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Review

Microbial Community Assembly, Interactions, and Flavor Formation During Huangjiu Fermentation: Current Understanding and Future Perspectives

Guangfa Xie [1], Caiqin Liu [1], Chao Han [1] and Xutao Hong [2,*]

[1] Zhejiang Collaborative Innovation Center for Full-Process Monitoring and Green Governance of Emerging Contaminants, School of Biological and Environmental Engineering, Zhejiang Shuren University, Hangzhou 310015, China; xiegf@zjsru.edu.cn (G.X.)
[2] Zhejiang California Nano-System Institute, Shaoxing Branch, Shaoxing 312000, China
* Correspondence: hongxt@zju.edu.cn

Abstract

Huangjiu (Chinese rice wine) is brewed through open mixed-culture fermentation in which saccharification and alcoholic fermentation proceed concurrently. Although multi-omics surveys have cataloged diverse microbial taxa and volatile compounds across regional starters (wheat Qu, Jiuyao, Xiaoqu, and Hongqu), translating taxonomic inventories into reproducible process control remains challenging. This narrative review connects process-dependent community assembly with a critical evaluation of observational multi-omics surveys, controlled functional interventions, computational metabolic predictions, and biochemical validation, while comparing reconstructed consortia across substrate pretreatments, sensory endpoints, strain safety, and experimental scales. Starter manufacture supplies both viable inocula—spanning filamentous fungi (*Aspergillus*, *Rhizopus*, *Monascus*), amylolytic and fermentative yeasts (*Saccharomycopsis*, *Saccharomyces*, non-*Saccharomyces* spp.), and bacteria (*Saccharopolyspora*, *Bacillus*, and lactic acid bacteria)—and preformed extracellular hydrolases that decouple early substrate release from contemporaneous microbial growth. Subsequent succession is shaped by thermal regimes, organic acid identity (e.g., lactate versus succinate), ethanol stress, colonization order (priority effects), and solid–liquid spatial heterogeneity. Cross-kingdom and intra-bacterial interactions govern the partitioning of grain-derived carbon and nitrogen into ethanol, organic acids, esters, and higher alcohols, while influencing safety-related metabolites including biogenic amines, ethyl carbamate precursors, purines. Finally, we evaluate environmental steering, bioaugmentation, and reconstructed synthetic consortia, outlining experimental strategies to bridge laboratory correlation with industrial fermentation predictability.

Keywords: Huangjiu (Chinese rice wine); microbial community assembly; priority effects; microbial interactions; flavor metabolism; synthetic microbial consortia; food fermentation safety

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1. Introduction

1.1. Huangjiu as a Traditional Mixed-Culture Fermentation System

Huangjiu is a family of cereal wines produced through mixed microbial fermentation. Saccharification and alcoholic fermentation are coupled: starter enzymes release sugars from grain while yeasts convert them into ethanol. Wheat Qu, Hongqu and regional Xiaoqu or Jiuyao introduce fungi, yeasts and bacteria together with accumulated enzymes [1,2].

Hydrolysis also supplies amino acids and aroma precursors. Fermentation consequently depends on the timing of resource release and consumption from the solid starter into the developing mash.

Cereals, starter preparation and brewing practices contribute to Huangjiu styles. Open or semi-open operations connect the inoculum with the production environment, while transfers, mixing and temperature management change microbial habitats [1,2]. These features link inheritance with environmental selection. Maintaining product consistency therefore requires attention to microbial functions, the enzyme reservoir and brewing conditions alongside the regional practices that sustain distinct flavors.

1.2. Progress and Limitations of Current Microbiological Research

Research on Huangjiu microbiology has expanded from inventories of microorganisms and flavor compounds toward the functions connecting them. Existing reviews describe contributions from starters, raw materials and brewing technology [1,2]. Metagenomic comparisons of successful and spoiled fermentations further link bacterial profiles with quality differences [3]. Across fermented foods, amplicon sequencing describes community patterns, shotgun metagenomics identifies encoded functions, and transcriptional or biochemical measurements address expression and activity [4,5]. These observations become ecologically informative when they explain how organisms establish, encounter partners and use the resources available during a particular process.

Tian et al. reviewed Huangjiu brewing, microbiota, flavor, safety, and microbial relationships [6]. Zhang et al. subsequently organized omics-guided defined consortia across fermented foods around core and supplementary microbiomes and an Assembly–Assessment–Redesign workflow [7]. These contributions already establish the importance of interactions and rational consortium design. The present review extends that foundation through a Huangjiu-specific comparison of three connected questions: how viable starter populations and inherited extracellular enzymes interact with process-dependent assembly; which interaction claims are supported by observations, interventions, predictions, or direct mechanistic tests; and what reconstructed communities achieve under their particular substrate treatments, sensory assessments, safety constraints, and experimental scales. The missing connection is often between a community pattern and a reproducible fermentation response. The same organism may benefit a partner in one medium and inhibit it in another because resource supply, acid exposure and local exchange differ. Reviews of fermented-food ecology identify competition, facilitation and metabolite exchange as conditional processes [6–8]. Huangjiu therefore needs comparisons that retain the biological context of an interaction: starter cultivation, mash fermentation and storage expose organisms to different substrates and stresses. Connecting treatment, recipient response and biochemical activity gives such studies value beyond another list of taxa associated with desirable compounds. It also makes transferability an explicit question: a useful effect in broth must persist under the conditions of the intended brewing stage.

This synthesis asks which detected organisms remain metabolically active after starter transfer, whether proposed donor–recipient exchanges operate in fermenting mash, how physical matrix structure and arrival order modulate partner compatibility, and which laboratory outcomes persist across independent brewing batches. By anchoring these ecological questions to the specific substrates, starter enzyme backgrounds, and validation scales of individual Huangjiu studies, this review complements broader fermented-food frameworks with brewing-specific mechanistic and translational criteria [6–8].

1.3. Objectives and Scope of This Review

This review examines how process-dependent assembly creates opportunities for microbial interactions and how those interactions affect flavor and quality. Recruitment, environmental selection and colonization history connect the organisms entering a fermentation with the functions expressed there [9–11]. Spatial organization determines which resources and partners those organisms encounter [12]. Together, these concepts organize comparisons among starter manufacture, mash fermentation and storage without treating them as one ecological setting.

The literature was identified through Web of Science Core Collection, Scopus, PubMed, and ScienceDirect and updated through 15 October 2026. Searches combined Huangjiu and starter-specific terms (wheat Qu, Maiqu, Jiuyao, Xiaoqu, and Hongqu) with descriptors covering microbial community assembly, succession, interspecies interactions, flavor metabolomics, fermentation safety (biogenic amines, ethyl carbamate, purines, and mycotoxins), and synthetic microbial consortia; selection emphasized original Huangjiu studies pairing community dynamics with measured biochemical activity or experimental intervention (Supplementary Note S1, supplied as a separate file). Studies of other fermented foods provide explicitly identified ecological or methodological comparisons within this narrative review. Because this work is a question-driven narrative synthesis, studies were selected and compared according to process stage, starter type, experimental intervention, and measured chemical or sensory endpoints, with divergent findings across studies interpreted in the context of strain identity, inoculum density, medium composition, enzyme supplementation, and sampling stage.

Because Huangjiu investigations span diverse analytical designs, findings throughout this review are weighed according to the level of causal resolution they provide. Observational amplicon or metagenomic surveys, co-occurrence networks, and genome-scale metabolic models identify candidate taxa, encoded pathways, and putative cross-feeding routes, whereas metatranscriptomic, metaproteomic, and enzymatic assays capture active physiological expression during starter maturation or mash fermentation. Establishing how specific organisms or environmental variables drive fermentation outcomes further requires controlled perturbations—such as pairwise co-cultures, arrival-order manipulations, strain omissions, or synthetic community reconstructions—paired with targeted biochemical or isotope-tracing validation when molecular transfer mechanisms are proposed. This comparative perspective connects microbial assembly, conditional interactions, and reproducible metabolic functions across the brewing process. Tables 1 and 2 classify the primary evidence for each reported finding into four functional categories: observational association or descriptive activity profiling (OA), experimental functional effect under a controlled intervention (EF), computational prediction (CP), and direct mechanistic validation (MV). Within this framework, transcriptional and enzymatic measurements provide evidence of gene expression and enzyme activity, while confirming partner-specific metabolite transfer or production-scale robustness further depends on targeted biochemical tracing, matched controls, and multi-batch validation.

Table 1. Representative studies on microbial sources, community assembly, and interspecies interactions in Huangjiu fermentation.

