BioDesign Research 8 (2026) 100089

Contents lists available at ScienceDirect

BioDesign Research

journal homepage: www.sciencedirect.com/journal/biodesign-research

Review Article

Ecology-microbiome engineering in Sauce-flavor Baijiu via Single-Cell Raman Spectroscopy

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ARTICLE INFO

Keywords:
Sauce-flavor Baijiu
Single-cell Raman Spectroscopy
Metabolic networks
Microbial community
Design-build-test-learn

ABSTRACT

Sauce-flavor Baijiu owes its layered aroma to a diverse microbial consortium fermenting under extreme, open, solid-state conditions. High-throughput sequencing has revealed marked spatiotemporal heterogeneity in community composition, yet a fundamental composition-function disconnect persists: bulk omics average signals across heterogeneous micro-niches and cultivation recovers only a minor fraction of total diversity, leaving "who is doing what" unresolved. Closing this disconnect is a prerequisite for rational design of fermentation microbiomes and demands cell-resolved functional tools within an iterative engineering framework. This review proposes that Single-Cell Raman Spectroscopy (SCRS) and its derivatives, including D_2O-Raman activity mapping, scRACS-Seq phenotype-to-genome linkage, scRACS-Culture recovery of rare functional strains, and Intra-Ramanome Correlation Analysis (IRCA) predictive metabolic phenotyping, collectively provide a label-free, culture-independent toolkit suited to this role in solid-state matrices. We delineate how these single-cell insights feed each stage of a Design-Build-Test-Learn (DBTL) cycle, from phenotype-informed strain selection through consortium assembly and functional validation to data-driven iterative optimization. This convergence of cell-resolved functional dissection, synthetic ecology, and process analytical technologies establishes the foundation for advancing Sauce-flavor Baijiu from experience-dependent craftsmanship toward intelligent brewing.

1. Introduction: from correlation to causation in complex fermentation

Sauce-flavor Baijiu owes its characteristic layered aroma to the metabolic activities of a highly diverse microbial community operating within an open, solid-state fermentation system [1–3]. Despite centuries of empirical craftsmanship, this system has long remained a "black box" in which the precise biological mechanisms underlying flavor formation are poorly understood. The field is currently undergoing a critical paradigm shift from descriptive cataloging of microbial inhabitants to mechanistic understanding of functional contributions. However, two fundamental challenges impede progress.

The first challenge is the composition-function gap. While high-throughput sequencing reveals "who is there", community composition alone cannot answer "who is doing what" [4]. Bulk DNA extraction has been shown to average signals across heterogeneous micro-niches, thereby obscuring the metabolic activities of individual cells [5]. Cultivation-based methods recover less than 1% of microbial diversity in most environments [6], excluding the vast "microbial dark matter" that may harbour key functional taxa [7,8]. The gap between genetic potential (what cells could do) and metabolic execution (what they are actually doing) remains unbridged by conventional approaches.

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https://doi.org/10.1016/j.bidere.2026.100089
Received 23 March 2026; Received in revised form 17 April 2026; Accepted 25 April 2026
Available online 5 May 2026

The second challenge is the disconnect between empirical knowledge and scientific understanding. Traditional production relies on the sensory judgment of master brewers with decades of experience. This judgment involves observation of foam texture, manual temperature assessment, and olfactory evaluation [9]. While this empirical expertise is remarkably effective, it also makes the process resistant to standardization, quantification, and scaling up [10]. The tacit knowledge embedded in craftsmanship lacks the biological vocabulary necessary for transmitting it across generations or geographic regions.

Together, these two challenges expose three critical knowledge gaps: (*i*) the absence of frameworks that link spatiotemporal heterogeneity to single-cell function; (*ii*) the lack of systematic approaches to resolve metabolic activity in solid-state matrices with high particle loads and fluorescence backgrounds; and (*iii*) the undefined pathways from mechanistic understanding to rational engineering application.

Single-Cell Raman Spectroscopy (SCRS) has emerged as a transformative bridge technology capable of resolving these bottlenecks. As a label-free, non-destructive, and culture-independent technique with true single-cell resolution, SCRS enables direct coupling of metabolic phenotype to cellular identity within intact fermentation ecosystems. This review progresses systematically from the ecological and metabolic context of Sauce-flavor Baijiu fermentation (Section 2), through methodological innovation at the single-cell level (Section 3), to engineering implementation via the Design-Build-Test-Learn (DBTL) framework (Section 4). Throughout, we emphasise how this integrated approach facilitates the transition from correlation-based inference to causal, mechanistic understanding, ultimately enabling data-driven intelligent brewing that preserves the essence of traditional craftsmanship while enhancing controllability and predictability.

2. Ecological and metabolic foundations: the composition-function gap

2.1. Spatiotemporal assembly and geographic variation of the core community

Sauce-flavor Baijiu relies on a Core Functional Community (CFC) defined by high prevalence, compositional stability, and validated functional evidence [11–14]. This CFC is shaped by rigorous environmental filtering, primarily through high-temperature Daqu production which stringently selects for thermotolerant, spore-forming bacteria like *Bacillus* [12,13]. SourceTracker analysis confirms that Daqu provides the majority of bacterial inocula, while the environment predominantly shapes the fungal communities (Fig. 1).

While these assembly mechanisms operate consistently, distinct local climatic conditions modulate taxonomic expression. Across all production regions, high-temperature Daqu ensures strict conservation of core thermotolerant bacteria (*Bacillus*, *Kroppenstedtia*, *Virgibacillus*), and *Lactobacillus* dominates pit fermentation [15–17]. Fungal communities, however, exhibit pronounced environment-dependent plasticity shaped by local climates.

For instance, in the quintessential river-valley climate of Guizhou (characterized by persistent high temperature and humidity), *Thermomyces* dominates the fungal community and is primarily sourced directly from Daqu [18,19]. Conversely, Beijing's cold and dry continental climate favors *Byssochlamys* and *Issatchenkia*, which are predominantly recruited from workshop surfaces rather than the starter culture [15,16]. In elevated subtropical regions like Sichuan, significant diurnal temperature variations and geographic gradients impose strong spatial filtering, producing pronounced workshop-level heterogeneity among fungi like *Monascus* [20]. Meanwhile, in the humid subtropical climate of Hunan, rigorous environmental filtering restricts taxa like *Escherichia-Shigella* while uniquely empowering *Kroppenstedtia* as a keystone bacterial taxon [17,21].

2.2. Spatiotemporal heterogeneity and synergistic metabolic networks

The long-cycle fermentation generates a continuously evolving physicochemical landscape, compartmentalizing metabolic labor across specific spatiotemporal niches (Fig. 2). While Fig. 2A presents a qualitative metabolic model typical of a 30-day cycle, the absolute temporal dynamics are subject to significant variation. These dynamics are driven by feedstock properties, seasonal conditions, and the specific stage of the production cycle, such as the 1st-7th fermentation rounds. Temporally, the process transitions from an aerobic initiation phase dominated by *Pseudomonas* and *Pichia*, to a thermal screening phase enriching *Bacillus*, and finally into an anaerobic esterification phase where *Lactobacillus* and fungi synthesize key flavors [18,22–26]. Spatially, radial oxygen gradients in open stacks create a microaerobic middle layer where *Bacillus* and *Lactobacillus* drive saccharification, and an anaerobic core where the oxygen-tolerant *Acetilactobacillus jinshanensis* dominates [27,28]. Within the anaerobic pit, the middle layer (35-40 °C, pH 3.0-3.5) functions as the optimal zone for *Lactobacillus* and *Issatchenkia* synergy, synthesizing approximately 60% of core esters [25,29].

