogenase, salicylate 1-monooxygenase, catechol 2,3-dioxygenases, catechol 1,2-dioxygenase, and quercetin 2,3-dioxygenase. Phenolic compounds are characteristic chemical components in teas and have a number of health benefit. The decrease of phenolic compounds contributes to the transformation of tea's taste from astringent to mellow.

5.5. Fermented soybean product

Da-jiang, a famous fermented soybean food in east Asian countries, has unique flavor and high nutritional value. The fermentation process of Da-jiang represents a highly complex microbiota ecosystem and microbial metabolism is pivotal to the product's quality. A metagenomic analysis identified 248 bacterial genera, including 841 species of

bacteria during da-jiang fermentation. The predominant species include *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Leuconostoc lactis*, *Acinetobacter lwoffii*, *Acinetobacter johnsonii*, *Enterobacter* sp. 638, *Enterococcus faecium*, and *Enterococcus faecalis*. KEGG functional annotation indicates that bacterial functional genes are primarily involved in carbohydrate metabolism, amino acid metabolism, membrane transport, inorganic ion transport and metabolism, signal transduction, and metabolism of coenzyme and vitamins. These bacterial species play a crucial role in shaping the characteristics of da-jiang. For example, *Pseudomonas Chlororaphis* is closely associated with the color of da-jiang and *Pseudomonas fluorescens* is closely related to the production of tyramine and histamine (Xie et al., 2020). Fungal community, primarily responsible for secreting a variety of enzymes that degrade starch and protein, is dominated by *Rhizopus*, *Penicillium*, and *Geotrichum* during da-jiang fermentation. In the early stages of fermentation, fungal species gradually grow, cover the soybean paste with hyphae, and subsequently secrete various enzymes. In the later stages, the growth of the fungal community stopped, but the enzymes they secrete continue to function. Metaproteomic analysis has identified the most active enzymes in carbohydrate metabolism. The enzymes involved in glycolysis/gluconeogenesis pathways predominantly belong to *Rhizopus* and *Penicillium*, such as phospho-beta-glucosidase, glucose-1-phosphatase, and phosphoglucomutase. This suggests that these two genera contribute to carbohydrate degradation, generating flavor in da-jiang-meju (Xie et al., 2019).

Metatranscriptomics analysis has uncovered the link between flavor formation and core microorganisms during da-jiang fermentation, identifying *Lactobacillus* and *Tetragenococcus* as the key microorganisms that determine chromaticity and flavor (An et al., 2021). Specifically, *Lactobacillus* expresses key enzymes related to flavor compound formation, including acetate kinase and pyruvate dehydrogenase involved in acetic acid production, alcohol dehydrogenase catalyzing ethanol production, and asparagine synthase and aspartate aminotransferase related to amino acid synthesis and metabolism. *Tetragenococcus*, on the other hand, expresses vital enzymes involved in the synthesis of aldehydes and ketones, including acetaldehyde dehydrogenase, alcohol dehydrogenase, and branched-chain amino acid aminotransferase.

6. Future directions for microbial function analysis in fermentations

(1) As complementary approaches to omics technologies, stable-isotope probing and single-cell technologies should be developed and applied to study microbial functions in fermentation systems. This will enhance the understanding of functional heterogeneity among microorganisms and key contributors with low-abundance populations. Consequently, the functions of complex microorganisms in fermentation systems can be more clearly elucidated, leading to a deeper comprehension of fermentation mechanisms toward the achievement of reproducible fermentation processes.

(2) Advanced cultivation strategies should be applied to the isolation of functional microorganisms. Despite recent progress, the majority of fermentation-relevant microorganisms remain unculturable. Innovative cultivation platforms from *in-situ* ecosystems are urgently needed, including: ① Membrane diffusion-based cultivation: Isolation Chip (iChip), Soil Substrate Membrane System (SSMS), and Hollow-Fibre Membrane Chambers (HFMC). ② Microfluidic systems: SlipChip and Nanoporous Microscale Microbial Incubators (NMMI). ③ Function-driven isolation: Fluorescence in situ Hybridization of Live Cells (Live-FISH), Raman-Activated Cell Sorting (RACS), and Reverse Genomics (Lewis et al., 2020). These innovative methods will facilitate construction of defined synthetic communities for controlled fermentation processes, potentially enhancing substrate conversion efficiency and process consistency while reducing fermentation time.

