



Decoding the synergistic mechanisms of functional microbial agents on the microecology and metabolic function in medium-high temperature *Daqu* starter for enhancing aromatic flavor

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ABSTRACT

Utilizing functional *Daqu* has emerged as an effective strategy to enhance aromatic compounds in Chinese Baijiu. However, research on how functional microbial agents enhance aromatics-producing enzymes and maintain community homeostasis in functional *Daqu* remains limited. Herein, we reveal the mechanisms of functional microbial agents for enhancing aromatic compounds through reducing interspecies interactions and simplifying the ecological network to drive the aggregated distribution of lactic acid bacteria, and inducing a localized microecology comprised of *Aspergillus*, *Pichia*, *Millerozyma*, *Pseudomonas*, *Paenibacillus*, and *Rhizomucor*, effectively boosting the expression of key enzymes for aromatic synthesis. Functional microbial agents significantly enhance the key enzyme activities (515.9 nmol/h/g and 6.1 U/g for PrAO and ALDH) compared with traditional *Daqu* (198.6 nmol/h/g and 0.9 U/g), improving the content of aromatic compounds with an increase of 185.57 %. These results revealed the mechanisms of functional *Daqu* in aromatic compounds production, thus contributing to improve *Baijiu* quality.

1. Introduction

Chinese *Baijiu*, with its rich history and profound brewing techniques, represents a significant symbol of Chinese traditional culture (Ma et al., 2022). Among the various types of *Baijiu*, strong-flavor *Baijiu* stands out as a prominent representative, commanding over 70 % of the market share. This category is typically characterized by its rich aroma, mellow sweetness, balanced flavor, and long-lasting aftertaste (Mu, Huang, Zhou, Zhang, Qin, Tang, et al., 2023). The unique brewing process of strong-flavor *Baijiu* includes the preparation of MHT *Daqu*, solid-state fermentation of *Zaopei*, distillation, aging, and blending (Zhu et al., 2022). MHT *Daqu* serves not only as the primary raw material for *Baijiu* brewing but also provides essential fermentation power and flavor precursors through its complex enzyme and microbial systems. Notably, *Daqu* contributes approximately 10 % to 20 % of the bacterial

community and 60 % to 80 % of the fungal community involved in fermentation (Zhu et al., 2024). These specific microbial strains, along with their metabolites and enzyme systems, directly influence the microbial community structure and metabolic activities within the *Zaopei* microecosystem, playing a crucial role in determining the fermentation quality of *Baijiu*.

The preparation of MHT *Daqu* typically occurs in an open production environment characterized by spontaneous fermentation, without the inoculation of exogenous microbes. The enrichment and adaptation of functional microbial strains primarily originate from raw materials, production tools, and the surrounding environment (Mu, Huang, Zhou, Zhang, Qin, Dong, et al., 2023; Zhu et al., 2022). However, this traditional approach can lead to an uncontrollable fermentation process, unstable product quality, and increased production costs (Ma et al., 2022; Mu et al., 2022). In response to these challenges, biofortification

Abbreviations: MHT, medium-high temperature; PrAO, primary amine oxidase; ALDH, aldehyde dehydrogenase; PCoA, principal coordinate analysis; PCA, principal component analysis.

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techniques, which involve the inoculation of functional agents, have emerged as an effective strategy for regulating microbial communities and their functions (Mu et al., 2022). This method has been shown to improve both the quality and flavor of fresh *Baijiu*. For instance, the inoculation of functional bacteria, yeasts, and molds isolated from *Zaopei* and *Daqu* has been demonstrated to enhance flavor and yield effectively (Hong et al., 2021). Additionally, *Aspergillus*, a prominent genus within the Eurotiales, secretes a variety of hydrolytic enzymes, including amylases, cellulases, and proteases, which facilitate the degradation of raw materials (Yang et al., 2021). This genus is closely associated with the generation of various flavor compounds (Wang, Quan, et al., 2023) and is frequently used as a starter strain for preparing functional *Daqu*.

Aromatic substances, such as phenylethanol, phenylacetaldehyde, and ethyl phenylacetate, impart rich floral, fruity, and sweet notes to strong-flavor *Baijiu*. These compounds are characterized by low odor thresholds, high aroma intensity, and long-lasting fragrance (Wang et al., 2014). The types and concentrations of these aromatic compounds directly affect the elegance and complexity of the liquor's aroma. In our previous research (Zhu et al., 2024), we validated the main synthesis pathways of aromatic compounds in sauce-flavor *Daqu* through metagenomics, metaproteomics, and targeted metabolomics combined with enzyme activity assays. Importantly, we identified that primary amine oxidase (PrAO) and aldehyde dehydrogenase (ALDH) are two key enzymes that promote the synthesis of aromatic compounds. Based on this, we conducted a detailed analysis of the relationship between enzyme proteins and target compounds, identifying potential key proteins belonging to these two enzymes. Through in vitro amplification experiments and heterologous expression, we confirmed the presence of critical functional genes, revealing that these genes are derived from *Aspergillus* genus. Consequently, we isolated high-yield strains of PrAO and ALDH from sauce-flavor *Daqu* (*A. flavus* C10 and *A. niger* IN2), providing excellent microbial resources for the development of functional *Daqu*. Despite recent advances, research on the role of functional microbial agents in enhancing key enzymes for aromatic production and