This spatiotemporal architecture supports complex, multi-species metabolic relays (Fig. 3). For example, ester synthesis operates via a cross-kingdom relay: bacteria like *Lactobacillus* produce organic acids, which are subsequently converted into signature esters (e.g., ethyl phenylacetate, ethyl caproate) by fungi such as *Wickerhamomyces* and *Issatchenkia* upregulating ester synthases [25,30–32]. Similarly, the roasted-aroma compound tetramethylpyrazine (TTMP) is synthesized through sequential catalysis: *Bacillus* generates α-aminoketone

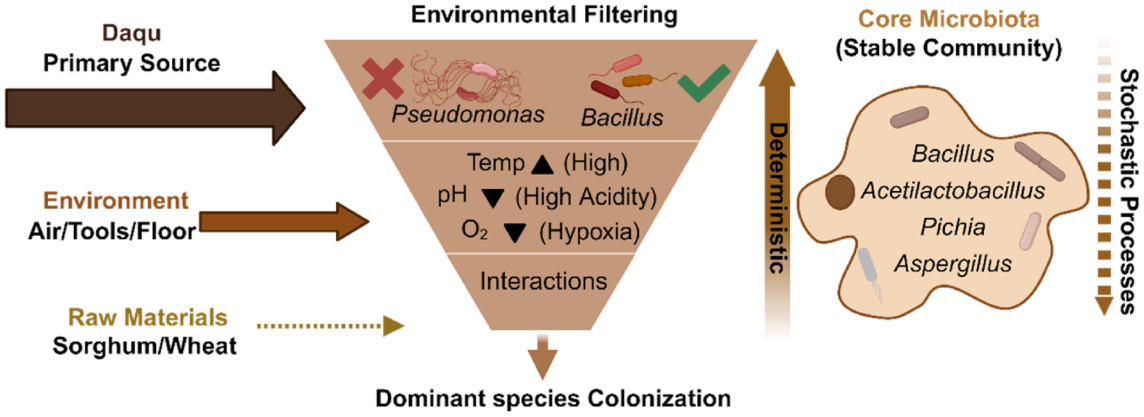

Fig. 1. Assembly mechanisms of the core microbiota in Sauce-flavor Baijiu. The microbial community is primarily sourced from Daqu (primary inoculum), the production environment, and raw materials. Driven by rigorous environmental filtering (high temperature, high acidity, and hypoxia) and microbial interactions, the community assembly shifts from stochastic processes during the initial stages to deterministic processes, ultimately forming a stable and functional core microbiota.

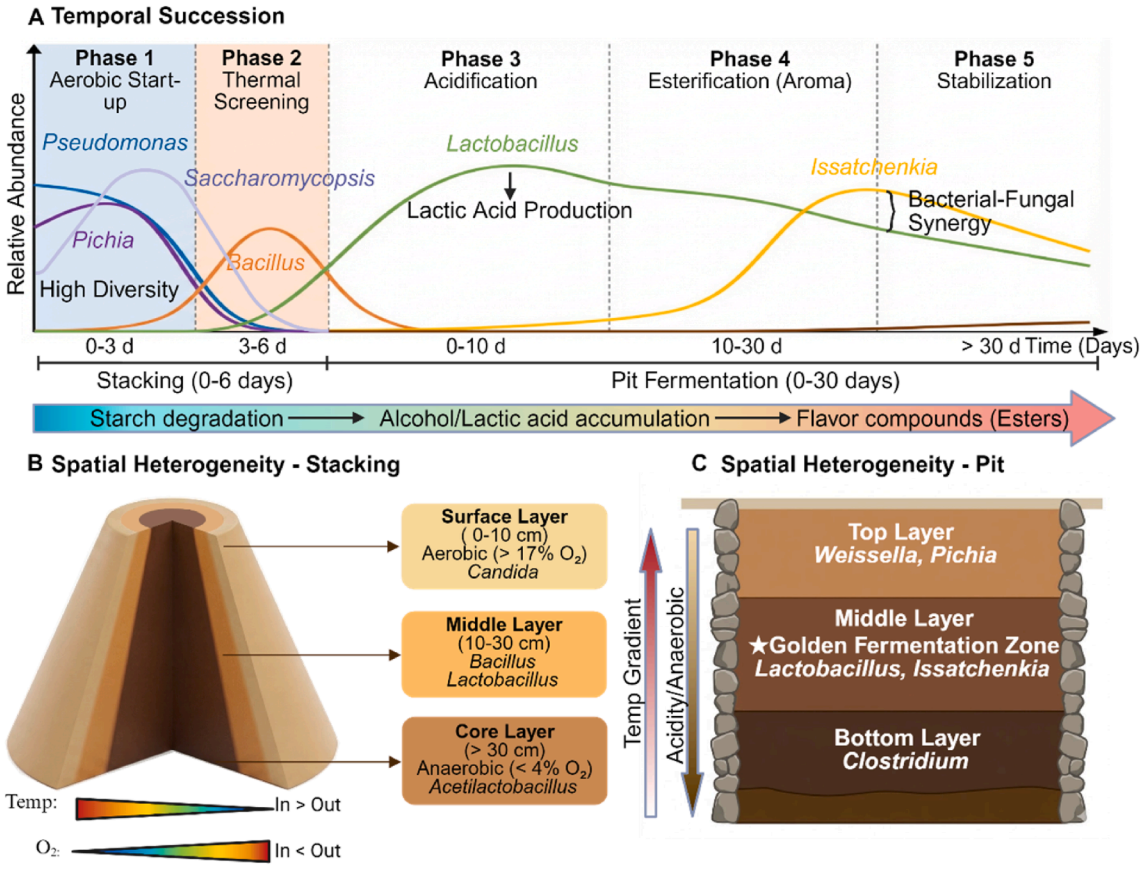

Fig. 2. Spatiotemporal heterogeneity and functional stratification of microbial communities during Sauce-flavor Baijiu fermentation. (A) Qualitative metabolic model of temporal succession across the stacking and pit fermentation stages, illustrating representative phased transitions from aerobic initiation and thermal screening to anaerobic esterification and stabilization. It should be emphasized that absolute durations vary significantly with feedstock characteristics, seasonal conditions, fermentation rounds (e.g., 1st-7th round), and spatial position within the matrix. (B) Spatial stratification within the open fermentation stack, driven by inwardly decreasing oxygen and temperature gradients to form distinct surface, middle, and core layers. (C) Vertical functional zonation within the anaerobic fermentation pit, shaped by gradients of temperature, acidity, and the continuous influence of pit mud microbiota.