(3) While stable-isotope probing and single-cell technology provide powerful and promising methods to investigate microbial function, their

Table 1
Comprehensive summary of microbial functional analysis techniques.

Methods	Research question	Key technical limitations	Industrial translation	Ideal use case / food matrix
PICRUSt	What is the predicted functional potential of the community?	Database-dependent. Indirect prediction. High error rate for rare taxa.	Low-resolution screening for hypothesis generation.	Initial and low-cost screening of microbial function in high-biomass samples (e.g., <i>Daqu</i>).
Metagenomics	What functional genes are present? What is the taxonomic and genetic potential?	Do not indicate activity. Cannot differentiate viable and non-viable cells. Host DNA contamination.	Identify all microbial players and their potential functions. Guides targeted isolation.	Profile community potential in food fermentations.
Metatranscriptomics	What genes are being actively transcribed?	Difficult extraction of high-quality RNA. Short mRNA half-life. Do not equate to protein activity.	Identify key active pathways under specific process conditions for optimization.	Study microbial response to environmental shifts (e.g., pH, salt).
Metaproteomics	What proteins are being synthesized and are active?	High host protein background. Complex extraction. Database dependency.	Confirm which enzymes are truly driving the process. Link function directly to taxa.	Link enzymes to flavor formation in cheese, soy et al.
Metabolomics	What metabolites are present and what are the net fluxes?	Difficulties in metabolite annotation due to the limited reference databases.	Directly monitor product formation/quality. Ideal for real-time process control.	Identify flavor compounds in all food matrices.
Multi-omics	What is the holistic view of the system from genes to metabolites?	Data integration is major challenge. Mask microbial heterogeneity. Ignore low-abundance populations.	Create digital twins of fermentation processes for in silico testing and optimization.	Hypothesis testing after initial omics discovery.
Stable-isotope probing	Who is actively using a specific substrate?	Isotope cross-feeding. Technical complexity.	Link specific taxa to specific functions. Key for starter culture design.	Track substrate utilization in <i>Baijiu</i> , kimchi.
Single-cell sequencing	What is the functional heterogeneity within a population? What do uncultured/rare taxa do?	Low input. High cost. Lack high-quality reference genomes.	Access genetic potential of uncultivable microbes.	Discover novel microbial functions.

application in complex fermentation systems can be resource-intensive and hypothesis-agnostic. As indispensable hypothesis-generating engines, in silico approaches, such as genome-scale metabolic models, can integrate genomic data to predict the metabolic potential and interaction networks of microbial communities. These predictions can strategically guide subsequent experimental design by identifying key target

taxa, proposing optimal isotopic tracer substrates (e.g., predicting which ¹³C-labeled compound would yield the most informative labeling patterns), and pinning out putative keystone species for further isolation and single-cell sequencing. Thus, the future of microbial functional research should integrate these techniques with sophisticated computational algorithms (Liu et al., 2023; Wu et al., 2024b; Sanchez et al.,