stabilizing the microecosystem in MHT *Daqu* remains limited. Therefore, this study aims to address this gap by applying *Aspergillus* agents with high enzyme activity for the preparation of industrial-scale functional *Daqu*. We comprehensively explore the regulatory effects of these functional agents on the MHT *Daqu* fermentation system from multiple dimensions, including physicochemical and enzymatic properties, the generation of key volatile flavors, adjustments to microbial structure and metabolic functions, and the critical driving factors involved in these processes. This study elucidates the regulatory effect of functional microbial agents on MHT *Daqu* and provides a practical strategy for the industry to optimize *Baijiu* production through microbial biotechnology.

2. Material and methods

2.1. *Daqu* manufacture and sample collection

Functional *Daqu* was prepared at a well-known distillery in Chengdu City, Sichuan Province, China. The process is illustrated in Fig. 1. Briefly, spore suspensions of *A. flavus* C10 (NMDC number: N0003GPP) and *A. niger* IN2 (NMDC number: N0003GPO) were prepared at a concentration of 10^6 spores/mL by adding physiological saline to the activated cultures. These suspensions were mixed in a 1:1 ratio to create a functional agent, which was inoculated into sterilized wheat to produce pure wheat *Qu*, following the preparation process detailed in our previous study (Zhu et al., 2024). Subsequently, MHT *Daqu* was prepared according to the standard process, with 3 % of the functional wheat *Qu* added to the function group (B). Traditional *Daqu* without inoculation served as the control group (K). *Daqu* was fermented in the *Qu*-room for one month and then stored in the stacking room for three months to obtain the final MHT *Daqu*. Samples were collected at various time points based on the temperature variation curves of past batches during *Daqu* production. A total of 18 samples were collected from the *Qu*-room on days 0, 3, 5, 8, 11, 18, and 30, and during storage on the 1st and 3rd months (M1 and M3, respectively). To ensure representativeness, samples were taken from three different sites (sites 1, 4, and 6) along the

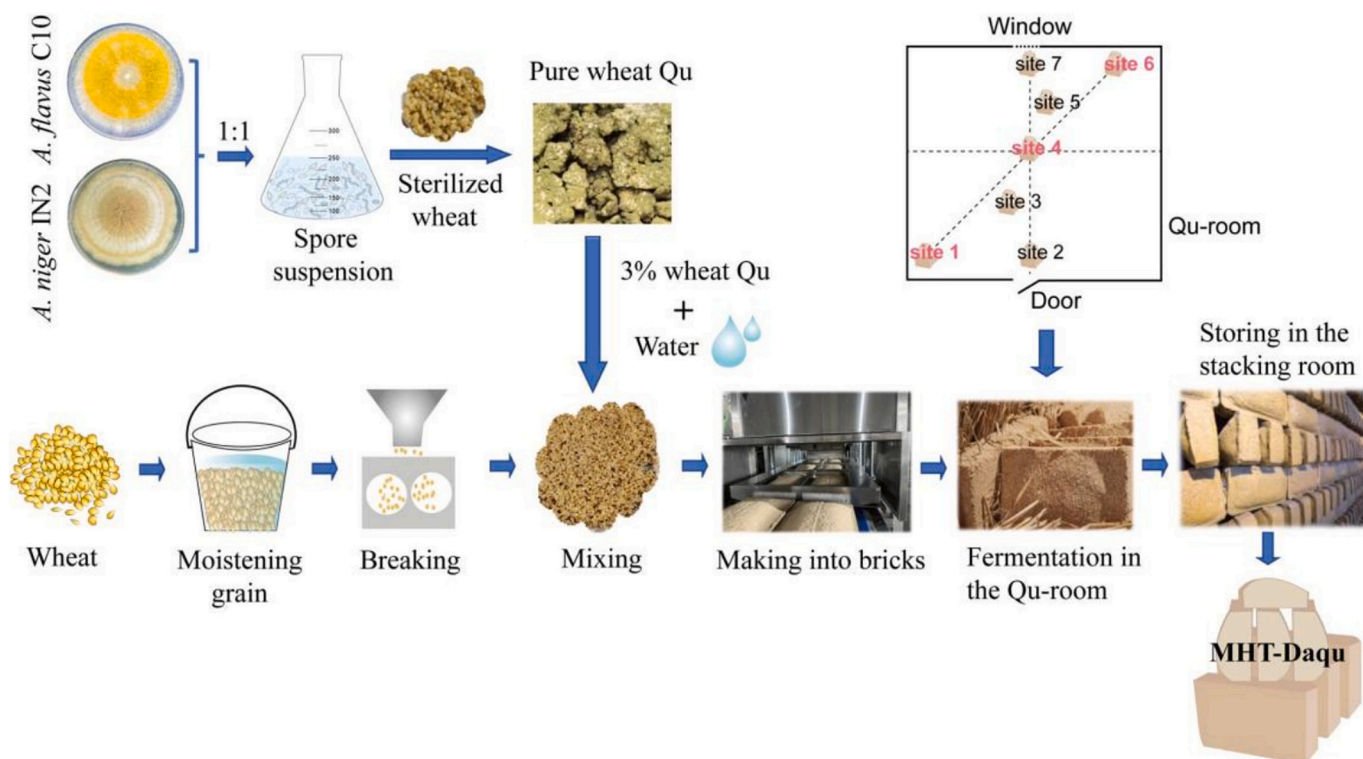


Fig. 1. Schematic diagram illustrating the functional *Daqu* preparation process.