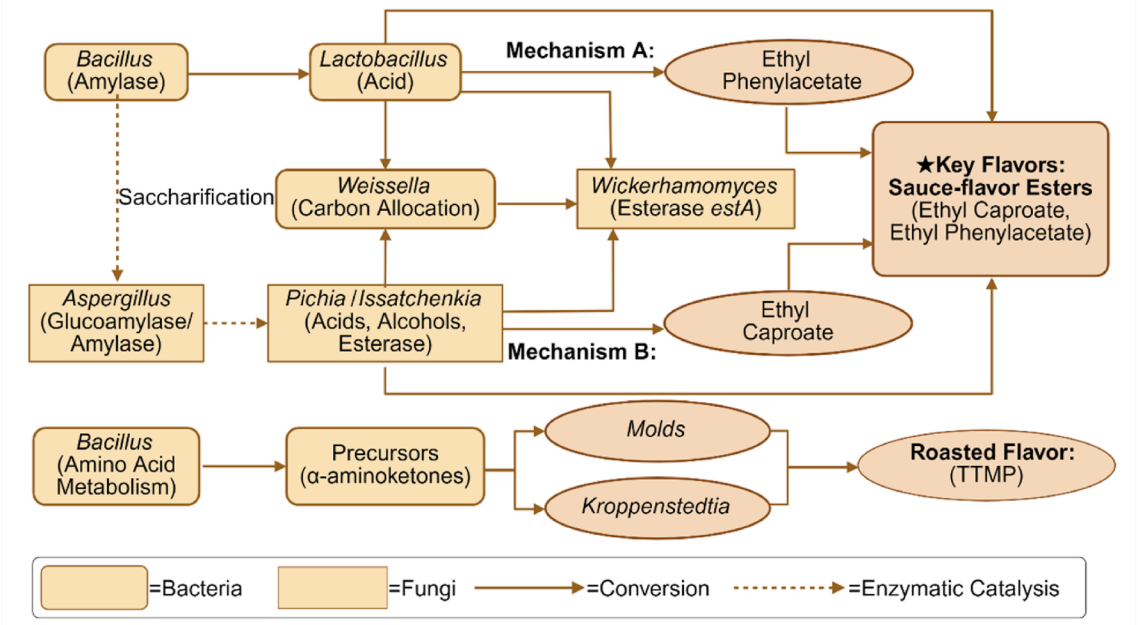

Fig. 3. Metabolic networks and synergistic interactions of core functional microbiota. The diagram illustrates the bioconversion of macronutrients into key flavor compounds through cross-kingdom metabolic relay. It highlights saccharification co-driven by *Bacillus* and *Aspergillus*, distinct ester synthesis pathways (Mechanism A via *Wickerhamomyces* and Mechanism B via *Pichia/Issatchenkia*), and the multi-step synergistic synthesis of the roasted flavor tetramethylpyrazine (TTMP) involving α-aminoketone precursors, *Kroppenstedtia*, and molds.

precursors that are subsequently condensed by *Kroppenstedtia* and molds [22,33,34].

2.3. The epistemic bottleneck: Why bulk methods cannot close the gap

Despite these ecological and metabolic insights, a fundamental composition and function disconnect remains unbridged [4]. Current understandings of microbial networks and keystone taxa are derived primarily from amplicon sequencing, bulk metagenomics, and co-occurrence network analyses [5,35]. These approaches report taxonomic presence and statistical associations, but they fundamentally average signals across heterogeneous micro-niches. They cannot distinguish metabolically active cells from dormant, senescent, or dead populations within the same niche. For instance, observing that *Lactobacillus* exceeds 70% relative abundance in the middle pit layer does not quantify what fraction of these cells is actively producing lactic acid [14,29]. Similarly, network topologies identify highly connected keystone taxa but fail to prove simultaneous *in situ* metabolic activity [36]. Resolving these ambiguities, such as identifying active cells, quantifying single-cell metabolic fluxes, and validating *in situ* syntrophic partnerships, strictly demands analytical tools with true single-cell resolution [14].

3. Single-cell Raman technologies: dissecting function in solid-state matrices

For decades, Baijiu microbiome research has been constrained by a persistent epistemic gap between correlation and causation. While high-throughput sequencing reveals community composition ("Who is there"), it provides only homology-based functional predictions rather than direct measurements of metabolic activity [37]. Cultivation-dependent methods capture merely the minor fraction of "Who can be grown", leaving fundamental questions regarding "Which cells are metabolically active" and "What are their functional phenotypes" unanswered [38].

Several single-cell technologies have been developed to close this gap, as summarized in Table 1. Single-cell transcriptomics (scRNA-seq) offers genome-wide expression profiling but requires cell lysis and is heavily biased toward organisms with well-annotated reference genomes, limiting its utility in poorly characterized, highly diverse communities [39]. Nanoscale secondary ion mass spectrometry (NanoSIMS) achieves sub-micrometre spatial resolution for isotope tracing, yet demands ultra-high vacuum conditions, destructive sample preparation, and extremely low throughput, making large-scale community surveys impractical [40]. Fluorescence-activated cell sorting (FACS) provides high throughput, yet its dependence on fluorescent reporters or stains introduces labelling artefacts and is particularly challenging when applied to cells embedded in grain-based matrices with strong autofluorescence [41]. Among these platforms, Single-Cell Raman Spectroscopy (SCRS) is uniquely suited to the constraints of solid-state fermentation. As a label-free, non-destructive, culture-independent technique with true single-cell resolution (0.5-1.0 μm), SCRS enables the direct coupling of genotype, phenotype, and metabolic activity within intact fermentation ecosystems without requiring genetic modification or exogenous probes [42]. Its compatibility with downstream sequencing (via scRACS-Seq) and cultivation (via scRACS-Culture) further enables a seamless phenotype-to-genotype and phenotype-to-isolate workflow that no other single-cell platform currently provides in a unified pipeline [43]. Nonetheless, limitations should be acknowledged: the throughput of spontaneous Raman imaging remains orders of magnitude lower than that of FACS, and its spatial resolution, diffraction-limited at approximately 0.5 μm, cannot match NanoSIMS for sub-cellular metabolite localisation [44]. Emerging advances in coherent Raman scattering and microfluidic integration are progressively narrowing these gaps [45], but at present SCRS is most accurately positioned not as a universal replacement for existing single-cell platforms, but as the most operationally compatible technology for the unique constraints of solid-state fermentation environments [46].

However, even for SCRS, the direct application to solid-state Sauce-flavor Baijiu fermentation presents distinct challenges when compared to liquid-phase systems. These matrix-associated hurdles include high particle loads (sorghum husks, starch granules) that entrap cells and confound optical detection [47]; intense fluorescence from Maillard reaction products; and pronounced spatial heterogeneity, such as temperature and oxygen gradients in stacks, and vertical stratification of pH and redox potential in pits [48,49]. Furthermore, extreme physiochemical stressors compromise cell viability, and metabolically active cells frequently represent a minor fraction of the total biomass. The specific technical challenges encountered in this complex ecosystem, along with the corresponding methodological adaptations required for successful *in situ* analysis, are systematically detailed in Table 2. The subsequent sections delineate the manner in which SCRS technologies address these constraints, with each tool explicitly mapped to specific research bottlenecks.