System and Stage	Organisms/ Guilds	Design and Comparison	Principal Ecological or Functional Findings	Evidence Class and Experimental Scope	References
Wheat Qu; mechanized Shaoxing mash	*Saccharopolyspora hirsuta* J2; *Saccharomyces cerevisiae* HJ	Meta-omics; isolation; biofortified-Qu brewing	*Saccharopolyspora* persists from wheat Qu into mash and supplies α-amylase, glucoamylase, and cellulase that support yeast alcoholic fermentation.	OA: source-community and meta-omics profiles; EF: isolate enzyme assays and biofortified-Qu brewing. Demonstrates hydrolytic support under the tested brewing conditions.	[13,14]
Shaoxing Jiuyao; 0–192 h	*Rhizopus*; *Saccharomycopsis*; *Bacillus*; *Aspergillus*; *Pediococcus*; *Weissella*	Amplicons; metatranscriptomics; enzyme assays	Liquefying and acid protease activities peak at 192 h; *Rhizopus* dominates amylase and glucoamylase transcription alongside *Saccharomycopsis* and *Bacillus*.	OA: time-resolved starter transcripts and enzyme activity assays capturing active hydrolysis during Jiuyao maturation.	[15]
Raw wheat Qu; surface/core and moisture	*Aspergillus*; *Thermomyces*; *Bacillus*; *Staphylococcus*	Spatial sampling; moisture treatments; community profiles	Surface *Aspergillus* and core *Thermomyces* populations partition along oxygen and metabolic heat gradients during raw Qu incubation.	OA: surface–core community profiles along oxygen and thermal gradients; EF: initial-moisture perturbation during raw Qu incubation.	[16]
Gutian/Wuyi Hongqu; Hongqu/Maiqu mash	*Monascus*; *Aspergillus*; *S. cerevisiae*; *Lactiplantibacillus*	Comparative metagenomics; volatile profiles	Starter provenance and Hongqu versus Maiqu functional gene repertoires establish distinct hydrolytic potentials and fungal-to-yeast transitions.	OA: comparative starter and mash metagenomics and volatile profiling across regional Hongqu and Maiqu brewing protocols.	[17,18]
Winery reservoirs; jar/tank mash	*Saccharopolyspora*; *Saccharomyces*; *Lactiplantibacillus*	10-winery, four-season survey; comparative metatranscriptomics	Winery equipment and surfaces harbor fermentation-associated taxa year-round; ceramic jars and mechanized tanks exhibit distinct glycolytic transcript profiles.	OA: four-season winery surface survey and comparative metatranscriptomic profiling of ceramic-jar versus mechanized-tank brewing.	[19,20]

Table 1. *Cont.*

System and Stage	Organisms/ Guilds	Design and Comparison	Principal Ecological or Functional Findings	Evidence Class and Experimental Scope	References
Black glutinous rice wine; 36, 120, 240 h	*S. cerevisiae*; *Rhizopus*; *Lactiplantibacillus*	Time-resolved metatranscriptomics; volatile profiles	*Saccharomyces*-dominated RNA profiles shift sequentially from amino acid metabolism (36 h) to glycolysis (120 h) and autophagy (240 h).	OA: time-resolved metatranscriptomic and volatile profiling demonstrating functional reprogramming within a numerically dominant genus.	[21]
Pre-colonization; supernatant/lees agar	*Lactiplantibacillus plantarum*; *Limosilactobacillus fermentum*; *S. cerevisiae*	25 LAB combinations; arrival order; supplementation; matrix comparisons	Selected LAB pre-colonization increases *S. cerevisiae* biomass by >30% and elevates free amino acids; pairwise LAB interactions are more inhibitory on lees agar than in liquid supernatant.	EF: arrival-order, co-culture, and amino acid supplementation assays across 25 synthetic LAB combinations in liquid supernatant versus lees agar.	[22]
Hongqu mash; single-acid broth assays	*Lactiplantibacillus*; *S. cerevisiae*	Starter comparison; acid dose–response	Mechanized Hongqu starter promotes LAB expansion, higher titratable acidity, and lower ethanol; in rice broth, 40 g/L lactate inhibits yeast whereas 50 mg/L succinate increases dry mass by 11%.	OA: mechanized versus traditional Hongqu mash comparison; EF: rice-broth single-acid dose–response assays (40 g/L lactate and 50 mg/L succinate).	[23,24]
Huangjiu LAB serial-transfer cultures	*Pediococcus pentosaceus* 1-D7; *L. plantarum* 271; *Weissella paramesenteroides* W; *Levilactobacillus brevis* 367	Cyclic versus transitive trio; spent media; metabolite assays	Cyclic three-species LAB consortium persists for ~200 generations, with nutrient-use and lactate-uptake observations suggesting possible explanations; substituting *L. brevis* 367 causes transitive exclusion (<8%).	EF: serial-transfer persistence and cyclic versus transitive species replacement; OA: spent-medium and metabolite-utilization profiles.	[25]

Table 1. *Cont.*

System and Stage	Organisms/ Guilds	Design and Comparison	Principal Ecological or Functional Findings	Evidence Class and Experimental Scope	References
Stepwise LAB additions to mold/yeast	*Aspergillus oryzae*; *S. cerevisiae*; added LAB	Reconstructed cultures; extracellular assays; metabolic model	Stepwise LAB addition raises extracellular phenylalanine from 19.00 ± 1.01 to 235.38 ± 10.47 mg/L and lactate from 3.47 to 6.26 g/L; metabolic model predicts 972 candidate exchanges including yeast α-keto acid supply.	EF: measured extracellular amino acid and lactate shifts after stepwise LAB additions; CP: 972 genome-scale model-predicted metabolite exchanges.	[26]

Note: Studies are grouped by starter system, brewing stage, and experimental design. Abbreviations: LAB, lactic acid bacteria; Qu, solid-state fermentation starter. Evidence categories: OA, observational association or descriptive activity profiling; EF, experimental functional effect under a controlled intervention; CP, computational prediction; MV, direct mechanistic validation.

Table 2. Experimental strategies for targeted microbial community regulation, bioaugmentation, and synthetic consortia in Huangjiu fermentation.

Strategy and Target	Manipulation	Substrate, Scale and Comparator	Quantitative Metabolic, Sensory and Safety Outcomes	Evidence, Sensory Endpoint, and Validation Scale	References
Starter biofortification; hydrolysis	*Saccharopolyspora rosea* A22; cultivation temperature	Raw wheat Qu; factory-Qu brewing reference	Increases core heating (52 °C at 36 h) and selected Qu hydrolase activities; resulting wine retains more reducing sugar and lower ethanol than factory-Qu wine.	EF: starter biofortification and laboratory brewing evaluation; highlights need to match starter enzyme gains with downstream mash consumption.	[27]
Environmental regulation; ester profile	28/28/20 °C benchmark; late stage 15 °C	Controlled rice mash; temperature-schedule controls	Late cooling to 15 °C increases long-chain FAEEs from 0.544 to 1.157 mg/L (+112.7%) while lowering terminal ethanol; 28/28/20 °C benchmark retains optimal sensory balance.	EF: controlled mash temperature scheduling evaluated by targeted ester quantification and trained sensory panel.	[28]
Nutrient regulation; recipient growth	Glutamine; Arg; Asp; Glu; equimolar Asp + Glu	Traditional starter mash, day 3; Huangjiu-derived broth; supplementation controls	Glutamine reduces neutral-model fit and alters volatile esters; Arg or equimolar Asp + Glu promotes yeast growth under lactate stress whereas Asp or Glu alone is inhibitory.	EF: amino acid supplementation and arrival-order assays in broth and day-3 starter mash; CP: neutral-community-model fit.	[22,29]

Table 2. *Cont.*

Strategy and Target	Manipulation	Substrate, Scale and Comparator	Quantitative Metabolic, Sensory and Safety Outcomes	Evidence, Sensory Endpoint, and Validation Scale	References
Selective yeast inoculation; sensory targets	*Rhodotorula mucilaginosa* H04 + *S. cerevisiae* SC01; *S. cerevisiae* SC-6	Mash; single-yeast or high-bitterness controls	Sequential H04/SC01 inoculation enhances fruity and honey notes and reduces alcoholic harshness; SC-6 modulates bacterial succession and lowers delayed bitterness.	EF: sequential and single-strain yeast bioaugmentation evaluated by volatile profiling and sensory panel; H04 requires strain-level safety qualification.	[30,31]
Bioaugmentation; selected metabolites	*Wickerhamomyces anomalus*; *Pantoea ananatis*	Fangxian mash with resident microbiota; bioaugmentation comparison	Co-addition elevates γ-aminobutyric acid (GABA) and creatine while mash chemical profiles converge across treatments.	EF: bioaugmentation and targeted metabolite enrichment within endogenous mash microbiota; *P. ananatis* requires strain-level food-safety verification.	[32]
Reconstruction; hydrolysis and fermentation	8-member Jiuyao consortium; omission-guided selection and assembly	1 kg steamed rice; traditional Jiuyao control; no exogenous enzymes	Reduces final total sugar to 1.81 ± 0.38 g/L (61.57% below traditional Jiuyao control) with higher ethanol and elevated saccharifying, protease, and cellulase activities.	EF: omission-guided reconstruction and 1 kg steamed-rice fermentation demonstrating self-sustained hydrolysis without exogenous enzymes.	[33]
Reconstruction; aroma retention	9 species retained from an 11-member candidate community	Rice medium; commercial α-amylase/glucoamylase pretreatment; larger assemblage reference	Retains principal volatile compounds and the sensory attributes evaluated by a trained panel after omitting two redundant species from the candidate consortium.	EF: bottom-up/top-down reconstruction in enzyme-pretreated rice medium evaluated by volatile profiling and a 12-member trained sensory panel.	[34]
Reconstruction and preservation; starter formulation	6-species artificial starter; optimized lyoprotectant	100 g rice, 30 h; traditional Jiuyao and commercial starter controls	Shows a similar ethanol trajectory and E-nose profile of traditional Jiuyao, while yielding 39 detected volatiles compared with 50 in traditional-starter wine.	EF: 100 g, 30 h laboratory fermentation evaluated by ethanol kinetics, GC-MS volatiles (39 vs. 50 compounds), and E-nose fingerprinting.	[35]

Note: Reported batch sizes and durations reflect the experimental scales tested in the cited studies. Abbreviations: Arg, arginine; Asp, aspartate; Glu, glutamate; GABA, γ-aminobutyric acid; FAEEs, fatty acid ethyl esters; E-nose, electronic nose. Evidence categories follow Table 1 (OA, EF, CP, MV). Analytical volatile profiles, E-nose fingerprints, trained-panel evaluations, and consumer acceptance represent distinct sensory endpoints, and the reconstructed consortia summarized here represent laboratory-scale demonstrations prior to multi-batch industrial validation.

2. Microbial Sources and Community Assembly in Huangjiu Fermentation

2.1. Microbial Sources and Initial Community Establishment

A Huangjiu starter supplies both a viable microbial inoculum and a biochemical legacy of substrate transformation. Organisms capable of establishing in mash arrive alongside extracellular hydrolases accumulated during solid-state starter cultivation, and these two contributions operate on distinct timescales. In commercial wheat Qu, abundant actinomycetes such as *Saccharopolyspora* extend the hydrolytic community beyond the filamentous fungi traditionally associated with starch liquefaction [13]. Shaoxing Jiuyao instead pairs *Rhizopus* and *Saccharomycopsis* with lactic acid bacteria (LAB), notably *Pediococcus* and *Weissella* [15]. These starter systems introduce distinct combinations of polymer-degrading and fermentative guilds into the mash (Table 1).