diagonal of the *Qu*-room. After thorough crushing and mixing, the samples were reduced to a composite sample of 500 g. The samples were then divided into three portions: one for analyzing physicochemical properties (stored at 4 °C), one for volatile compounds and enzyme activity (stored at −20 °C), and one for DNA extraction (stored at −80 °C).

2.2. Detection of physicochemical, enzymatic properties and volatile compounds

Temperature changes in the *Qu*-room and *Daqu* core were recorded using electronic temperature sensors. The methods of direct drying and acid-base titration were used to measure moisture content and total acidity, respectively (Zhu et al., 2022). The saccharifying, liquefying, fermenting, and esterifying abilities were assessed using previously described methods (Wang, Du, et al., 2023). Additionally, PrAO and ALDH activities were measured according to previous studies (Zhu et al., 2024). A detailed definition of enzyme activity can be found in Supplementary Section 1. Volatile compounds in *Daqu* were analyzed using a GC–MS (Wang, Du, et al., 2023; Zhu et al., 2024). For a detailed description, refer to Supplementary Section 2.

2.3. Microbial community analysis

The E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) was used to extract total microbial genomic DNA in *Daqu* (Ma et al., 2022). The quality and concentration of DNA were assessed by 1.0 % agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, U.S.), respectively (Zhang et al., 2024). The method of PCR amplification was carried out in accordance with our previous study (Zhu et al., 2022). The PCR products were purified by PCR Clean-Up Kit (Yuhua, Shanghai, China) and then sequenced by Illumina PE300 platform (Illumina, San Diego, USA).

The software fastp (version 0.19.6) was used to perform quality filtering on the original sequences (Chen, Zhou, et al., 2018). Meanwhile, the software FLASH (version 1.2.7) was employed to merge (Magoč & Salzberg, 2011). Low-quality sequences were removed based on criteria including ambiguous bases >0, primer mismatches >2, average quality score < 20, mononucleotide repeats >8, and length < 150. The software UPARSE 7.1 was used to cluster optimized sequences into operational taxonomic units (OTUs) with a 97 % similarity threshold (Edgar, 2013; Stackebrandt & Goebel, 1994; Li, Yi, et al., 2024). Taxonomy of OTUs was assigned using the RDP Classifier v2.2 against the Silva v138 and UNITE v8.0 databases, with a confidence threshold of 0.95 (Wang et al., 2007).

2.4. Statistical analysis and visualization

Line graphs, histograms, and radar charts were generated using OriginPro v9.0 (Northampton, MA, USA) and Visio v24.0 (Chicago, IL, USA) to illustrate variations in physicochemical indicators, flavor profiles, and enzyme activity. Differences in PrAO and ALDH activity were assessed using Student's *t*-test with the stats package in R v3.3.1. Alpha diversity indices, including Chao and Shannon indexes, were calculated with Mothur v1.30.1 to compare microbial community richness and diversity. Principal Coordinate Analysis (PCoA) and hierarchical clustering using the unweighted pair-group method with arithmetic mean (UPGMA) were performed in R v3.3.1 with the vegan v2.5–3 package, using the Bray-Curtis distance algorithm to determine similarities and differences among microbial communities in different samples. The PERMANOVA test (999 permutations) was used to assess significant differences among microbial communities using the adonis package in R v3.3.1. A bar plot in R v3.3.1 was used to identify the most abundant microbial communities at the genus level, with groups representing less than 1 % relative abundance in all samples combined into “others.” A co-occurrence network was constructed based on $|p| > 0.7$ and $p < 0.05$ and

visualized with Gephi v0.9.2. A ternary plot, generated with the ggtern package (Hamilton & Ferry, 2018) in R v3.3.1, was used to visualize the distribution of microbial communities across different stages. The Mantel test was performed to explore correlations between microbial communities and environmental factors using the vegan package in R v3.3.1. The functional composition of the microbial community in MHT *Daqu* was analyzed by PICRUSt2 (Douglas et al., 2020).

2.5. Data availability

All data confirming the results of this study have been provided in this article on request. The raw sequencing reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Numbers: PRJNA1152505 and PRJNA1152506).