3.1. D_2O-Raman: mapping the active minority

To address the critical bottleneck of distinguishing metabolically active cells from dormant or dead populations in high-fluorescence matrices and identifying rare active taxa that frequently evade bulk detection, D_2O-Raman labeling is employed to resolve metabolic activity at the single-cell level. In Moutai Daqu fermentation, this approach successfully distinguished metabolically active *Bacillus* cells (exhibiting strong C-D peaks) from dormant spores in high-temperature (55 °C) samples, revealing that only 15-30% of detected cells were actively dividing despite high total cell counts [43]. Metabolically active cells incorporate deuterium from environmental D_2O into newly synthesized biomacromolecules such as lipids and proteins, generating distinct C-D bond characteristic peaks (2040-2300 cm^{-1}) within the Raman "silent region" (1800-2800 cm^{-1}) [54]. This culture-independent method enables the differentiation of active, dormant, and dead cells with high

Table 1
Comparison of single-cell technologies for microbiome research in solid-state fermentation.

Technology	Primary Capability	Key Limitations in Solid-State Matrices (e.g., Baijiu)	Suitability for Baijiu Ecosystem	Ref.
scRNA-seq (Single-cell transcriptomics)	Genome-wide expression profiling	Requires cell lysis; heavily biased toward organisms with well-annotated reference genomes	Low: Ineffective for poorly characterized, highly diverse "dark matter" communities	[39]
NanoSIMS	Sub-micrometre spatial resolution for isotope tracing	Demands ultra-high vacuum conditions; destructive sample preparation; extremely low throughput	Low: Impractical for large-scale, dynamic community surveys	[40]
FACS (Fluorescence-activated cell sorting)	High-throughput single-cell sorting	Dependence on fluorescent reporters introduces labelling artefacts; strong interference from matrix autofluorescence	Moderate/Low: Severely limited by the intense autofluorescence of grain-based matrices	[41]
SCRS (Single-Cell Raman Spectroscopy)	Label-free, non-destructive, culture-independent metabolic phenotyping with true single-cell resolution (0.5-1.0 μm)	Throughput is lower than FACS; spatial resolution is lower than NanoSIMS; susceptible to high particle loads (requires adaptation)	High: Uniquely suited; seamlessly couples *in situ* phenotype to genotype/isolate without exogenous probes	[42]

Table 2
Application of single-cell Raman technologies in Sauce-flavor Baijiu.

Technology	Target Resolution/ Output	Throughput	Time-to-Result	Target Application in Baijiu	Key Matrix Challenge	Required Adaptation Strategy	Ref.
SCRS	Whole-cell metabolic phenotype	Low	Fast (Seconds/cell)	Rapid phenotyping of dominant microbiota	High fluorescence (from Maillard melanoidins & humic acids)	SERDS optimization, laser photobleaching, ML background correction	[50–55]
D_2O-Raman	Metabolic Activity (Active vs. Dormant)	Low	Medium (Requires D_2O incubation)	Identifying rare active cells in high-fluorescence pit mud	Strong spatial heterogeneity	Micro-scale sampling grids, *in situ* condition preservation	[50, 56–58]
scRACS-Seq	Phenotype + Genotype	Low	Slow (Sorting + WGA + Sequencing)	Linking active "dark matter" to spatial niches	High particle load (cell entrapment in sorghum husks)	Differential centrifugation, enzymatic digestion	[47,56, 57,59, 60]
scRACS-Culture	Phenotype + Viable Isolate	Low	Slow (Sorting + Cultivation days)	Targeted recovery of rare active yeasts (e.g., under heat stress)	Extreme physiochemical stress (viability loss during extraction)	Rapid processing workflows, protective microencapsulation	[42,51, 61]
IRCA	Predictive Kinetic Modeling	N/A (Analytical Framework)	Real-time prediction (post-modeling)	Predicting flavor flux (e.g., TTMP) prior to accumulation	Kinetic misalignment (intracellular vs. extracellular lag)	Time-series co-profiling, kinetic mathematical modeling, PLS-R	[44,62]
FlowRACS	High-throughput Phenotype Sorting	High (Up to 600 events/ min)	Fast (Continuous flow)	Large-scale screening of high-performance starter cultures	High particle load (Microfluidic channel clogging)	Size-selective filtration, pDEP parameter optimization	[47,52, 59]
SCIVVS	Viability & Vitality Profiling	Medium	Rapid (~5 h total)	Rapid, culture-independent yeast quality control in Daqu	Cell fragility in high solid content	Optimization of flow conditions to minimize shear damage	[50,52]

precision, making it particularly effective for high-fluorescence environments like pit mud where traditional optical detection is often confounded [54].

Regarding Baijiu-specific adaptations, melanoidins from Maillard reactions and humic acids in pit mud generate intense background fluorescence. Methodological solutions include Shifted-Excitation Raman Difference Spectroscopy (SERDS) with optimized wavelength shift (e.g., 2.4 nm at 830 nm excitation) [55]; brief laser pre-exposure to quench autofluorescence; and machine learning-based spectral reconstruction following SERDS subtraction [63]. These adaptations enable direct visualization of metabolic activity in high-fluorescence pit mud and anaerobic core layers, where acid-tolerant bacteria predominate.

3.2. scRACS-seq: function-to-genome linkage

A persistent challenge in microbiome research is linking phenotypic activity to genomic identity for rare, uncultured populations, the so-called functional "dark matter" absent from cultivation-based databases. Raman-activated cell sorting combined with sequencing (scRACS-Seq) addresses this gap by establishing a direct phenotype-to-genome linkage at single-cell resolution [42,56,60]. In a proof-of-concept study on phosphate-solubilizing bacteria in wastewater, scRACS-Seq linked high metabolic activity phenotypes to specific genomic identities, yielding isolates with 3-fold higher solubilization efficiency than conventional plating methods [42]. This is particularly relevant for Sauce-flavor Baijiu, where metabolically active cells often constitute only a minor fraction of the total biomass. By identifying microbial populations whose specific metabolic activities correlate with spatial niche differentiation, scRACS-Seq provides the physiological evidence needed to elucidate how individual phenotypes contribute to community-scale processes.

For Baijiu-specific adaptations, the approach involves micro-scale sampling with preservation of *in situ* physiochemistry across steep spatial gradients, followed by gentle pre-enrichment to remove coarse particles without compromising cellular integrity. Post-sorting, rigorous whole-genome amplification is performed with stringent contamination controls [42]. This strategy enables the identification of microbial populations whose metabolic activities correlate with spatial niche differentiation. Rather than attributing niche formation to individual cells, scRACS-Seq provides physiological evidence at the single-cell level that, when integrated with community-level ecological data, helps elucidate how individual metabolic phenotypes contribute to the emergent, community-scale process of niche construction. For example, single cells exhibiting high C-D ratios in specific spatial zones can be linked to localized substrate depletion or metabolite accumulation patterns, thereby connecting cellular-level activity measurements to the community-environment feedbacks that ultimately shape micro-niche architecture [60,64].

3.3. scRACS-culture: targeted recovery of functional dark matter

To overcome the bottleneck of translating identified signals into biological resources and recovering rare but active cells from extreme environments (pH < 3.5, >50 °C) for cultivation and functional validation, scRACS-Culture utilizes a "screen-first, culture-second" strategy. This integrated pipeline combines Raman sorting with Raman-Activated-Gravity-driven Encapsulation (RAGE) to isolate single cells exhibiting high metabolic activity (high C-D ratio) [42,51].