Differences among starter types reflect both their cultivation substrates and the enzymatic repertoires they assemble. In Jiuyao, transcriptional and enzymatic profiles link the maturing fungal–bacterial consortium directly to starch-liquefying and proteolytic capacities, whereas comparative metagenomics of wheat Qu and Baijiu Daqu reveals shared carbohydrate- and amino-acid-metabolic potential despite divergent taxonomic composition [15,36]. In traditional Fangxian Xiaoqu manufacture, placing seed Qu within or around rice-flour doughs propagates adapted microbiota across consecutive production cycles [37]. Hongqu systems introduce further regional differentiation: *Monascus* and *Saccharomyces* predominate in Gutian brewing series, whereas certain Wuyi starters are dominated by *Aspergillus* [17], and parallel Hongqu and Maiqu fermentations establish distinct hydrolytic and volatile trajectories under their respective brewing protocols [18]. Across these systems, initial mash performance reflects the combined inheritance of viable starter populations and preformed extracellular enzymes.

Botanical additives and winery environments further shape the initial microbial pool. Adding *Polygonum orientale* L. flowers during Fangxian Xiaoqu preparation modifies microbial diversity and elevates acid protease activity while preserving core dominant genera [38]. In open or semi-open brewing workshops, equipment and facility surfaces act as persistent microbial reservoirs: a four-season survey across ten Shaoxing wineries detected fermentation-associated bacteria and yeasts on tools and ground surfaces throughout the year [19]. Tracking strain-level overlap between these environmental reservoirs, starters, and fermenting mashes is therefore essential to distinguish ambient recruitment from starter propagation.

2.2. Microbial Succession Across Fermentation Stages

Microbial succession begins during solid-state starter preparation, where metabolic heat and moisture loss progressively remodel the growth matrix. During raw wheat Qu incubation, shifts in temperature, moisture, and oxygen availability drive coordinated bacterial and fungal replacement [16]. Similarly, Shaoxing Jiuyao maturation couples community succession with enzyme accumulation: liquefying amylase and acid protease activities peak at 192 h, supported by dominant *Rhizopus* amylase and glucoamylase transcription alongside contributions from *Saccharomycopsis*, *Bacillus*, and *Aspergillus* [15]. When the mature starter is pitched into steamed rice mash, both the selected microbial populations and the accumulated enzyme pool enter a high-moisture, oxygen-limited habitat where early saccharification can proceed even before newly inoculated cells reach peak biomass.

Once mash fermentation begins, ecological succession follows physiological resource transitions rather than a single chronological schedule across Huangjiu styles. Quantitative profiling of Hakka Huangjiu captured shifting *Saccharomycopsis* and *Rhizopus* gene abundances alongside an early *Pediococcus* expansion during saccharification, whereas an in-

dependent Hakka production series highlighted prominent non-*Saccharomyces* yeasts [39,40]. In longer Hongqu and Maiqu brewing cycles, succession is marked by expanding *Saccharomyces* and declining *Aspergillus* populations [18], while starter-borne *Saccharopolyspora* persists from wheat Qu into mechanized Shaoxing fermentation mashes [13,14]. Functional succession also occurs when a single genus remains numerically dominant: in black glutinous rice wine, *Saccharomyces* dominated RNA-assigned communities throughout fermentation while shifting transcription from early amino acid metabolism (36 h) to mid-stage glycolysis (120 h) and late autophagy-related pathways (240 h) [21]. Community trajectories thus encompass stage-specific metabolic reprogramming as well as taxonomic turnover (Figure 1; Table 1).

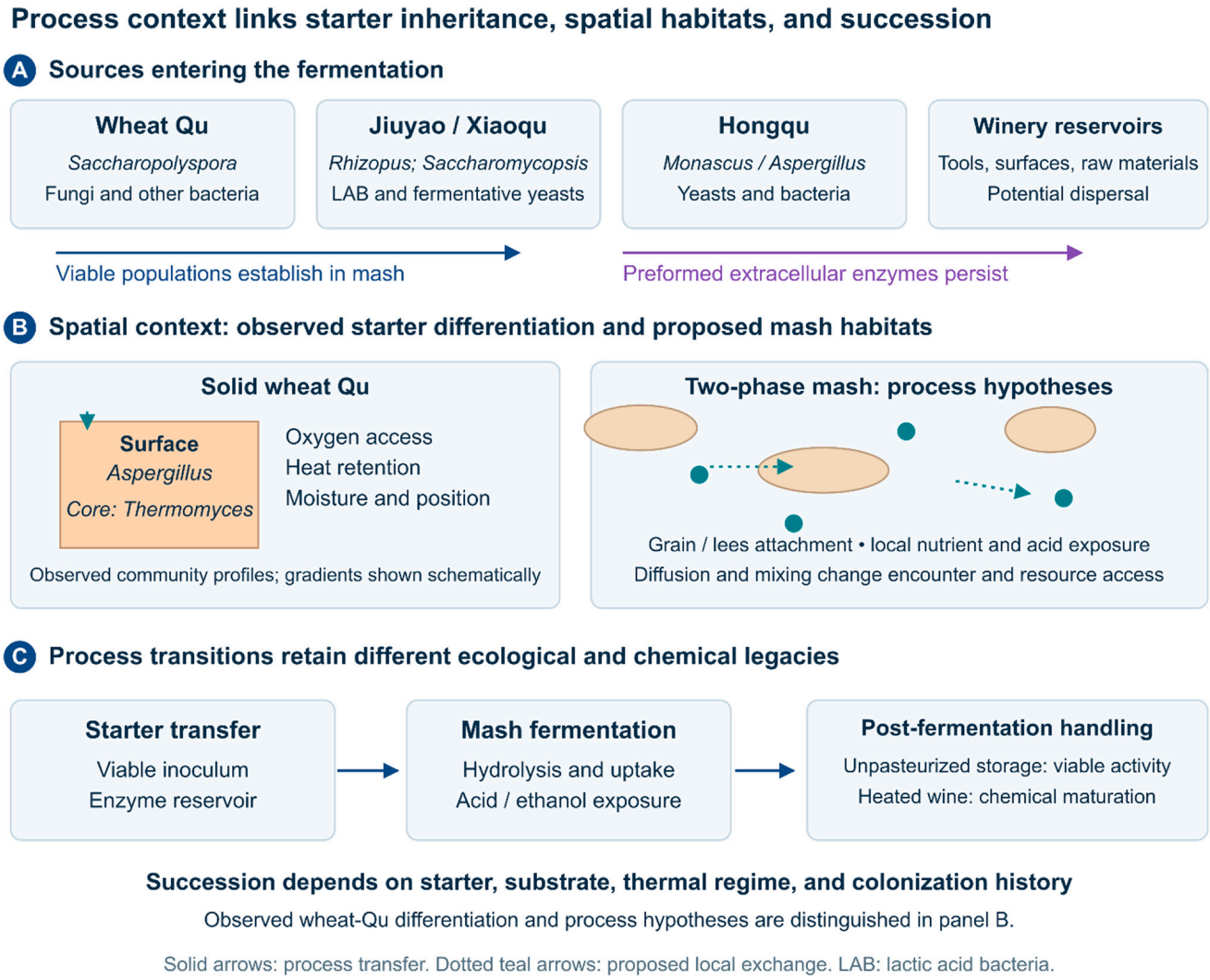


Figure 1. Ecological framework of microbial sources, spatial microenvironments, and stage transitions in Huangjiu community assembly. (**A**) Regional starters—including wheat Qu, Jiuyao/Xiaoqu, and Hongqu—introduce distinct guilds of filamentous fungi, yeasts, and bacteria alongside preformed extracellular hydrolases into the mash, while winery tools, surfaces, and raw ingredients represent potential environmental reservoirs [19]. (**B**) Spatial habitats in solid wheat Qu and two-phase mash: observed surface-associated *Aspergillus* and core-associated *Thermomyces* profiles motivate a process model connecting oxygen access, heat retention, grain/lees attachment, and local resource exchange [16]. (**C**) Coupled substrate hydrolysis, microbial uptake, and metabolite accumulation drive physiological succession across mash fermentation, followed by post-fermentation differentiation between unpasteurized fresh-wine cold storage and post-sterilization chemical maturation [2,13,15,20,41]. Solid navy and purple arrows indicate process transfer of viable populations and

preformed enzymes, respectively; dotted teal arrows indicate proposed local exchange in mash; tan ovals and teal dots in panel (B) schematically represent suspended grain/lees particles and microbial cells, respectively. LAB, lactic acid bacteria. Panel (**B**) distinguishes observed Qu differentiation from proposed mash microenvironments; panel (**C**) summarizes process transitions whose timing and community trajectories vary among brewing systems.

Brewing vessel geometry and mechanization further modulate these successional trajectories. Parallel comparisons of traditional ceramic-jar and mechanized tank fermentations revealed distinct thermal histories, mixing regimes, and glycolytic transcription profiles alongside diverging microbial succession [20]. In Hongqu rice wine, substituting mechanized starter for traditional starter accelerated saccharification but promoted excessive LAB proliferation and mash acidification [23]. Rapid sugar release in modified processes can therefore shift the competitive balance between bacterial acidogenesis and yeast alcoholic fermentation.

2.3. Environmental Filtering and Ecological Drivers

Environmental filtering and microbial niche construction act reciprocally during brewing because early metabolic activity reshapes the physicochemical habitat encountered by subsequent populations [9,11]. In biofortified wheat Qu, inoculating *Saccharopolyspora rosea* A22 intensified core self-heating (reaching 52 °C at 36 h) and accelerated *Saccharopolyspora* dominance, while controlled incubation-temperature comparisons differentially selected bacterial and fungal guilds [27]. During liquid mash fermentation, staged temperature scheduling similarly steered bacterial succession toward *Lactiplantibacillus* dominance and altered downstream ester accumulation [28]. Together, these studies demonstrate how endogenous metabolic heat during starter cultivation and exogenous thermal control during mash fermentation act as primary physical filters.