3. Results and discussion

3.1. Effect of functional agents on physicochemical and enzymatic properties during MHT-*Daqu* production

Physicochemical and enzymatic properties are essential indicators of environmental conditions and microbial metabolic activity within the *Daqu* fermentation system. As shown in Fig. 2A, the temperature trends in both the core of the *Daqu* and the *Qu*-room were similar for functional and traditional *Daqu*, respectively. This suggests that the inoculation of functional agents does not interfere with the normal heating process during *Daqu* fermentation. The temperature followed a typical parabolic pattern: it increased from days 0 to 8 (ascending temperature), peaked between days 8 and 14 (peak temperature), and then decreased after day 14 (descending temperature). The temperature on days 8 and 14 corresponded with the first and second *Daqu*-turning (moving the position of the *Daqu* to balance temperature and humidity), respectively. This pattern is consistent with typical temperature changes observed in MHT-*Daqu* production (Yang et al., 2021). Notably, as this batch of *Daqu* was produced in winter, the temperature in the *Qu*-room was significantly lower than the core temperature due to external environmental conditions. Additionally, the moisture content in both functional and traditional *Daqu* decreased consistently (Fig. 2B). This decline was primarily due to the high moisture content in the *Daqu* blocks during the initial stage. At this phase, active microbial metabolism generated biological heat (Xia, Luo, et al., 2023), which rapidly evaporated the surface moisture. As a result, the moisture content decreased more rapidly during the first 18 days of fermentation. As surface moisture diminished and microbial metabolic activity decreased, the moisture content declined slowly after 18 days, eventually stabilizing around 12 %. The acidity changes in both types of *Daqu* were similar (Fig. 2C), but the acidity of functional *Daqu* was higher than that of traditional *Daqu* after 3 days of fermentation, particularly in the mature *Daqu* (M3). This indicates that the inoculation of functional agents can increase the organic acid content of *Daqu*, providing important precursors for ester formation.

Daqu serves as a saccharification and fermentation agent as well as a complex flavor enhancer. During production, it accumulates a large number of beneficial microbes and various enzymes, endowing *Daqu* with multiple functions such as saccharification, liquefaction, fermentation, and esterification abilities. The activity levels of these enzymes are crucial in determining whether *Daqu* can be effectively used in the brewing process. Specifically, saccharifying and liquefying abilities are key indicators of *Daqu*'s capacity to convert starch into fermentable sugars (Fan et al., 2020; Xia, Zhou, et al., 2023). During *Daqu* production, both functional and traditional *Daqu* exhibited similar levels of saccharifying power (Fig. 2D), with higher levels observed at the initial stage due to the high saccharifying enzyme content in the wheat (Zhu et al., 2023). As fermentation progressed, the first *Daqu*-turning on day 8 (Fig. 2A) increased oxygen exposure, promoting the growth of aerobic fungi such as *Rhizopus* and *Aspergillus niger* (Zhu et al., 2022), which led

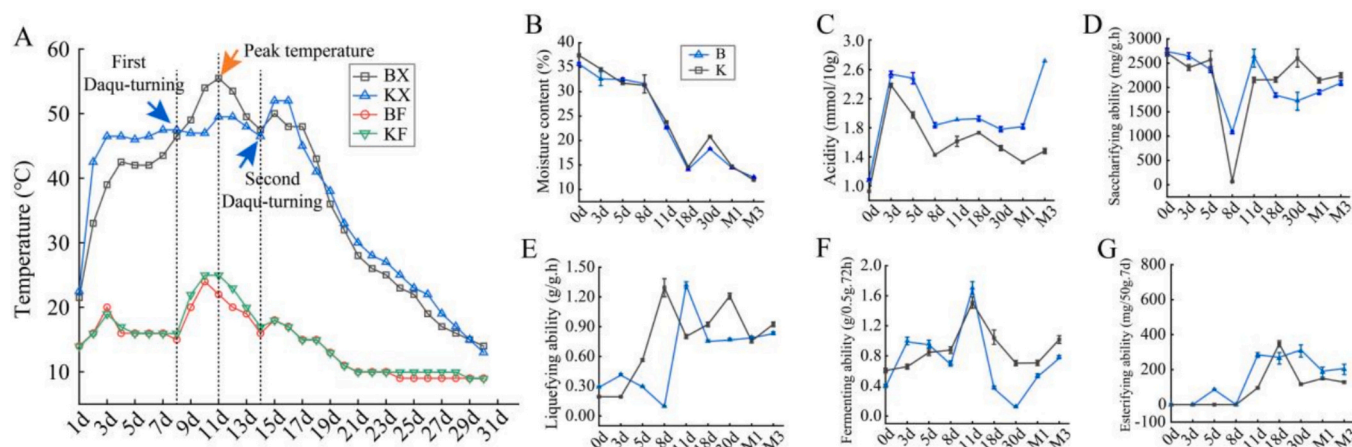


Fig. 2. Effects of functional agents on physicochemical and enzymatic properties during MHT-*Daqu* preparation, including temperature (A), moisture (B), acidity (C), saccharifying ability (D), liquefying ability (E), fermenting ability (F), and esterifying ability (G). B and K represent functional and traditional *Daqu*, respectively. BX and KX denote core temperatures of functional and traditional *Daqu*, while BF and KF indicate *Qu*-room temperatures for functional and traditional *Daqu*.