When applied to Moutai high-temperature Daqu [51], this approach revealed that only 10-32% of detected yeast species remained metabolically active under thermal stress, correcting the assumption that abundance correlates with functional contribution. Single-cell phenotyping uncovered a temperature-dependent "metabolic relay": *Saccharomycopsis* and *Wickerhamomyces* predominate at moderate temperatures, while thermotolerant *Pichia* maintains elevated activity during heat stress (45 °C or higher), ensuring fermentation continuity. Analogous strategies isolated phosphate-solubilizing bacteria from wastewater [42], demonstrating broad applicability across diverse matrices.

3.4. IRCA: predictive phenotyping for flavor flux

Aiming to overcome the lag between metabolic activity and extracellular product accumulation and enable real-time process regulation, Intra-Ramanome Correlation Analysis (IRCA) constructs correlation models linking fermentation time, intracellular Raman phenotypes, and extracellular flavor precursors [62]. Because intracellular metabolic shifts serve as leading indicators of extracellular product formation, IRCA captures these early intracellular signals to provide predictive insights. This early warning capacity allows for the timely adjustment of parameters, such as stacking duration or temperature, to optimize conditions for flavor formation prior to product accumulation.

Intracellular storage materials and metabolic intermediates (e.g., starch granules, polyhydroxyalkanoates, proteins, lipids) exhibit specific fingerprint peaks in Raman spectra [65–67]. Alterations in these intracellular fingerprints frequently precede extracellular flavor compound accumulation by hours to tens of hours [44,62], with the exact lag dependent on metabolite class, diffusion rates, and physiological state. He et al. [44] demonstrated in beer fermentation that intracellular Raman spectral evolution precedes extracellular metabolite accumulation by 6-12 h, accurately predicting 19 phenotypes (higher alcohols, esters, amino acids, organic acids, saccharides) and enabling proactive process intervention before flavor deviations manifest. In the context of Sauce-flavor Baijiu, continuous monitoring of Raman signals associated with pyrazine-related metabolic intermediates in *Bacillus* during stacking fermentation can predict the potential for TTMP synthesis before product accumulation becomes detectable by conventional means. This early warning capacity enables timely adjustment of stacking duration or temperature to optimize conditions for pyrazine formation. It should be noted that the IRCA framework has been principally validated in beer fermentation systems [44], and its direct application to the more complex solid-state Baijiu matrix remains to be comprehensively demonstrated. Nevertheless, the underlying principle (that intracellular Raman signatures serve as leading indicators of extracellular metabolite accumulation) is matrix-independent, and preliminary observations in Baijiu-relevant organisms support its transferability.

Regarding Baijiu-specific kinetic challenges, GC-MS relies on delayed extracellular product accumulation, whereas SCRS captures immediate intracellular precursor signals. Kinetic misalignment arises because intracellular Raman signals precede extracellular GC-MS peaks by time lags that depend on metabolite class and diffusion rates. Adaptations include time-series co-profiling with stringent temporal alignment (6-h sampling intervals), kinetic modeling to mathematically determine delay constants, and multivariate calibration (PLSR) against spiked reference compounds in authentic matrices [44,62].

Furthermore, the predictive power of IRCA can be significantly amplified by integrating advanced computational models. While traditional multivariate calibrations like PLSR are effective, the high-dimensional and non-linear nature of Raman spectra in complex solid-state matrices necessitates the application of machine learning (ML) and deep learning algorithms. Convolutional Neural Networks (CNNs) and Random Forest classifiers can be trained on large-scale Ramanome datasets to deconvolve overlapping spectral peaks caused by matrix interference. By coupling these AI-driven spectral decoders with dynamic metabolic flux models, IRCA can transition from simple linear correlations to robust, non-linear predictive engines, enabling real-time, in silico simulation of flavor compound trajectories based on instantaneous single-cell snapshots.

3.5. FlowRACS and SCIVVS: high-throughput and rapid assessment

To meet industrial-scale screening requirements and provide rapid quality control without the delays of cultivation, FlowRACS enables label-free, high-throughput screening of up to 600 events per minute for sorting and up to 3000 events per minute for analysis based on metabolic phenotypes [59,68]. FlowRACS has been applied to screen lipid-rich yeast mutants at a throughput of about 270 events/minute (40 μL/min flow rate, $\sim 6.75 \times 10^3$ cells/mL), isolating strains with 30.85% higher triacylglycerol content than parental lines [68].

SCIVVS (Single-Cell Raman-based Viability and Vitality Profiling) is a rapid, culture-independent method for assessing cell physiological state that utilizes D_2O labeling [50,52]. This workflow, when executed within a 5-h timeframe (excluding the sequencing process), facilitates the execution of species-resolved live-cell counting and metabolic vitality measurement [52]. The method has been demonstrated to detect metabolically active yet non-culturable cells that evade traditional plating. Its application to yeast quality control in Daqu has been validated [50–52].

Regarding Baijiu-specific adaptations, the high solid content and cell fragility necessitate optimization of positive dielectrophoresis (pDEP) parameters and flow conditions to minimize shear damage while preserving sorting efficiency [59].

3.6. Current limitations and future optimization

While the SCRS toolkit provides unique capabilities, targeted development is required for three frontier areas.

Cell extraction without physiological alteration: the physical complexity of fermented grains (Jiupei), namely recalcitrant sorghum husks and dense particulate matter, has been shown to cause significant microbial cell entrapment. Current protocols have been shown to achieve dual release of spores and vegetative cells; however, the efficiency of this process varies according to the specific fermentation stage. In order to meet the needs of the future, it is essential to develop stage-specific protocols and real-time metabolic activity validation via D_2O-Raman. This will ensure physiological preservation.

Standardization and automation: The prevailing SCRS analysis remains operator-intensive, thereby limiting throughput and reproducibility. The following critical needs have been identified: (*i*) automated sample preparation workstations; (*ii*) reference databases of Raman spectra from Baijiu-relevant strains under diverse stress conditions; (*iii*) machine learning classifiers trained on Baijiu-specific spectral features [69–71]; and (*iv*) inter-laboratory validation studies establishing quality control standards.

Integration with industrial Process Analytical Technology (PAT): at-line SCRS modules are required to interface with existing Process Analytical Technology frameworks. Raman spectroscopy has been successfully employed as a PAT tool for real-time monitoring of biomass, metabolites, and substrates in fermentation processes [69,72,73]. The following requirements must be met: (*i*) robust sampling interfaces are required for heterogeneous matrices; (*ii*) rapid sample preparation must be compatible with industrial timelines; (*iii*) data pipelines must translate SCRS phenotypes into actionable process parameters; (*iv*) pilot-scale validation must be conducted in collaboration with production facilities.