Organic acid accumulation imposes a compound-specific chemical filter on mash communities. While rapid LAB expansion in mechanized Hongqu brewing elevated titratable acidity and suppressed ethanol yield, controlled dose–response assays in rice broth demonstrated that yeast physiology depends on both acid identity and concentration [23,24]. In the reported single-acid assay, 40 g/L lactic acid strongly inhibited *Saccharomyces cerevisiae*, whereas 50 mg/L succinic acid increased dry cell mass by 11% at 48 h relative to the unsupplemented control (Table 1). Because these single-acid assays were conducted in sterilized, agitated rice medium (30 °C, 180 rpm), translating their thresholds to solid–liquid Huangjiu mashes requires accounting for local pH buffering, ethanol accumulation, and substrate heterogeneity.

Physicochemical inputs from brewing water, exogenous agrochemical residues, and recipe formulation further modify habitat selectivity. Adjusting initial moisture during raw wheat Qu preparation or varying regional water chemistry in Shaoxing brewing alters both substrate dissolution and community structure [16,42]. Controlled spiking experiments also show that agrochemical residues such as dinotefuran act as selective pressures that perturb fungal–bacterial succession and volatile generation relative to untreated control mashes [43]. Similarly, substituting brewing water with finished wine in Jiafan processes reshapes sugar and ethanol baselines alongside starter-associated enzymatic and volatile profiles [44].

2.4. Priority Effects, Spatial Structure, and Assembly Mechanisms

Colonization history shapes community assembly through priority effects, in which early-arriving populations modify resource availability or inhibitory conditions for subsequent colonists [9,10]. In Huangjiu-derived media, pre-colonization by individual LAB isolates frequently suppressed subsequent *S. cerevisiae* growth, whereas a defined pre-culture

combining *Lactiplantibacillus plantarum* and *Limosilactobacillus fermentum* elevated extracellular free amino acids and increased yeast biomass by more than 30% [22] (Table 1). These contrasting outcomes demonstrate that early bacterial establishment can either facilitate or impede yeast fermentation depending on resident strain composition and pre-fermentation nutrient remodeling.

Physical matrix structure governs the spatial scale over which such interactions operate [12]. In solid wheat Qu blocks, limited gas diffusion and internal heat retention partition aerobic *Aspergillus* populations to the surface and thermotolerant *Thermomyces* to the core, accompanied by depth-dependent bacterial distributions [16]. In two-phase rice mashes, suspended grain solids and lees similarly create micro-niches with distinct local nutrient and metabolite concentrations before mechanical mixing or pressing homogenizes the liquid phase. Attachment to grain particles and lees separates immobilized populations from freely suspended cells, where polymer accessibility, restricted diffusion, and spatial proximity between hydrolytic producers and fermentative consumers modulate local substrate supply and acid or ethanol exposure [12,16]. Because bulk pH and metabolite measurements average across these microhabitats, combining depth- or phase-resolved sampling with matched-matrix perturbations will clarify how spatial heterogeneity shapes mash succession alongside the established surface–core differentiation in wheat Qu.

Viewed through the ecological framework of dispersal, selection, diversification, and drift [9], current Huangjiu evidence highlights strong deterministic filtering driven by temperature, acidity, and nutrient supply. For example, exogenous glutamine supplementation on day 3 of fermentation reduced neutral-community-model fit across both bacterial and fungal assemblages, consistent with strengthened nutritional selection [29]. In traditional grain vinegar fermentation, ecological null modeling further revealed distinct assembly partitioning between abundant and rare bacterial sub-communities [45], suggesting that separately tracking dominant fermenters and low-abundance accessory taxa could refine assembly models in Huangjiu.

3. Microbial Interactions and Ecological Relationships

3.1. Interactions Between Filamentous Fungi and Yeasts

Cross-kingdom hydrolytic complementarity between filamentous fungi, amylolytic yeasts, and fermentative yeasts forms the biochemical backbone of Huangjiu brewing. Secretome profiling of *Aspergillus oryzae* SU16 in Shaoxing koji identified abundant α-amylases, glucoamylases, and acid proteases [46], while metatranscriptomic tracking of Jiuyao confirmed coordinated starch-degrading gene expression by *Rhizopus* and *Saccharomycopsis* [15]. These extracellular depolymerases convert intact cereal starch and storage proteins into maltose, glucose, peptides, and free amino acids assimilated by *S. cerevisiae* (Figure 2; Table 1). Because preformed starter hydrolases remain catalytically active after transfer into mash, initial sugar and amino nitrogen release is partially decoupled from the immediate growth rate of the enzyme-producing molds.

Synthetic omission experiments demonstrate that individual hydrolytic partners contribute differentially to fermentation completion and volatile formation. In a reconstructed Jiuyao community, omitting *Rhizopus delemar*, *Saccharomycopsis fibuligera*, or *S. cerevisiae* significantly reduced saccharifying and proteolytic activities as well as ethanol output [33]. In a separate pour-over rice-wine consortium, omitting *Rhizopus arrhizus* lowered terminal ethanol concentration without significantly altering the principal aroma compounds [47]. Evaluating fungal–yeast complementarity therefore requires monitoring both bulk substrate conversion and secondary volatile profiles across the complete fermentation.

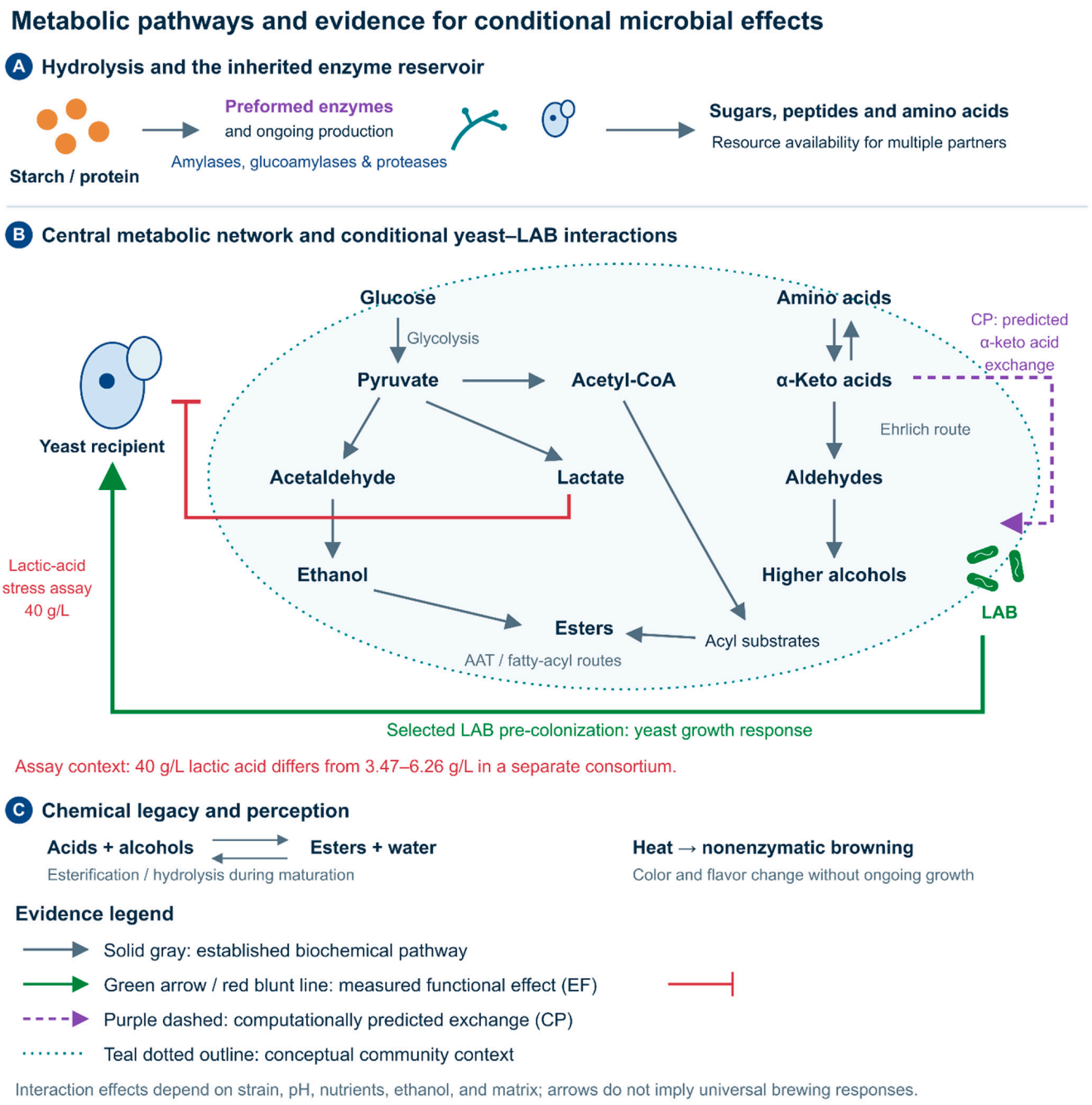


Figure 2. Metabolic resource coupling, conditional yeast–bacterial interactions, and chemical maturation in Huangjiu flavor formation. (**A**) Preformed starter hydrolases and enzymes secreted by growing molds, amylolytic yeasts, and actinomycetes hydrolyze cereal starch and proteins into fermentable sugars, peptides, and free amino acids [15]. (**B**) Central metabolic network linking glycolysis, pyruvate partitioning, acetyl-CoA-mediated ester synthesis, and Ehrlich-pathway higher alcohol formation with experimentally tested yeast–LAB interactions: selected LAB pre-colonization promotes subsequent *S. cerevisiae* growth (green arrow) [22], a 40 g/L lactic-acid challenge inhibits yeast growth in rice-broth assays (red blunt line, distinct from the 3.47–6.26 g/L lactate range measured in reconstructed co-cultures) [24,26], and genome-scale metabolic modeling predicts candidate yeast-to-LAB α-keto acid provision (purple dashed arrow) [26]. (**C**) Non-enzymatic esterification, ester hydrolysis, and thermally induced browning continue to reshape volatile and color profiles during post-fermentation processing and aging [2,34]. Solid gray arrows denote established biochemical pathways; green arrows and the red blunt line denote experimentally observed functional effects (EF); the purple dashed arrow denotes a computationally predicted exchange (CP); the dotted teal outline denotes the community context. AAT, alcohol acetyltransferase; LAB, lactic acid bacteria.