to a rise in saccharifying power. The liquefying ability of *Daqu* was initially low (Fig. 2E). However, as fermentation progressed, the active growth and metabolism of bacteria and molds led to increased secretion of α -amylase, causing the liquefaction capacity of both types of *Daqu* to peak on the 11th and 8th days, respectively. Fermentation capacity, which characterizes *Daqu*'s ability to produce ethanol from fermentable sugars, showed a trend of increasing and then decreasing in both types of *Daqu* in the *Qu*-room (Fig. 2F), consistent with previous research (Wang, Du, et al., 2023). It is hypothesized that during the early to mid-fermentation periods (from days 0 to 11), environmental conditions were favorable for the abundant growth and enrichment of yeast. However, as microbial metabolic activity intensified and the *Daqu* blocks warmed, the yeast, unable to tolerate high temperatures, either died or entered a dormant state (Xiao et al., 2017), leading to a decrease in fermentation ability. Esterification ability, a key indicator of *Daqu*'s esterification and aroma-producing capacity, showed a fluctuating increase over time, eventually stabilizing during the storage period (Fig. 2G). Notably, after the first *Daqu*-turning on day 8, the temperature of the *Daqu* blocks peaked at 56 °C on day 11 (Fig. 2A), with the activities of various enzymes also reaching their peak. This suggests that these enzymes exhibit a certain tolerance to temperature. Overall, fortification with dual functional agents did not affect the fundamental properties of *Daqu*, allowing functional *Daqu* to be effectively utilized in subsequent brewing processes. Moreover, the increased acidity provides a foundation for the formation of flavor compounds in the *Zaopei* microecosystem.

3.2. Effect of functional agents on volatile flavors and aromatics-producing enzymes during MHT-*Daqu* production

To assess the impact of aromatics-producing agents on the flavor profiles of MHT *Daqu*, we compared the volatile flavor expression patterns of functional and traditional *Daqu* throughout the preparation process. Principal component analysis (PCA) of 83 identified volatile compounds revealed significant changes in flavor composition on days 5, 8 and 11 of fermentation (Fig. 3A), suggesting that *Aspergillus* agents influenced volatile metabolite patterns during the heating period. Previous studies (Li, Yan, et al., 2024) have shown that *Aspergillus* secretes amylases and proteases to catalyze the formation of reducing sugars and amino acids, promoting the generation of pyrazines in *Daqu* through the Maillard reaction. *Aspergillus* is also closely associated with the production of esters and aromatic compounds (He, Jin, et al., 2022; Wang, Quan, et al., 2023; Zhu et al., 2024), playing a key role in enhancing *Daqu*'s flavor. Further PCA of 20 aromatic compounds (Fig. 3B) revealed similar distribution patterns across the *Daqu* samples. Combining these

findings with Fig. 3C, we concluded that the high content of aromatic compounds significantly contributes to the differences in volatile metabolites throughout the fermentation process.

The impact of *Aspergillus* strains on the volatile flavors of MHT *Daqu* mainly involves esters, alcohols, acids, and aromatic compounds. This study analyzed the changes in these compounds during the *Daqu* preparation process (Fig. 3C and Table S1). For ester content, both functional and traditional *Daqu* peaked on day 3, after which they continuously decreased. During the peak temperature period (day 11), ester levels in both types rapidly dropped to lower levels. This phenomenon suggests that the metabolic activity of ester-producing microorganisms is highly sensitive to temperature fluctuations, leading to sharp variations. The microbial ester synthases reported primarily belong to four enzyme families: Alcohol acyltransferases (AATs, EC 2.3.1.-), alcohol dehydrogenases (ADHs, EC 1.1.1.-), esterases (ESTs, EC 3.1.1.-), and Baeyer-Villiger monooxygenases (BVMOs, EC 1.14.13.-). These enzymes catalyze the formation of esters from alcohols, aldehydes, acids, and ketones as precursors in *Daqu* (Wang, Quan, et al., 2023). Temperature affects the activity of AATs and ADHs, thus influencing ester levels in MHT *Daqu* (Wang, Du, et al., 2023). Furthermore, at the genetic level, the Acetolactate synthase gene (*JqALS1*) correlates closely with changes in ester content in *Daqu*, and is highly temperature-dependent (Wang, Zhou, et al., 2024). More importantly, lower temperatures induce the up-regulation of some ALS genes, which aligns with the higher ester content in medium-temperature *Daqu* compared to high-temperature *Daqu* (Wang, Zhou, et al., 2024). The alcohol content in functional *Daqu* showed a trend of first increasing, then decreasing, and later rising again, in contrast to the traditional *Daqu*, which followed a pattern of increasing and then decreasing. Throughout the fermentation process, alcohol levels in functional *Daqu* remained at higher levels, maintaining a superior concentration (12.78 mg/kg) in the final product. The volatile acid content in traditional *Daqu* remained low throughout the fermentation, while in functional *Daqu*, acids levels peaked on day 8, fluctuated thereafter, and maintained a higher level (6.76 mg/kg) in the mature *Daqu*. Furthermore, the inoculation of microbial agents significantly increased aromatic compound levels. Specifically, the contents of aromatic compounds in matured *Daqu* reached 100.59 mg/kg, which increased significantly by 185.57 % compared with traditional *Daqu*. Notably, the pattern of changes in aromatic compounds in functional *Daqu* was similar to that of alcohols and acids, with peaks on day 11 and day 8, respectively. These findings explain the deviations of functional *Daqu* from traditional *Daqu* on days 8 and 11 (Fig. 3A), which can be attributed to the higher levels of alcohols, acids, and aromatic compounds resulting from functional strain regulation. Additionally, for monomeric aromatic compounds, functional *Daqu* exhibited