4. From single-cell insights to engineered consortia: A DBTL framework for Sauce-flavor Baijiu

Having established the capabilities and current limitations of the SCRS toolkit (Section 3), we now address how these single-cell insights can be systematically translated into engineering outcomes. SCRS and its derivative technologies can resolve metabolic phenotypes, link them to genotypes, and recover functional strains from the complex solid-state matrices of Sauce-flavor Baijiu. However, translating these single-cell insights into tangible improvements in fermentation performance requires a systematic engineering logic. The Design-Build-Test-Learn (DBTL) cycle, originally formalized in synthetic biology for iterative strain and pathway engineering [74], provides such a framework. In the context of Sauce-flavor Baijiu, each DBTL iteration aims to progressively refine a synthetic functional consortium whose metabolic output approximates, or ultimately exceeds, the flavor complexity generated by the native open fermentation community. Ultimately, this framework

serves the grand vision of rationally controlling microbiota in spontaneous food fermentations, forming a continuous, data-driven optimization loop (Fig. 4).

4.1. Design: from Ramanome-guided strain selection to consortium architecture

The Design phase confronts a fundamental tension in synthetic ecology: distilling the metabolic complexity of a native community into a minimal, controllable consortium for spontaneous fermentation. Traditional cultivation-based screening is inherently biased toward fast-growing taxa. SCRS reframes this by defining strains through *in situ* metabolic phenotypes. We propose a multi-tiered selection pipeline: (*i*) Activity gating via D_2O-Raman to identify the metabolically active subpopulation, excluding dormant cells [43,75]; (*ii*) Functional classification using Ramanome fingerprints (e.g., starch, lipid, or protein biomarkers) to map phenotypic guilds [43]; (*iii*) Rarity assessment to prioritize highly active but low-abundance taxa via scRACS-Culture, capturing trace aroma producers often lost in conventional enrichment [42].

Drawing on "defined community" principles [76], a minimal functional consortium (e.g., 10-15 species) should satisfy three criteria: metabolic coverage, metabolic complementarity, and ecological robustness. To achieve true rational design, this phenotypic selection must be integrated with computational systems biology. Genomic data acquired via scRACS-Seq provides the blueprint to reconstruct Genome-Scale Metabolic Models (GEMs) for each candidate strain. Rather than relying on empirical assumptions, researchers can utilize these GEMs, alongside IRCA-derived phenotype correlation networks [62], to perform in silico simulations of cross-feeding networks and metabolic bottlenecks. This ensures the selected strains exhibit synergistic rather than competitive interactions, while maintaining functional redundancy at critical trophic nodes to withstand fluctuating pit conditions.

Finally, determining initial inoculation ratios shifts from empirical mixing to a rigorous computational optimization problem. We propose a "metabolic-vitality-weighted" design approach. Specifically, quantitative metabolic activity indices (e.g., C-D ratios) derived from D_2O-Raman serve as in vivo physiological constraints for Flux Balance Analysis (FBA). By employing dynamic FBA (dFBA) on the constructed GEMs, researchers can computationally predict the optimal initial stoichiometry that maximizes flux toward target flavor compounds (e.g., pyrazines or esters). For instance, rate-limiting taxa (e.g., pyrazine-producing Bacillus) can be rationally over-represented based on model predictions, while fast-growing fermenters are inoculated at lower ratios. This approach fully leverages SCRS data to construct a predictive and robust consortium architecture, establishing a rational starting point that will be continuously refined by the subsequent Test and Learn phases. This approach fully leverages SCRS data to construct a predictive and robust consortium architecture, establishing a rational starting point that will be continuously refined by the subsequent Test and Learn phases. The rational design of minimal functional consortia for extreme environments further draws on principles established in thermophilic synthetic microbial community engineering, where metabolic complementarity and functional redundancy at critical trophic nodes are essential for consortium stability under thermal stress [77].

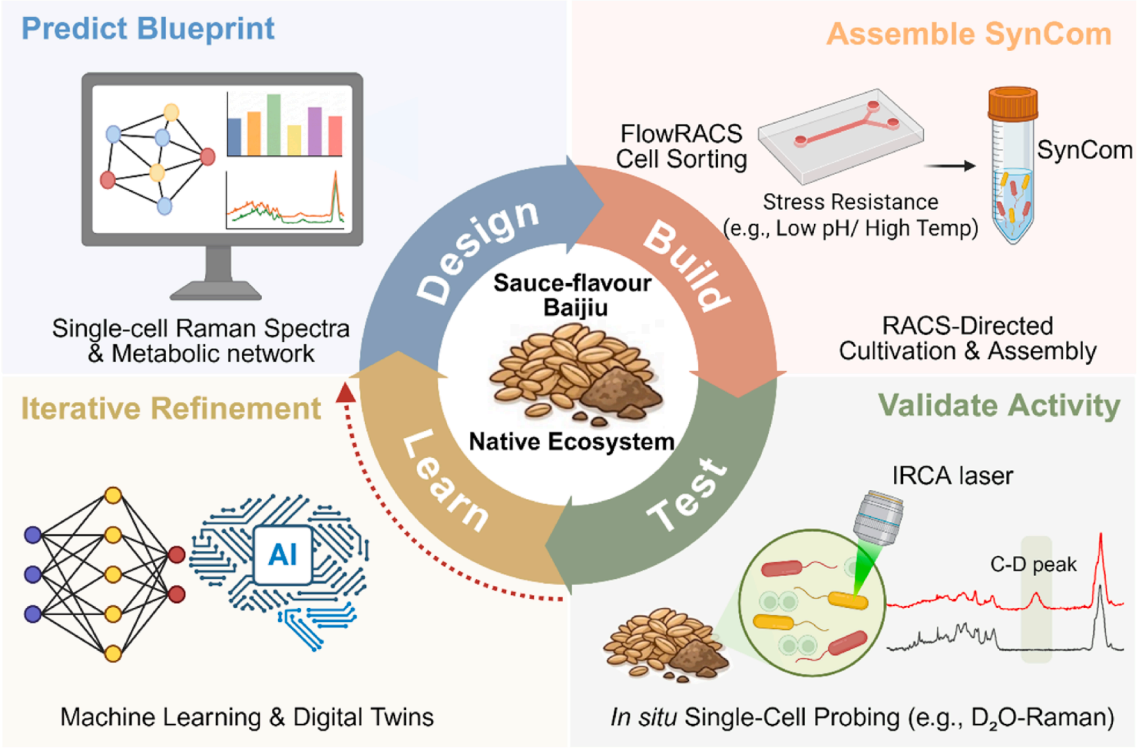

Fig. 4. The DBTL framework for rational microbiome engineering in Sauce-flavor Baijiu fermentation. The cycle integrates single-cell Raman spectroscopy (SCRS) technologies to overcome the unique constraints of complex solid-state environments (high stress and high heterogeneity). Design (Predict Blueprint): Single-cell Raman spectra and metabolic networks guide the in silico modeling to predict the blueprint of the target synthetic consortium (SynCom). Build (Assemble SynCom): FlowRACS cell sorting and RACS-directed cultivation are used to assemble the SynCom, selectively recovering resilient cells capable of withstanding extreme physicochemical stresses (e.g., low pH and high temperature). Test (Validate Activity): *In situ* single-cell probing (e.g., D_2O-Raman equipped with an IRCA laser) validates the real-time metabolic activity of the introduced SynCom within the native ecosystem by tracking distinct spectral markers, such as the C-D peak. Learn (Iterative Refinement): Extracted data are used to train machine learning models and construct Fermentation Digital Twins. This creates a data-driven feedback loop for the iterative refinement of the SynCom architecture and macroscopic parameters for the next design cycle.