3.2. Interactions Between Yeasts and Lactic Acid Bacteria

Interactions between yeasts and lactic acid bacteria shift dynamically as fermentable sugars are depleted and organic acids accumulate. Overlapping requirements for carbon and assimilable nitrogen generate competition, whereas bacterial proteolysis or metabolite secretion can facilitate yeast physiology under moderate acidity [6,8]. As shown by single-acid dose–response assays in rice broth, 4 g/L lactic acid did not significantly impair *S. cerevisiae* proliferation, whereas 40 g/L lactic acid strongly inhibited yeast growth [24]. Notably, the 40 g/L lactic acid challenge represents a severe stress level compared with the lower lactate concentrations measured in multi-species co-cultures (Section 3.4), underscoring that organic acid inhibition in brewing depends on undissociated acid fraction, mash pH, ethanol co-stress, and nutrient buffering [22–24].

Physical matrix structure and colonization order further modulate yeast–LAB compatibility. Pairwise assays among Huangjiu-derived LAB isolates revealed a higher frequency of growth-promoting interactions in liquid culture supernatant than on solid lees-containing agar, showing matrix-dependent interaction outcomes [12,22]. When yeast was introduced after bacterial pre-colonization, selected *L. plantarum* and *L. fermentum* combinations enhanced yeast biomass recovery, and targeted amino acid additions modulated yeast tolerance to lactic acid stress (Table 1). Because culture supernatant and lees-based agar differ simultaneously in physical immobilization, mass diffusion, and retained lees metabolites, pairing pH- and nutrient-matched controls with controlled mixing or particle-attachment assays will help decouple physical matrix structure from chemical composition.

3.3. Interactions Among Bacteria and Other Functional Microorganisms

Bacterial coexistence can depend on the organization of competition, not simply on the prevalence of positive pairwise effects [8,22]. A Huangjiu-derived cyclic trio of *Pediococcus pentosaceus* 1-D7, *L. plantarum* 271 and *Weissella paramesenteroides* W persisted through approximately 200 generations; replacing *Weissella* with *Levilactobacillus brevis* 367 produced a transitive configuration in which the weak competitor fell below 8% [25] (Table 1). Nutrient profiles, lactate consumption and spent-medium responses suggested resource-based explanations, while aspartate supplementation inhibited all three members. Persistence therefore arose in a community containing competitive relationships, and the benefit of a resource depended on the recipient and conditions rather than its general classification as a nutrient.

Intraspecific genotypic diversity can further reshape bacterial interaction networks without altering species-level taxonomic inventories. In chemically defined co-cultures of *Lactobacillus acetotolerans* isolated from cereal fermentations, distinct conspecific genotype groups exhibited asymmetric fitness responses during co-cultivation [48]. This observation underscores why strain-resolved selection is critical when assembling functional bacterial consortia for traditional fermented beverages [8,25,48].

Bacterial contributions also connect starter preparation with downstream resource use and safety. *Saccharopolyspora hirsuta* J2 cultivated in wheat Qu supported a later *S. cerevisiae* HJ fermentation, extending hydrolytic complementarity beyond fungal partners [13] (Table 1). A different design combined low-amine-producing *L. plantarum* ACBC271 with amine-degrading *Staphylococcus xylosus* CGMCC1.8382 and reduced amines relative to uninoculated fermentation and either single inoculation [49]. The first system evaluates a prepared starter's resource contribution; the second joins complementary strain phenotypes affecting undesirable products. Both illustrate why bacterial selection should begin with the function needed in the full fermentation rather than a uniform label of cooperation.

3.4. Metabolic Cross-Feeding and Functional Complementarity

Metabolic cross-feeding links community diversity to the extracellular pool of amino acids and organic acids [8,22]. In reconstructed mold–yeast–bacterial fermentations, stepwise addition of LAB species to an *A. oryzae* and *S. cerevisiae* baseline elevated extracellular phenylalanine from 19.00 ± 1.01 to 235.38 ± 10.47 mg/L and final lactate from 3.47 to 6.26 g/L, while genome-scale metabolic modeling predicted 972 directional metabolite exchanges, including yeast-to-LAB α-keto acid supply [26] (Figure 2; Table 1). Here, the elevated extracellular amino acid and lactate pools provide experimental functional evidence (EF) at the consortium level, whereas the 972 modeled exchanges represent computational predictions (CPs) of candidate donor–recipient pathways whose net flux and directionality in mash require confirmation via targeted uptake perturbation or stable-isotope tracing.

Targeted nutrient supplementation and spent-medium assays help dissect how specific nitrogen sources mediate these partner responses. In Huangjiu-derived broth, exogenous arginine or an equimolar mixture of aspartate and glutamate promoted *S. cerevisiae* growth and alleviated lactic acid stress, whereas aspartate or glutamate supplied individually inhibited yeast proliferation [22]. This non-linear mixture effect explains why total free amino nitrogen alone cannot predict yeast fermentation performance. Similarly, computationally guided LAB pairings from fermented foods supported recipient growth in spent-medium assays under nutrient-limited or osmotically stressed conditions [50].

Complementary substrate utilization and organic acid turnover also stabilize multispecies bacterial coexistence in Huangjiu [22,25,26]. Because conditioned media simultaneously alter pH, carbon availability, and inhibitory byproducts, pairing pH-matched controls with time-resolved metabolite tracking and stable-isotope labeling offers a rigorous pathway to confirm directional cross-feeding in complex brewing matrices.

3.5. Methodological Evidence for Microbial Interactions

Different experimental designs resolve different parts of an interaction [5,33]. Profiling twelve Fangxian Xiaoqu preparations associated communities with enzyme activities and wine composition, identifying candidates for functional tests [51]. Co-culture compares a partner's effect with monoculture; omission tests its contribution among remaining community members. Both require attention to total inoculum and potential compensation. A changed product after omission supports a dependency under the tested conditions, while add-back and targeted loss-and-rescue experiments can examine whether a specific function explains it. These comparisons separate a useful community phenotype from the stronger claim of molecular necessity.

Aligning ecological terminology with experimental design strengthens comparative synthesis across fermented-food studies [8]. Functional complementarity describes partner contributions to an aggregate process outcome (such as coupled starch hydrolysis and ethanol formation), facilitation denotes a verified fitness or growth benefit to a recipient population, and mutualism requires reciprocal benefits to both partners under the same brewing conditions. While metabolite supplementation and genome-scale metabolic models generate testable hypotheses, spatial metabolomics and purified-metabolite bioassays—such as the demonstration that fungal penicillic acid inhibits *Brachybacterium alimentarium* in cheese-rind communities [52]—illustrate how chemical imaging and targeted perturbations can resolve micro-scale interaction mechanisms in solid and semi-solid fermentations [9].

Across these studies, interaction outcomes are conditional on the resource pool and habitat in which partners meet. Enzyme carryover supplies an initial resource contribution; nutrient mixtures, acid concentration and local retention modify later responses; arrival order determines the conditions already present when a recipient establishes. A strain combination can consequently support fermentation without reciprocal mutualism, or

remain persistent despite competition. This context explains why a favorable broth response may change after transfer to grain mash and why useful consortia must be compared by both partner responses and product outcomes.

4. Microbial Functions and Flavor Formation

4.1. Starch and Protein Hydrolysis as Metabolic Foundations

Enzymatic depolymerization of cereal starch and proteins governs the rate at which fermentable carbon and assimilable nitrogen enter microbial metabolism. Liquefying α-amylases and saccharifying glucoamylases release maltooligosaccharides and glucose, while acid proteases and carboxypeptidases liberate peptides and free amino acids required for yeast growth and higher alcohol synthesis [2,15,46]. Metagenomic comparisons of Hongqu and Maiqu starters reveal distinct glycoside hydrolase and protease gene repertoires, and omission experiments in reconstructed Jiuyao confirm that hydrolytic output is distributed across fungal, yeast, and bacterial guilds [18,33] (Figure 2).

Increasing starter enzyme activity does not necessarily increase ethanol yield [2,27]. When raw wheat Qu was biofortified with *S. rosea* A22, selected starter hydrolase activities increased, yet the resulting Huangjiu retained higher residual reducing sugar and lower ethanol and free amino acid concentrations than wine brewed with factory Qu [27] (Table 2). Optimizing starter hydrolysis therefore requires balancing substrate release rates with yeast glycolytic consumption and nitrogen uptake in the fermenting mash.

Raw grain composition and thermal liquefaction protocols further shape substrate accessibility prior to inoculation. Mass spectrometry imaging across three brewing rice cultivars mapped sucrose, glucose, and key amino acids to distinct anatomical grain regions, while comparative fermentations across five cereal substrates (rice, millet, corn, sorghum, and wheat) and diverse glutinous rice varieties demonstrated that grain chemistry and enzymatic liquefaction treatments substantially remodel microbial succession and volatile ester profiles [53–55] (Supplementary Table S1).

4.2. Alcohol and Organic Acid Metabolism

Pyruvate serves as the central metabolic node partitioning carbon between alcoholic fermentation and organic acid synthesis. In yeast, pyruvate decarboxylation and acetaldehyde reduction regenerate NAD^+ while supplying ethanol for subsequent esterification [2,56]. Time-resolved metatranscriptomics in black glutinous rice wine captured a pronounced mid-fermentation peak in glycolytic gene expression followed by late-stage stress-response and autophagy transcription, whereas comparative profiling of traditional ceramic-jar and mechanized tank brewing revealed process-dependent shifts in carbohydrate and pyruvate pathway expression [20,21].