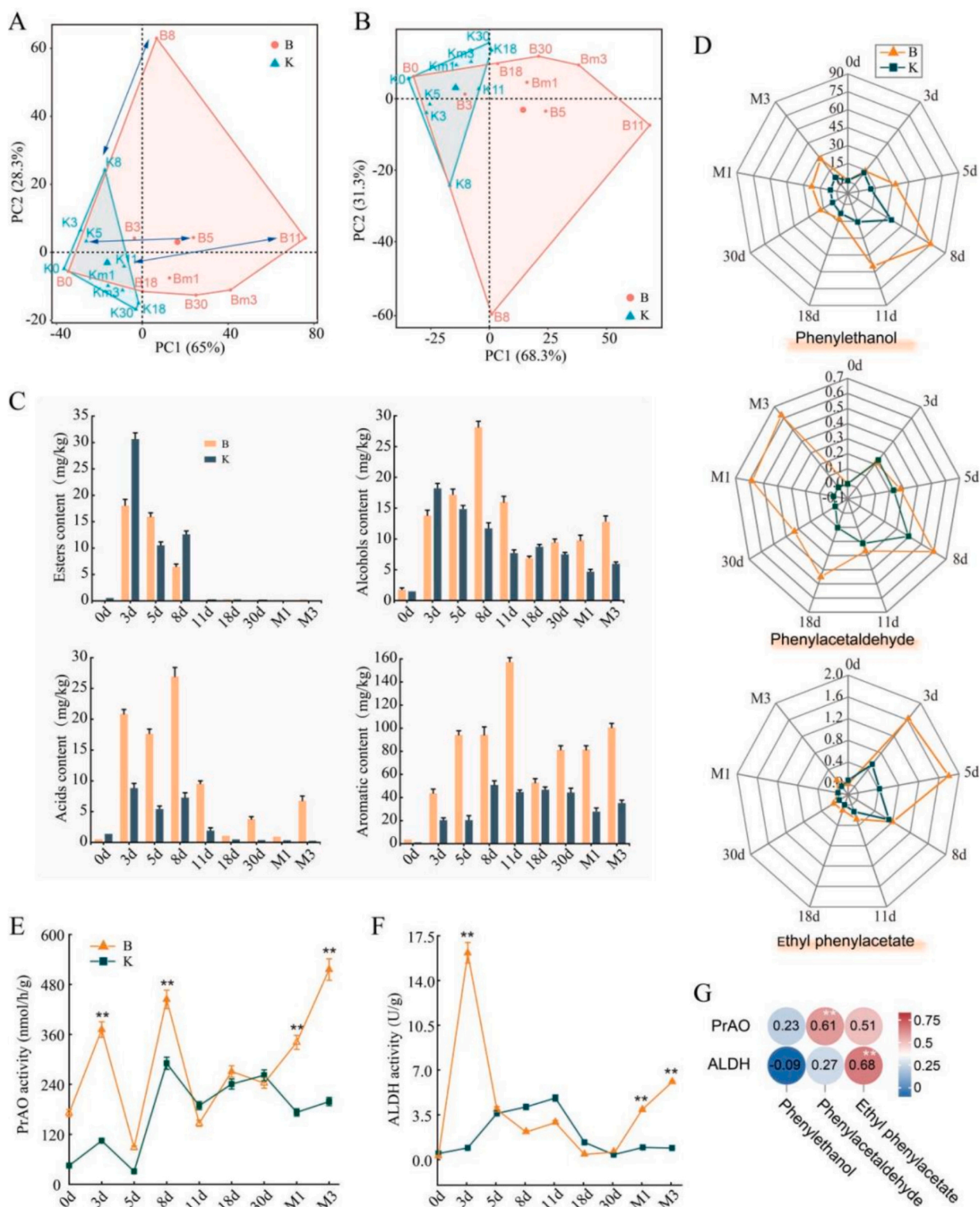


Fig. 3. Effects of functional agents on volatile flavors and aroma-producing enzymes during MHT-Daqu preparation. Principal component analysis of all volatile metabolites (A) and aromatic compounds (B), the total content of each chemical category (C), the content of phenylethanol, phenylacetaldehyde, and ethyl phenylacetate (D), primary amine oxidase activity (E), aldehyde dehydrogenase activity (F), and Spearman's correlations between enzyme activity and target metabolites (G). (* 0.01 < p ≤ 0.05, ** 0.001 < p ≤ 0.01, *** p ≤ 0.001).

significantly higher levels of phenylethanol, phenylacetaldehyde, and ethyl phenylacetate during Daqu preparation (Fig. 3D). Notably, in mature Daqu, phenylethanol, phenylacetaldehyde, and ethyl phenylacetate contents in functional Daqu were 27.48 mg/kg, 0.628 mg/kg, and 0.131 mg/kg, respectively. Phenylethanol showed a 285.85 %

increase compared to traditional Daqu, while phenylacetaldehyde and ethyl phenylacetate were not detected in traditional Daqu (Table S1). This further confirms that *Aspergillus* agents effectively promote the production of aromatic compounds.