4.2. Build: RACS-directed cultivation and assembly for bioaugmentation

Constructing a Synthetic Consortium (SynCom) for open, extreme environments faces a critical bottleneck: the "potential-to-function gap" [78]. Traditional cultivation often yields strains that possess the genomic potential but fail to perform *in situ*. Therefore, the "Build" phase must pivot from merely assembling isolates to engineering viable and functional SynComs explicitly designed for bioaugmentation. Importantly, given stringent regulatory frameworks for traditional fermented foods [79] and consumer acceptance criteria [80], this phase prioritizes rational assembly and adaptive laboratory evolution of wild-type strains, avoiding extensive genetic engineering.

scRACS-Culture and advanced cell sorting platforms (e.g., FACS) are indispensable here. They facilitate the targeted recovery of rare yet highly active "microbial dark matter" that retains metabolic vigour under the intense physicochemical stresses (high ethanol >10% v/v, pH < 3.5, temperature >50 °C) [42]. A critical innovation at this stage is the use of microencapsulation technologies, such as RAGE, which encapsulates sorted single cells in hydrogel microdroplets. This provides a protective micro-niche that prevents fragile, stress-adapted cells from lysing or entering viable-but-non-culturable (VBNC) states upon abrupt transfer to artificial media [81]. However, RAGE exhibits low throughput (~2 events/minute) [60,81], necessitating a tiered assembly strategy: FlowRACS for primary broad-spectrum enrichment (~300-600 events/minute) [59], RAGE reserved for rare, high-value isolates, and standard cultivation for biomass generation.

Once stable isolates are recovered, the physical assembly of the SynCom relies on establishing robust "metabolic handoffs". Guided by the in silico models developed in the Design phase, the Build phase physically co-cultures specific partners, such as pairing an amylolytic *Bacillus* strain sorted for rapid starch degradation with a highly active *Wickerhamomyces* yeast sorted for robust esterase activity. Crucially, before deployment, these engineered assemblies must undergo "pre-adaptation". Rather than being cultivated solely in liquid broth, the SynCom is sequentially adapted in progressively complex matrices (from liquid media to sterilized solid-state sorghum grains) [82]. This ensures that the synergistic interactions and spatial organization within the SynCom are fully established and phenotypically stable prior to facing the hostile Daqu or Jiupei environment.

4.3. Test: in situ validation of colonization and emergent metabolism

Testing engineered SynComs under isolated laboratory conditions, such as liquid shake-flask cultures or sterilized grain substrates, is fundamentally insufficient for open, solid-state fermentation systems. The reason is twofold. First, laboratory setups eliminate the very biotic pressures that define the real fermentation niche: intense colonization resistance from the indigenous microbiota, competitive exclusion via nutrient sequestration and antimicrobial metabolite secretion, and physical barriers imposed by the dense, heterogeneous solid-state matrix itself [78]. Second, simplified media fail to replicate the steep physicochemical gradients of temperature, moisture, acidity, and oxygen availability that characterize Daqu and Jiupei environments, gradients that exert strong selective pressures on microbial survival and metabolic expression [83]. A SynCom that performs optimally on defined media may therefore collapse rapidly once introduced into an ecologically saturated and thermally dynamic fermentation stack. Consequently, the true "Test" phase within the DBTL cycle must shift from controlled monoculture assessment to direct, ecologically realistic evaluation of the survival, spatial colonization, metabolic viability, and functional output of bioaugmented strains within the native Daqu or Jiupei ecosystem.

Before introduction into the actual Jiupei, the pre-adapted SynCom must pass quantitative readiness benchmarks defined relative to the in silico model predictions from the Design phase (Section 4.1) and native community baselines, rather than universal constants. The first criterion is survival and metabolic viability, assessed via D_2O-Raman profiling as the percentage of cells exhibiting the active C-D vibrational band relative to the total sampled population: if profiling of the native community reveals that 20-40% of dominant taxa maintain metabolic activity under characteristic pit stressors (pH 3.0-3.5, accumulating ethanol, 40-45 °C) [25,51], the SynCom must achieve a comparable or higher active fraction to ensure competitive viability. The second is compositional stability, requiring that the consortium resist competitive exclusion and maintain a stable species ratio close to the FBA-optimized design over sustained solid-state co-cultivation, verified through strain-specific quantitative PCR or single-cell Raman fingerprint classification via FlowRACS. The third is enzymatic retention, demanding that target extracellular enzyme activities such as amylase and esterase remain at levels comparable to or exceeding those of native Daqu, detected using standard colorimetric assays on extracellular extracts from the solid-state sorghum matrix [84].

Once a SynCom satisfying these pre-deployment criteria is introduced into the fermentation stack, comprehensive *in situ* validation requires a dual-domain monitoring strategy bridging microscopic cellular behavior and macroscopic fermentation outcomes. On the solid-phase side, spatially resolved SCRS profiling tracks the intracellular metabolic status of individual cells across different niches within the Daqu or Jiupei matrix. On the liquid-phase side, metabolomic profiling of the fermentation liquid (Huangshui) via GC-MS or HPLC captures the cumulative extracellular flux of flavor-active compounds. Correlating these two datasets enables researchers to verify whether the intracellular metabolic shifts detected by SCRS effectively translate into the desired macroscopic product profiles [44,62], thereby closing the loop between single-cell phenotype and process-level performance.

SCRS combined with D_2O-Raman isotope probing addresses this need by providing a label-free, non-destructive, and genuinely *in situ* verification method [46]. By tracking the intensity and kinetics of C-D band emergence at the single-cell level, researchers can determine whether introduced functional taxa, for example, high-pyrazine-producing *Bacillus* spp. or ester-synthesizing yeasts, have successfully colonized the target micro-niche, maintained active anabolism under the prevailing physicochemical stresses, and overcome competitive exclusion by resident populations [43,57,85]. Beyond simple survival confirmation, Raman spectral profiling enables a deeper layer of metabolic inference. The broader Ramanome of each cell, encompassing bands associated with carotenoids, cytochromes, poly-hydroxyalkanoates, amide conformations, and other biomolecular signatures, provides a phenotypic fingerprint that can be compared against reference spectra collected during the "Build" phase. Significant deviations may indicate that the introduced strain has shifted its metabolic programme in response to ecological interactions, such as substrate competition, cross-feeding, or quorum-sensing crosstalk with indigenous species, a phenomenon best described as emergent metabolism arising from community context rather than monoculture genetics alone. This definitive validation separates active functional contributors from merely persisting dormant DNA [62].

In summary, the integration of SCRS and D_2O-Raman isotope probing into the Test phase transforms validation from a correlative exercise reliant on DNA detection and bulk chemistry into a mechanistic, spatiotemporally resolved assessment of single-cell function within an ecologically intact fermentation matrix. The resulting data, documenting which introduced strains are active, where they reside, and how their metabolism responds to community context, feed directly into the subsequent Learn phase, closing the DBTL loop with empirical evidence of unprecedented cellular resolution.

4.4. Learn: data-driven process control and iterative optimization

Derived performance data from *in situ* SCRS probing must inform subsequent design iterations to close the DBTL loop. Cross-system insights from gut and soil microbiome studies offer transferable principles for community assembly [86,87]. However, Baijiu fermentation is distinctly oriented towards the domain of secondary metabolism and the

regulation of flavor flux, underscoring the necessity for a learning framework that directly couples cellular physiology with macroscopic process control [88,89].