Organic acid profiles reflect the concurrent activity of bacterial fermentation and tricarboxylic acid cycle branches. Lactic acid bacteria reduce pyruvate to lactate and generate acetate via phosphoketolase or acetyl–phosphate pathways, while fungal and yeast populations contribute succinate, malate, and citrate [23,24,56]. Stepwise addition of LAB species to a mold–yeast consortium nearly doubled terminal lactate from 3.47 to 6.26 g/L [26], whereas excessive LAB proliferation in mechanized Hongqu brewing elevated titratable acidity at the expense of ethanol production [23] (Table 1).

Accumulated organic acids reciprocally regulate community physiology and sensory balance. In rice-medium dose–response assays, 40 g/L lactic acid inhibited *S. cerevisiae* growth and 50 mg/L succinic acid increased biomass under the tested conditions [24]. Furthermore, under staged mash temperature control, treatment rankings for total titratable acidity diverged from those for lactic acid concentration, underscoring the value of quantifying individual organic acid species alongside bulk acidity [28].

Ethanol imposes a parallel physiological constraint during main and late fermentation [21,24]. A Huangjiu-derived *Bacillus velezensis* isolate showed delayed growth and membrane-associated stress responses under ethanol exposure in broth [57]. Such responses illustrate how alcoholic fermentation changes the conditions for bacterial activity. The functions sustained later in brewing depend on tolerance to the developing habitat as well as on the metabolic capabilities present in the initial starter.

4.3. Ester Formation and Higher Alcohol Biosynthesis

Ester profiles integrate microbial precursor production with later chemical reactions. Acetyl-CoA-dependent alcohol acetylation and fatty-acyl routes generate acetate esters and fatty acid ethyl esters, respectively; Huangjiu metagenomic and transcriptional annotations identify candidate functions across these pathways [18,21,56]. Esterification and hydrolysis can change the mixture during maturation, while thermal processing can induce nonenzymatic browning [2]. Communities therefore influence later flavor through precursor supply even when cells cease activity after processing. Final concentrations reflect biological synthesis, chemical conversion and retention, so microbial attribution must identify the relevant process stage and activity rather than rely on taxon-concentration correlations alone (Figure 2).

Metabolomics is most informative when precursor availability and product formation are measured in the same process. Starch hydrolysis supplies fermentable sugars for glycolysis, while proteolysis supplies amino acids for growth and higher-alcohol formation. Organic-acid production changes both the precursor pool for ethyl esters and the physiological environment of yeasts. Thus, a community intervention can alter ester abundance through several routes: changing alcohol or acyl-substrate supply, changing enzyme expression, or changing subsequent chemical conversion. Huangjiu time-series transcriptomics, metagenomic pathway mapping, and regional metagenomics–volatilomics comparisons provide complementary evidence for these links [20,21,56,58].

Higher alcohols arise from amino acid catabolism via the Ehrlich pathway and from de novo synthesis using carbohydrate-derived precursors [2,21,56]. In Tartary buckwheat Huangjiu, fortifying wheat Qu with bacterial isolates *Granulicatella* T3 or *Acidothermus* T4 selectively elevated phenylethyl alcohol, isoamyl alcohol, and isobutanol relative to unfortified Qu fermentations [59] (Supplementary Table S1). These starter biofortification trials demonstrate that bacterial additions during Qu manufacture can steer downstream higher alcohol profiles in the finished wine.

Non-*Saccharomyces* yeast co-fermentation and staged thermal control provide complementary routes to modulate the ester-to-alcohol ratio. Co-inoculating *S. cerevisiae* with high-erythritol *Yarrowia lipolytica* F4 increased total volatile esters by 78.2%, whereas co-fermentation with low-erythritol strain 167 increased total higher alcohols by 43.8% [60] (Supplementary Table S1). Similarly, cooling the late mash fermentation stage from 20 to 15 °C increased long-chain fatty acid ethyl esters from 0.544 to 1.157 mg/L (+112.7%) while lowering terminal ethanol, whereas combined early and late cooling favored medium-chain esters [28] (Table 2). Managing aroma synthesis therefore requires balancing targeted ester enrichment against overall alcoholic fermentation efficiency.

Sensory evaluation determines which of these chemical changes matters to the perceived mixture. Huangjiu reconstitution and omission identified influential alcohols and esters alongside compounds whose removal caused no perceptible change, and Hakka rice wine showed similarly compound-specific responses [34,40]. The benchmark temperature schedule also retained the most balanced sensory profile despite other schedules enriching selected esters [28] (Table 2). Concentration changes, biological attribution and perceptual

contribution thus answer different parts of the same ecological question: how an altered community or habitat changes the wine experienced by the consumer.

Analytical profiles, instrumental fingerprints, trained-panel judgments, and consumer responses resolve different endpoints. Untargeted metabolomics can identify candidate precursor and product changes, but peak annotation and relative signal require confirmation by standards and targeted quantification where a mechanistic claim depends on compound identity. Time-resolved transcript or protein measurements and enzyme assays can connect those changes to active pathways; Stable-isotope tracing can help quantify metabolic flux and test proposed metabolite-transfer routes. Aroma reconstitution and omission test perceptual contributions within a defined mixture, while preference and acceptance require consumer studies. Huangjiu studies combining volatile analysis with reconstitution or omission illustrate this progression, and aging metabolomics additionally requires separation of chemical maturation from vintage and process effects [34,40,61].

4.4. Microbial Communities and Fermentation Quality

Fermentation quality depends on whether a community sustains conversion while producing an acceptable sensory balance. Successful and spoiled Huangjiu differed in microbiota and chemistry [3], whereas selective yeast interventions tested specific sensory objectives. Sequential *Rhodotorula mucilaginosa* H04 and *S. cerevisiae* SC01 inoculation increased fruity and honey notes and reduced alcoholic harshness, whereas *S. cerevisiae* SC-6 reduced bitterness and delayed bitterness in a high-bitterness-strain fermentation [30,31] (Table 2). Because inoculation timing and dosage are integral to the H04 protocol, the sensory improvement reflects the complete co-fermentation regime, and advancing such adjunct isolates toward production requires pairing trained-panel evaluation with consumer preference testing, multi-batch validation, and strain-level safety verification (Section 4.5).

Regional Huangjiu styles embed these metabolic networks within distinct cereal substrates and thermal schedules. Multi-omics tracking of Shanxi broomcorn millet and proso-millet wines linked fungal-to-bacterial succession with characteristic organic acid and volatile ester profiles [62,63] (Supplementary Table S1), while cross-regional comparisons of Shaoxing, Jimo, and Fangxian fermentations demonstrated how soaking duration, starter type, and mash temperature jointly shape core metabolic networks [58].

Post-fermentation storage and aging further differentiate microbially active fresh wines from thermally pasteurized, chemically maturing products. Comparative profiling of Xiecun Huangjiu across aging stages revealed progressive shifts in volatile esters and amino acids [64], while unpasteurized Xiangyang fresh Huangjiu exhibited continuous microbial succession and volatile evolution over 22 days of refrigerated storage at 4 °C [41] (Supplementary Table S1). In multi-year jar-aging series, untargeted metabolomics and electronic-tongue analyses captured systematic changes in non-volatile taste compounds that reflect the combined effects of long-term chemical maturation and vintage-specific brewing history [61].

4.5. Safety-Related Metabolites and Undesirable Fermentation Outcomes

Nitrogen metabolism during mixed-culture fermentation can also generate safety-relevant compounds, most notably biogenic amines (BAs). While metagenomic profiling of Hongqu rice wine associates bacterial amino acid decarboxylase genes with tyramine, putrescine, and cadaverine accumulation [65], inoculating a reverse-screened, amine-negative *Enterococcus durans* AR315 isolate lowered final total BAs by 27.6% relative to uninoculated controls [66]. Furthermore, in situ substitution and community modeling in simulated rice wine demonstrated that final BA levels depend sensitively on initial LAB inoculation ratios and amino nitrogen availability [67] (Supplementary Table S1).

In biofortified wheat-Qu brewing trials, *S. hirsuta* J2 inoculation similarly lowered total biogenic amines by approximately 45% relative to the control fermentation [13]. This strain-specific functional effect (EF) complements low-amine LAB starter strategies [49,66], while motivating biochemical characterization of the putative amine-oxidizing or degrading enzymes encoded in the *S. hirsuta* genome.

Mitigating ethyl carbamate (EC) precursors and purines similarly requires strain-level functional screening coupled with terminal metabolite verification. In reconstructed Huangjiu consortia, multi-omics tracking revealed altered arginine, citrulline, ornithine, and urea trajectories (reaching 0.70 ± 0.11 mg/L urea on day 20), highlighting the need to verify whether precursor shifts translate into reduced terminal EC after thermal pasteurization and storage [68]. For purine control, amplicon-based inference initially linked bacterial succession to rising purine pools [69], and subsequent metagenomic pathway mapping paired with isolate co-culture demonstrated that adding *L. brevis* W4 reduced total assayed purines by 9.4% compared with the single-yeast *S. cerevisiae* N85 control [70] (Supplementary Table S1).

Phase partitioning across the solid–liquid mash matrix also governs the fate of mycotoxins and exogenous residues during processing [43]. In sterigmatocystin-spiked rice-wine trials, starter addition rates and solid–liquid pressing significantly altered toxin recovery in the liquid wine fraction and yielded hydroxylated transformation products, demonstrating why safety audits must monitor both fermented grain lees and clarified wine [71] (Supplementary Table S1).