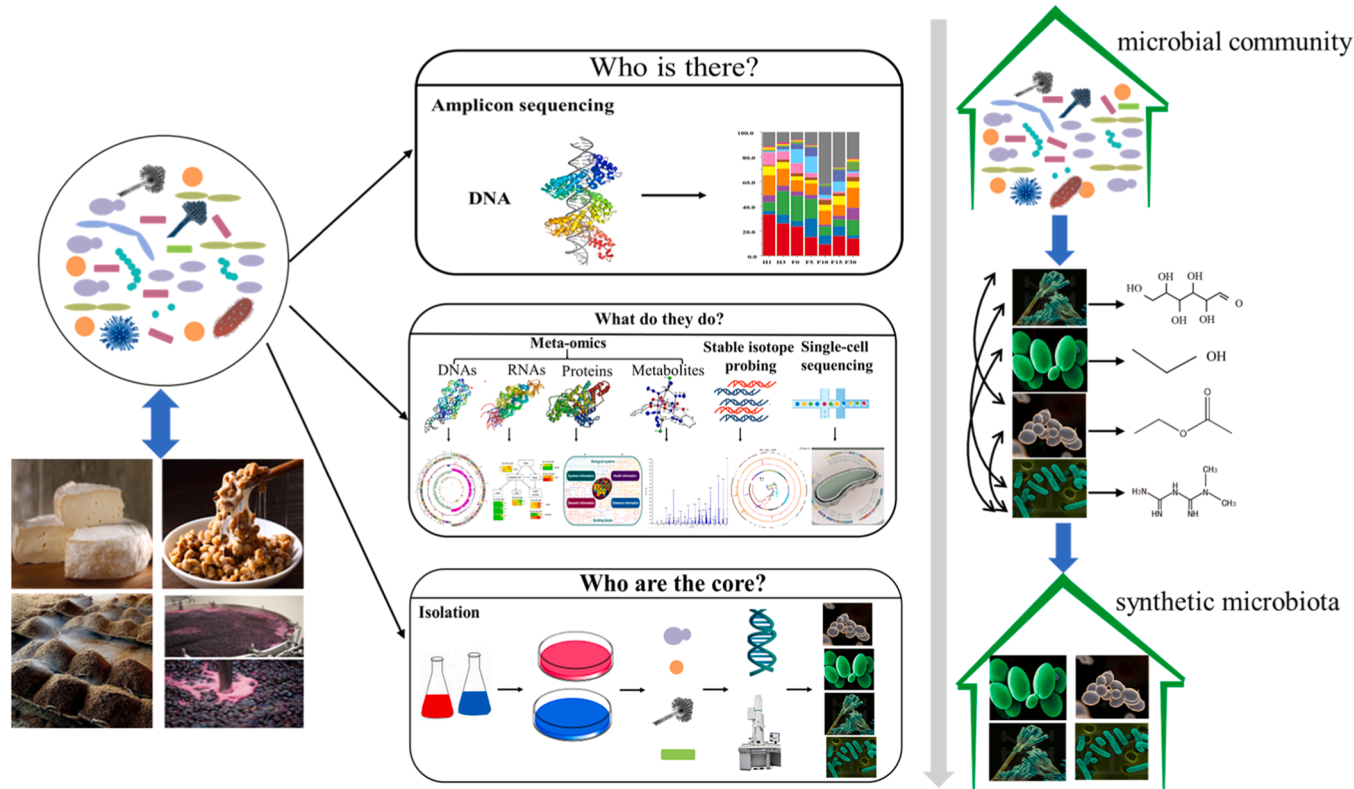


Fig. 4. A roadmap for developing synthetic microbiota via functional analysis to optimize spontaneous food fermentation management.

2023). The convergence of these technological advances will transform our ability to understand, predict, and engineer microbial communities for next-generation fermentation biotechnology. By integrating traditional fermentation practices with modern systems biology, it is possible to develop precision fermentation systems that combine advanced process control with the preservation of artisanal product qualities.

7. Conclusion

The inherent variability of spontaneous food fermentations, driven by complex and uncontrolled microbial dynamics, often results in inconsistent product yield and quality. To achieve consistent production while preserving desirable characteristics, understanding the functional roles of complex microbial consortia is essential. This review provides a critical assessment of current methodologies for deciphering microbial function in fermentation ecosystems, highlighting both their capabilities and limitations (Table 1). While conventional approaches offer broad functional insights, they frequently fail to resolve functional heterogeneity among microorganisms, the function of individual microbial taxa, and key contributors with low-abundance populations. Emerging techniques—stable isotope probing and single-cell sequencing—have demonstrated remarkable success in dissecting microbial function within other complex ecosystems. Through targeted adaptation and optimization, these advanced methodologies hold significant promise for application in fermentation systems. Ultimately, comprehensive elucidation of complex microbial community function in spontaneous fermentations will pave the way for rationally designed synthetic communities. Such engineered consortia could enable precise control over fermentation processes, ensuring both production consistency and superior product quality while maintaining the artisanal attributes of traditional fermented foods (Fig. 4).

Declaration of competing interest

We declare that we 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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Data availability

Data will be made available on request.

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Further reading

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