Collectively, the phenotypic data streams generated through iterative DBTL cycles, including single-cell metabolic activity indices (C-D ratios from D_2O-Raman), intracellular precursor kinetics (IRCA-derived temporal profiles), and *in situ* survival rates, constitute the high-resolution biological input necessary for constructing "Fermentation Digital Twins". Without such cell-resolved functional data, digital twin models remain constrained to physicochemical proxies (temperature, pH, moisture content) that capture process symptoms rather than underlying biological causation.

The integration of fermentation liquid data significantly enhances the predictive power of the "Learn" phase. Digital twins reach optimal accuracy when they are constrained by both high-resolution single-cell Ramanomes and absolute metabolite concentrations from the liquid phase. This multi-scale data fusion ensures that the re-design of consortium architectures is grounded in an exhaustive understanding of both metabolic potential and actual chemical execution within the heterogeneous fermentation matrix.

This multi-scale data fusion ensures that the re-design of consortium architectures is grounded in an exhaustive understanding of both metabolic potential and actual chemical execution within the heterogeneous fermentation matrix. The construction of Fermentation Digital Twins and the deployment of machine learning for iterative consortium optimization align with broader strategies in fermentation intelligence, where ML-driven process design translates high-dimensional biological data into actionable control parameters [90].

Data-to-design translation mechanisms operationalize this phase. For instance, when ML models detect premature yeast dormancy induced by acidification, they trigger dual-pathway corrections: (*i*) Consortium re-design, tightening FlowRACS gating for acid-tolerant fungi or reducing bacterial-to-fungal inoculation ratios; and (*ii*) Process adjustment, via digital twins recommending modified stacking height or pit-entry moisture to optimize oxygen and thermal profiles [91]. This closed-loop system converts single-cell phenotypes into continuous optimization of both SynCom architecture and process parameters.

The integration of SCRS-derived phenotypic streams with ML algorithms creates a powerful, predictive feedback loop [91]. As a conceptual illustration, consider a scenario where *in situ* D_2O-Raman testing reveals that an engineered ester-producing yeast population enters premature dormancy due to unexpectedly rapid lactic acid accumulation in the pit [51,60], the ML algorithm captures this specific phenotypic shift [46,61]. The "Learn" module then directly informs the next "Design" cycle to either mathematically adjust the initial *Lactobacillus*-to-yeast inoculation ratio to delay the acidification peak, or prompts the "Build" phase to re-sort for a more acid-tolerant yeast variant using tightened FlowRACS gating parameters [68].

Ultimately, this data-driven feedback loop transforms empirical troubleshooting into a predictive science, iteratively refining both the SynCom architecture and the macroscopic fermentation controls (e.g., stacking temperature, moisture content). By converting centuries of tacit brewing knowledge into computable, mechanistic datasets [9,14], the Learn phase establishes the definitive bridge from single-cell analytical dissection to fully controllable intelligent brewing [91].

5. Future directions: critical challenges for intelligent brewing

Transitioning from single-cell dissection to data-driven, intelligent brewing requires overcoming fundamental technical and conceptual barriers. Although the DBTL framework offers a structured approach, four interconnected challenges require ongoing attention.

Technological maturation. Current SCRS applications face substantial constraints imposed by the complex Baijiu matrix, including melanoidin-induced fluorescence interference, elevated particulate loads derived from sorghum husks, and extreme physicochemical conditions (pH < 3.5, >50 °C) [92]. Methodological adaptations initially developed at the proof-of-concept scale, specifically SERDS protocols and protective-agent microencapsulation, now require comprehensive standardization and automation to ensure industrial-grade reliability [61].

Process integration. It is imperative that at-line SCRS modules exhibit robust interfacing capabilities with existing Process Analytical Technology frameworks [93]. It is imperative to address the following critical needs: the development of sampling interfaces for heterogeneous matrices that do not disrupt fermentation processes [94]; the creation of kinetic models that correlate instantaneous Raman signals with delayed metabolite accumulation [95,96]; and the establishment of data pipelines that translate phenotypes into actionable parameters that are compatible with industrial timelines [97].

Synthetic ecology validation. Synthetic consortia, which are assembled through activity-based sorting, must maintain functionality against competitive native microbiota in open solid-state systems [79,98]. The validation of scale, the regulatory frameworks that have been established, and the strategies that have been developed for consumer acceptance must be developed in parallel with technical advances [80].

Cultural-technological balance. The ultimate measure of success is not determined by the extent of technical sophistication; rather, it is defined by the preservation of the heritage of empirical craftsmanship [9,10]. Single-cell technologies are designed to function as decoding tools, thereby rendering traditional fermentation practices comprehensible and transferable across generations. Rather than serving as replacement technologies, these tools are intended to complement human expertise [14,38]. The sensory assessment conducted by the master brewer plays a pivotal role in the quality evaluation process [9,10]. The SCRS provides a comprehensive biological vocabulary that enables the articulation and substantiation of this expertise [4,14,53].

It is evident that no single discipline can adequately address these intertwined challenges independently. It is imperative for optical physicists to advance Raman signal recovery from high-fluorescence matrices. In addition, microbial physiologists must map single-cell phenotypes to volatile flavor outcomes. Furthermore, AI practitioners must build interpretable models that distill mechanistic insight from centuries of empirical craftsmanship.

6. Conclusions

The transition from empirical craftsmanship to intelligent brewing in Sauce-flavor Baijiu necessitates a fundamental shift from descriptive cataloging to mechanistic control of the fermentation microbiome. This review establishes that the core functional community, while shaped by conserved environmental filtering, is driven by intricate spatiotemporal heterogeneity and cross-kingdom metabolic relays that define the spirit's aromatic complexity.

We demonstrate that Single-Cell Raman Spectroscopy (SCRS) and its derivative toolkit (e.g., D_2O-Raman, scRACS-Seq, scRACS-Culture, and IRCA) provide the essential "biological vocabulary" to bridge the long-standing composition-function disconnect. By enabling label-free metabolic phenotyping, phenotype-to-genome linkage, and the targeted recovery of "functional dark matter" directly from complex solid-state matrices, these technologies transform our understanding of microbial activity from population averages to single-cell-resolved functional insights.

The integration of these single-cell insights into a systematic Design-Build-Test-Learn (DBTL) framework provides a rational pathway for microbiome engineering. This iterative cycle facilitates the assembly of functional synthetic consortia and the construction of Fermentation Digital Twins grounded in mechanistic causation. Ultimately, SCRS does not seek to replace the heritage of traditional craftsmanship; rather, it decodes and preserves the tacit knowledge of master brewers,

establishing a data-driven foundation for a new era of predictable, controllable, and mechanistically informed intelligent brewing.

Author contributions

J.Z. designed and led this study. X.S., X.Y., and J.Z. performed all the analyses and wrote the manuscript. All the authors approved the final manuscript.

Funding

This work was supported by the National Key Research and Development Program of China (Grant No. 2022YFF0713104), the National Key Research and Development Project of China (2023YFA0916100), and the National Science Foundation of China (31701569).

Declaration of competing interest

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

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