Citrinin warrants dedicated monitoring in Hongqu-based brewing systems because its biosynthesis depends on the specific *Monascus* strain and solid-state cultivation conditions [17,18]. Analytical comparisons demonstrated that acid or enzymatic hydrolysis released additional measurable citrinin from citrinin-positive Hongqu samples, indicating that conventional free-toxin extraction can underestimate matrix-associated material [72]. Because citrinin production varies widely across *Monascus* isolates and starter-level detection reflects raw-material hazard potential rather than universal presence in finished wine, quality control should combine non-toxigenic *Monascus* starter selection with matrix-validated quantification across Hongqu, mash lees, and aged Huangjiu [17,72].

Evaluating candidate functional strains for food application also requires integrating technological performance with strain-level biosafety verification. Although isolates of *Pantoea ananatis*, *R. mucilaginosa*, and *E. durans* have been investigated in fermented foods for flavor or metabolite modulation, clinical infections involving opportunistic *P. ananatis* [73] and *R. mucilaginosa* strains in immunocompromised patients [74] underscore why species-level identity or a single favorable metabolic trait (such as the amine-negative phenotype of *E. durans* AR315 [66]) must be complemented by comprehensive safety qualification. Prior to pilot or industrial deployment, non-conventional adjunct isolates should undergo whole-genome taxonomic confirmation and screening for virulence determinants, transferable antimicrobial resistance, and toxigenic potential under the intended brewing conditions.

5. Community Regulation and Synthetic Microbial Consortia

5.1. Ecological Principles for Targeted Community Regulation

Community regulation encompasses different interventions. Environmental regulation changes temperature, resources or other growth conditions; selective inoculation specifies strains, ratios or arrival order. Bioaugmentation adds selected organisms to an existing microbiota, whereas full reconstruction replaces that background with a defined consortium. Temperature schedules act during mash fermentation, and cultivation temperature shapes biofortified wheat Qu [27,28]. Glutamine supplementation likewise changes composition and metabolic output within a traditional starter system [29]. These strategies act

at different stages and should be chosen against a defined functional target, not treated as interchangeable ways to increase favorable taxa.

Timing and initial density further distinguish inoculation protocols. Huangjiu-derived LAB pre-colonization experiments support testing establishment before yeast introduction [22]; sequential yeast inoculation changes timing together with dose and membership [31]. LAB ratios also affect growth and amine outcomes in simulated rice wine [67]. Comparing arrival sequences at matched starting biomass would isolate timing more clearly. The engineering objective is to establish a useful resource and product trajectory that persists as acids accumulate and substrates are consumed.

5.2. Functional Strain Selection and Synthetic Community Design

Top-down design begins with a functioning community and simplifies membership while testing retention of a chosen phenotype, whereas bottom-up design combines cultured isolates selected for measured activities and compatibility [7,33,34]. An integrated approach uses community profiles to choose candidates, cultivation to obtain testable strains, and omissions or additions to resolve their contributions. Neither high abundance nor a predicted pathway makes a member indispensable; the definition of a sufficient consortium depends on the target function, substrate, and reference community. Figure 3 places both routes within an iterative Design–Build–Test–Learn (DBTL) cycle, adapting the omics-guided core and supplementary microbiome framework of Zhang et al. [7] to compare Huangjiu-specific substrate pretreatments, controls, and multi-endpoint validation criteria.

The target determines what must be retained during simplification. A hydrolysis-oriented design tests resource release and alcoholic fermentation, whereas an aroma-oriented design also requires chemical and sensory comparisons. Pairwise screens identify interactions in the tested medium; whole-community omissions reveal contributions amid potential compensation. Similar total inoculum, substrate pretreatment and enzyme background make alternative designs more interpretable. The Test step should measure conversion, sensory performance and undesirable metabolites together; the Learn step should use discrepancies to revise membership, inoculation timing or cultivation conditions. Industrial predictability then requires testing whether the improved design retains its function in independent fermentations.

Strain selection should preserve tested phenotypes when a community is simplified [47,48]. The six-species artificial starter specified individual isolates and inoculum concentrations, including added *S. cerevisiae* for alcoholic fermentation [35]. The strain-level dependencies discussed in Section 3.3 explain why substituting a conspecific isolate still requires functional testing. Species-level similarity alone would discard the compatibility information on which reconstruction depends.

Selection can also target a specific nutritional or chemical phenotype within an endogenous mash community [7,31]. In Fangxian Huangjiu, bioaugmentation with *Wickerhamomyces anomalus* and *P. ananatis* elevated γ-aminobutyric acid (GABA) and creatine concentrations while overall chemical profiles converged [32]. Because bioaugmented isolates operate alongside resident starter microbiota, candidate strains such as *P. ananatis* must combine persistent metabolic activity in mash with verified sensory compatibility and strain-level food-safety qualification prior to commercial use (Section 4.5).

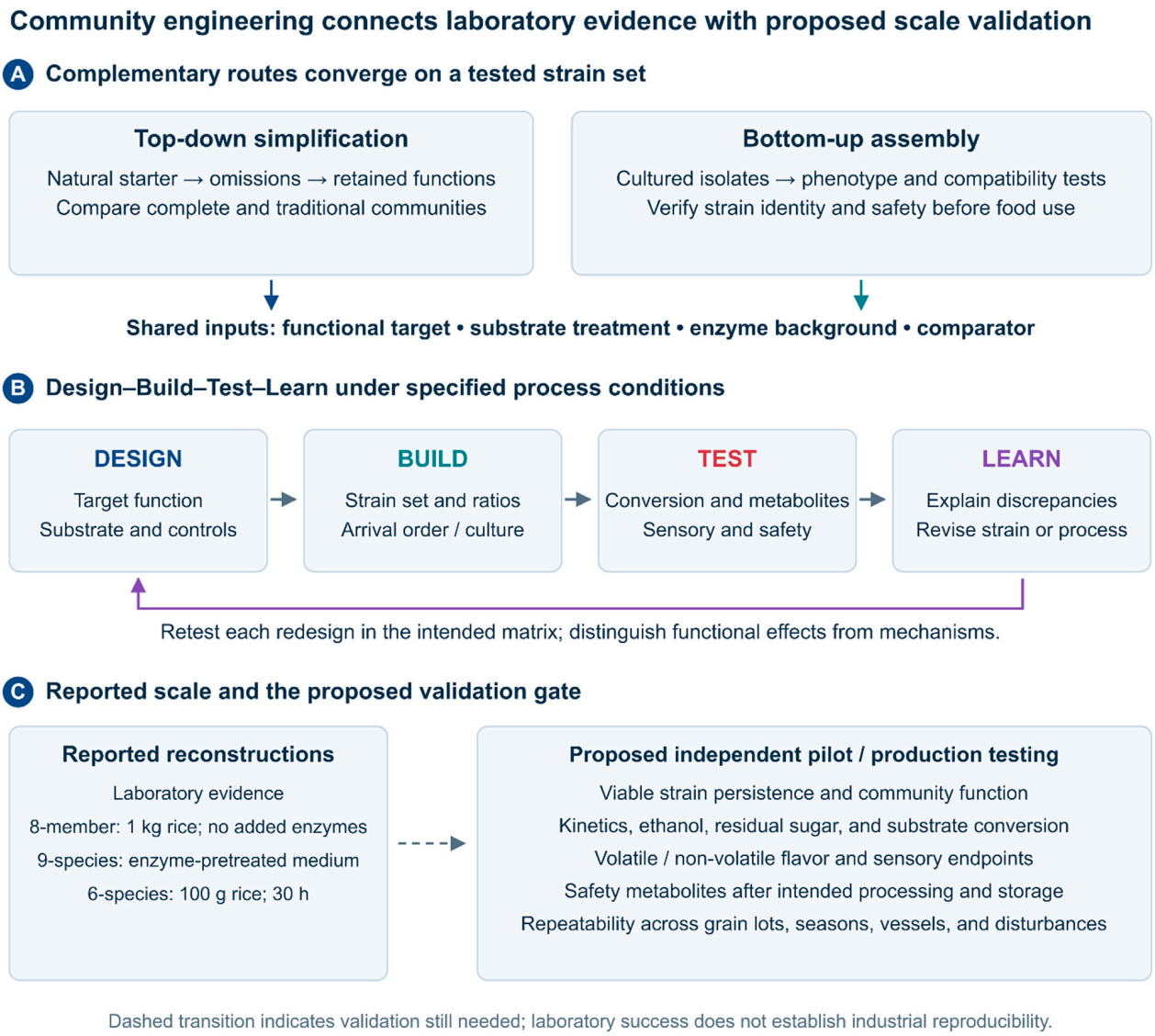


Figure 3. Function-oriented microbial community engineering and multi-scale validation workflow for Huangjiu fermentation. (**A**) Complementary top-down community simplification (omission-guided core taxon identification from natural starters) and bottom-up assembly (combining phenotyped, compatible isolates). (**B**) Iterative Design–Build–Test–Learn (DBTL) cycle integrating substrate pretreatment, strain ratios, arrival order, and thermal scheduling with multi-endpoint laboratory evaluation of substrate conversion, sensory balance, and safety-related metabolites [33–35]. (**C**) Scale-up validation pathway connecting reported laboratory reconstructions (100 g to 1 kg rice) to independent pilot and production batches across five core endpoints: viable strain persistence, fermentation kinetics, flavor and sensory quality, product safety, and batch-to-batch repeatability across raw-material lots, seasons, and vessel geometries. Solid navy and teal arrows in panel (**A**) indicate the convergence of top-down and bottom-up routes; solid gray arrows and the purple return arrow in panel (**B**) denote sequential workflow progression and iterative redesign feedback, respectively; the dashed gray arrow in panel (**C**) indicates the proposed transition from laboratory reconstruction to independent pilot- and industrial-scale validation.