However, the contribution of these flavor compounds in Daqu to the

flavor profile of fresh *Baijiu* remains uncertain due to the spatial and temporal heterogeneity of *Zaopei* fermentation (Cai et al., 2022) and the complexity of substance migration and transformation during distillation (He, Yang, et al., 2022). Additionally, *Daqu*, which is enriched with numerous enzymes from microbial flora metabolism (Xiao et al., 2017), also functions as a crude enzyme preparation in the brewing process. In the present study, dual functional agents that highly produce PrAO and ALDH were applied to prepare functional *Daqu*. Therefore, we further validated the improvement of enzyme activity in functional *Daqu*. Fig. 3E reveals that the dual-strain agents significantly increased PrAO activity of *Daqu*, which is consistent with our expectations. Additionally, while the ALDH activity of functional *Daqu* was generally lower than that of traditional *Daqu* during fermentation, it was significantly higher in the mature functional *Daqu* (Fig. 3F). Notably, after entering the storage period (30 days and beyond), the activity of the two enzymes in functional *Daqu* (515.9 nmol/h/g and 6.1 U/g for PrAO and ALDH) significantly higher than those of traditional *Daqu* (198.6 nmol/h/g and 0.9 U/g), suggesting that the environmental conditions in the stacking room are more conducive to the accumulation of key aromatic-producing enzymes. This aligns with the notion that at the end of fermentation, the abundance of key genes and enzyme proteins involved in aromatic compounds synthesis is highest in sauce-flavor *Daqu* (Zhu

et al., 2024). we further investigated the relationship between PrAO, ALDH, and target flavor compounds (Fig. 3G). The results showed that PrAO and ALDH were significantly positively correlated with phenylacetaldehyde and ethyl phenylacetate, respectively. Thus, the role of PrAO and ALDH as key enzymes in the synthesis of aromatic compounds is validated in MHT *Daqu*.

3.3. Effect of functional agents on microbial communities during MHT-*Daqu* production

To elucidate the regulatory effects of functional agents on the microbial community within MHT *Daqu* fermentation systems, we investigated the microbial composition and succession patterns using Illumina MiSeq sequencing. After quality control, the raw reads obtained for bacteria and fungi in 14 *Daqu* samples totaled 888,039 and 1,296,876, respectively, with high-quality sequences amounting to 816,454 and 1,275,356. Species richness and diversity of microbial communities were assessed using the Chao and Shannon indices, respectively. The bacterial communities in MHT *Daqu* exhibited higher richness and diversity than the fungal communities (Fig. 4A). Notably, functional agents significantly reduced bacterial richness and diversity while having a minor impact on fungal communities. This suggests that