5.3. Experimental Outcomes of Reconstructed Microbial Communities

Reconstructed synthetic consortia have successfully reproduced core hydrolytic or aromatic functions under laboratory conditions, while illustrating how substrate pretreatment defines the scope of each design. An omission-guided eight-member Jiuyao consortium fermented 1 kg of steamed rice without exogenous enzymes, reducing final total sugar to 1.81 ± 0.38 g/L (61.57% below the traditional Jiuyao control) while elevating ethanol yield

and saccharifying, protease, and cellulase activities [33]. By contrast, a nine-species aroma-oriented consortium simplified from an eleven-member candidate pool retained principal volatile esters, higher alcohols, and sensory scores evaluated by twelve trained panelists in a rice medium pretreated with commercial α-amylase and glucoamylase [34]. These two architectures therefore address complementary engineering goals—self-sustained grain depolymerization versus aroma reproduction on pre-hydrolyzed substrates (Table 2)—and provide bench-scale foundations that can next be evaluated for consumer preference and multi-batch pilot reproducibility.

Preserving primary fermentation kinetics can nevertheless coincide with fewer detected volatile compounds [34,35]. A freeze-dried six-species starter showed ethanol trajectories and electronic-nose fingerprints similar to those obtained with traditional Jiuyao in a 100 g, 30 h laboratory fermentation, while yielding 39 detectable volatiles compared with 50 in the traditional-starter wine [35]. Because electronic-nose sensors capture bulk headspace similarity rather than human odor-threshold perception or post-aging bouquet, pairing instrumental fingerprinting with trained sensory panels, consumer testing, and longer maturation trials is essential when evaluating simplified starters.

Defined reconstruction and bioaugmentation can also target specific safety endpoints [49,66,67]. Co-inoculating *L. plantarum* ACBC271 and *S. xylosus* CGMCC1.8382 lowered total biogenic amines more effectively than either single inoculum or uninoculated controls [49]. Together with reconstructed hydrolytic and aroma starters, these trials demonstrate that functional improvements can be achieved through distinct community architectures tailored to specific brewing objectives.

5.4. Challenges for Industrial Applications

Industrial translation requires functions to survive culture manufacture, storage and repeated fermentation. Lyoprotectant optimization addresses one preservation step, but the six-species starter's brewing comparison used 100 g rice over 30 h [35]. LAB persistence during laboratory serial transfer tests a different aspect of stability [25]. Neither alone establishes stable production-scale performance. Viable composition matters when losing a member disrupts a needed activity; function may nevertheless remain reproducible despite some compositional variation. Both population trajectories and product outcomes should therefore be followed through storage and brewing.

Environmental recruitment remains relevant during scale-up because winery tools and facility surfaces harbor fermentation-associated microbes year-round [19,20]. Larger vessels also alter heat dissipation and the spatial distribution of solid substrates, requiring concurrent monitoring of population trajectories and product consistency [20]. Replicated batches across raw-material lots and seasons can verify whether an inoculated consortium retains its target functions under workshop conditions, while balancing the operational cost of separate mold, yeast, and bacterial propagation against quality stability.

Rigorous biosafety qualification must accompany this scale-up pipeline. Whether deploying a reverse-screened low-amine isolate such as *E. durans* AR315 [66], a metabolite-enriching co-culture containing *P. ananatis* [32], or an aroma-modulating non-*Saccharomyces* adjunct such as *R. mucilaginosa* H04 [31], industrial translation requires maintaining strain genetic identity, documenting the absence of transferable resistance or virulence traits, and verifying terminal safety endpoints in the finished product.

6. Current Limitations and Future Perspectives

6.1. Limitations of Current Analytical Approaches

Resolving causal mechanisms in Huangjiu ecosystems requires overcoming key methodological limitations in current multi-omics workflows. Amplicon primer bias, DNA

extraction efficiency, and reference database coverage influence taxonomic profiles, while relative abundance data can mask shifts in absolute microbial load and extracellular relic DNA can persist long after cell death [4]. Integrating spike-in quantitative sequencing, flow cytometry, or strain-resolved metagenomics with enzymatic assays can separate active proliferation from carried-over starter biomass. Viability assays must also be validated for acidic, ethanol-rich matrices, as propidium monoazide (PMA)-qPCR estimates became unstable during prolonged organic acid accumulation in simulated rice wine [67].

The biological uncertainty should determine the comparison. Matched-biomass arrival-order experiments test historical effects; controlled temperature or nutrient perturbations test environmental responses. Strain-resolved tracking addresses the within-species variation discussed in Section 3.3. Networks and assembly models nominate relationships, while metagenomes identify potential functions and transcript or biochemical measurements characterize activity [5]. Following populations, resources and products through the same intervention connect establishment with a functional response. Whether phage-mediated selection alters Huangjiu community trajectories remains to be tested [9].

6.2. From Associations to Experimentally Validated Mechanisms

Bridging statistical co-occurrence with biochemical causation requires experimental designs that isolate candidate mechanisms under fermentation-relevant conditions. While species omission and add-back experiments identify functional dependencies at the consortium level, comparing pathway-deficient mutants with parental and genetically complemented strains can establish whether a specific enzymatic or transport activity is required. In parallel, pairing metabolite supplementation with pH-matched controls separates nutritional facilitation from acid-stress relief, and stable-isotope tracing (e.g., ^{13}C- or ^{15}N-labeled substrates) can verify directional carbon or nitrogen transfer between molds, yeasts, and LAB.

Mechanistic tests must also preserve the physical and chemical context of the brewing matrix [12,22]. For example, a defined Sauce-aroma Baijiu consortium paired solid-substrate phenotyping with transcriptomic analysis in a separate liquid medium [75]. In Huangjiu, coupling time-resolved enzyme and metabolite measurements with controlled perturbations in rice mash would allow proposed interactions to be tested under brewing-relevant conditions.

6.3. Toward Predictable and Stable Fermentation Microbiomes

Translating mechanistic insights into industrial predictability requires evaluating consortium stability across independent production batches and scales. Dynamic Lotka–Volterra and resource-consumer models fitted to controlled LAB co-cultures provide a quantitative starting point, although model parameters must be calibrated across the wider pH, ethanol, and temperature ranges encountered during full-length brewing [67]. Because metabolomic profiles can converge despite taxonomic variation—as observed in bioaugmented Fangxian Huangjiu [32]—process control frameworks should define quantitative acceptance windows for sugar depletion, ethanol, titratable and individual acids, key aroma compounds, and safety endpoints alongside viable strain recovery.

Prediction must cover the stages where inocula can lose useful function. Freeze-drying and serial-transfer studies address preservation and population maintenance [25,35]. Independent pilot batches should test establishment, disturbance recovery and resident competition, recording starter preparation, vessel geometry and temperature history. Grain lots and seasons add variation, including recruitment from winery reservoirs [19]. Evaluation on held-out batches would distinguish explanation of development data from useful production prediction. Population trajectories should be interpreted alongside conversion,

sensory and safety outcomes to define the operating range over which a community retains its function.

To guide industrial translation, we propose a set of validation endpoints across independently prepared pilot and production batches: (i) community stability, tracking viable strain persistence, member replacement, and activity retention after starter storage and pitching; (ii) fermentation kinetics, quantifying starch liquefaction, residual sugar depletion, ethanol yield, and individual organic acids; (iii) flavor and sensory quality, integrating targeted volatile/non-volatile quantification with trained-panel profiling and consumer acceptance testing; (iv) product safety, monitoring biogenic amines, citrinin (for Hongqu-based processes), and terminal ethyl carbamate after thermal sterilization and jar aging; and (v) batch-to-batch repeatability across cereal lots, seasonal regimes, and vessel geometries. Integrating these multi-tier criteria provides an operational benchmark for advancing the laboratory consortia summarized in Table 2 toward commercial brewing.

6.4. Research Priorities

Future research on the Huangjiu microbiome can be anchored around three interconnected priorities: (i) mapping strain-resolved dispersal and spatial niche partitioning across solid starters and two-phase mashes; (ii) validating cross-kingdom and intra-bacterial metabolic fluxes through targeted genetic, enzymatic, and isotope-tracing perturbations; and (iii) establishing multi-batch, pilot-to-industrial validation protocols for freeze-dried and reconstructed synthetic starters. Developing shared reference strain banks and standardized time-series multi-omics datasets across major regional Huangjiu styles will accelerate the transition from descriptive profiling to rational, predictable fermentation engineering.

7. Conclusions

Huangjiu communities assemble through process-dependent combinations of inherited inocula, environmental selection and colonization history. Preformed starter enzymes add a functional legacy that can persist independently of the organisms producing them. Wheat Qu, Jiuyao, Xiaoqu and Hongqu therefore provide different starting conditions, while traditional and mechanized brewing modify subsequent habitats. Ecological transitions in resource availability, acid exposure and ethanol accumulation offer useful comparisons across these systems without implying a universal succession timeline.

Interactions connect those trajectories with flavor and quality, but their direction depends on strain identity, nutrients, stress, physical structure and timing. Hydrolytic complementarity, recipient facilitation and competition can all contribute to fermentation performance. Co-culture, omission and supplementation experiments identify functional effects; reciprocal mutualism, direct metabolite transfer and molecular necessity require evidence matched to those stronger claims. Chemical concentration changes must also be separated from biosynthetic attribution and sensory significance. This distinction preserves useful treatment responses while clarifying which biological explanations remain to be tested.

Community engineering is most useful when it links a defined intervention to the reproducible conversion, sensory performance and control of undesirable metabolites. Substrate pretreatment, controls and scale determine what a reconstructed consortium has demonstrated: preserving aroma in an enzyme-pretreated system differs from retaining starter hydrolysis. Functional reproducibility must be evaluated across independent fermentations while retaining strain safety and compatibility as constraints. Alternative communities may achieve similar product objectives through different regional processes, but the operating range of each fermentation must be established. The central challenge is to determine which ecological relationships can reproducibly deliver desirable Huangjiu traits across fermen-

tation conditions. For Hongqu-derived designs in particular, strain-dependent citrinin potential and measured finished-wine occurrence require separate evaluation. Reliable application will depend on satisfying conversion, sensory, safety, and batch-reproducibility criteria together.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12120561/s1, Note S1: Literature identification and selection; Table S1: Substrate conversion, regional quality variation, and safety-related metabolite outcomes across Huangjiu fermentation systems.

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