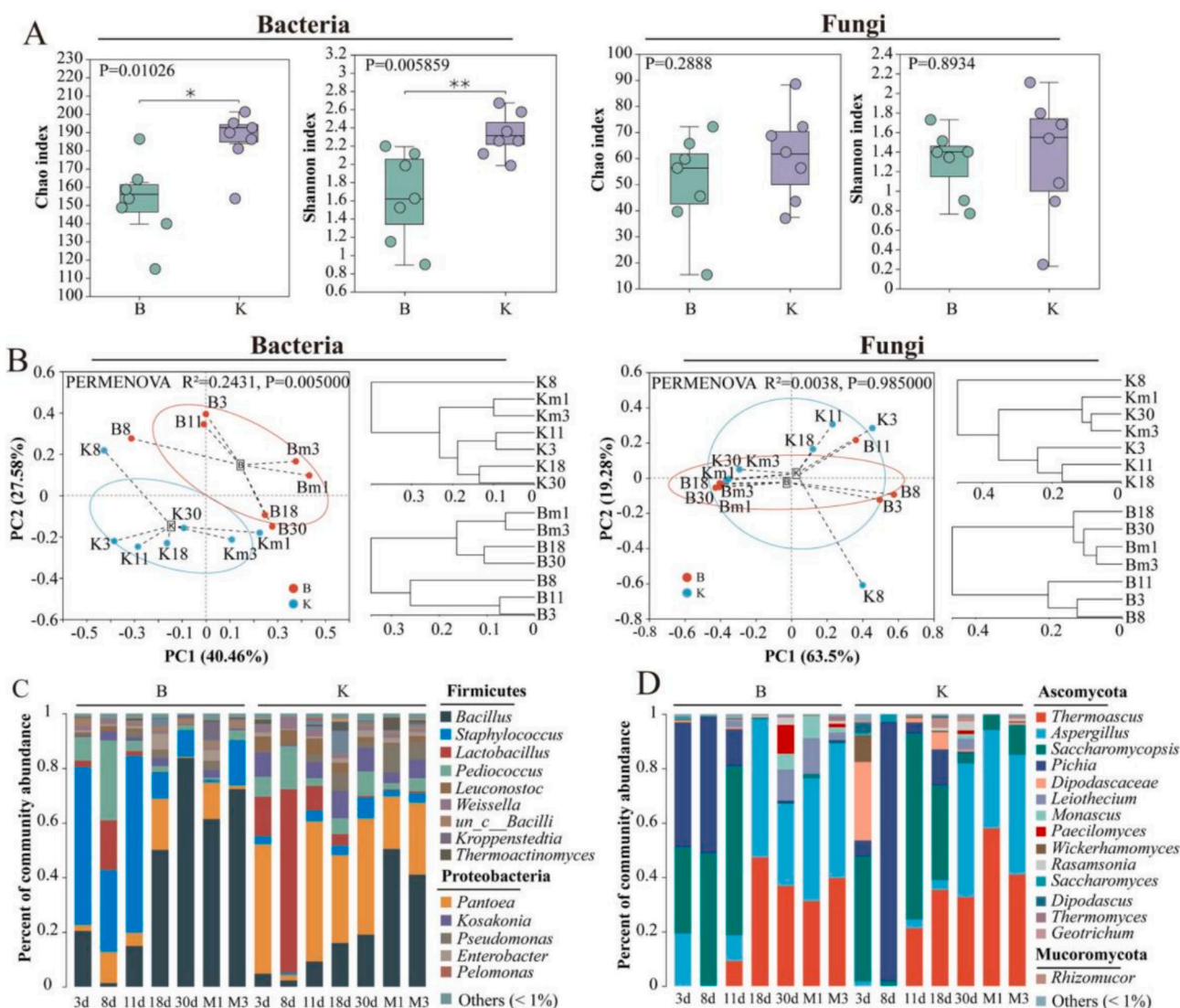


Fig. 4. Effects of functional agents on α -diversity (A), β -diversity (B), bacterial community structure (C), and fungal community structure (D) during MHT-*Daqu* preparation. (* $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$).

functional agents primarily influence bacterial composition and distribution, thereby regulating the *Daqu* microecosystem. PCoA analysis revealed clear separation between the bacterial communities of functional and traditional *Daqu*, with a PERMANOVA test confirming significant differences in their structures. In contrast, differences in fungal communities were relatively minor, particularly after 18 days of fermentation, when the fungal structures showed high similarity. Hierarchical clustering analysis identified three distinct fermentation stages during MHT *Daqu* production: the first stage (days 3 to 11), the second stage (days 18 to 30), and the storage period (M1-M3). Compared to traditional *Daqu*, functional agents enhanced stage-specific and temporal distribution, particularly by reducing specific variations observed on day 8.

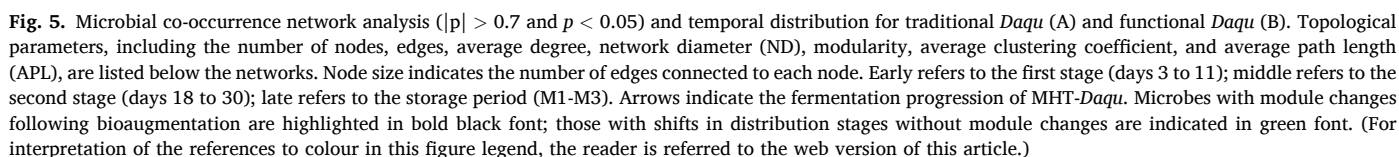
For bacteria, we identified 14 dominant genera with relative abundances greater than 1 % in both types of *Daqu*, primarily belonging to Firmicutes and Proteobacteria. During the first stage (days 3–11) of MHT *Daqu* production, the dominant genera in functional *Daqu* included *Pediococcus*, *Bacillus*, and *Staphylococcus*. In contrast, traditional *Daqu* was dominated by *Pantoea* from Proteobacteria and *Lactobacillus* from Firmicutes, with *Lactobacillus* abundance reaching 67.18 % on day 8, consistent with previous research (Yang et al., 2021). As fermentation progressed into the second stage (days 18–30), *Staphylococcus* and *Pediococcus* rapidly decreased in functional *Daqu*, while *Bacillus* significantly increased, reaching 83.74 % on day 30 and becoming the dominant genus. Conversely, traditional *Daqu* remained dominated by *Pantoea*, followed by *Bacillus*, *Kosakonia*, and *Pediococcus*. During the storage period (M1–M3), *Bacillus* remained predominant in functional *Daqu*, while in traditional *Daqu*, *Bacillus* also became dominant, accompanied by a decrease in *Pantoea* abundance. For fungi, we identified 15 dominant genera in MHT *Daqu*, primarily belonging to Ascomycota and Mucoromycota. In the first stage, functional *Daqu* was dominated by *Saccharomycopsis* and *Pichia*, with *Aspergillus*, the functional agents used in this study, also present. However, in traditional *Daqu*, significant differences were observed on day 3, *Saccharomycopsis* and *Dipodascaceae* dominated with abundances of 45.86 % and 28.89 %, respectively. By day 8, *Pichia* rapidly increased to 95.46 %, becoming the main dominant fungus. By day 11, *Saccharomycopsis* re-emerged as the dominant species (68.29 %), followed by *Thermomyces* (21.29 %). The dominant fungal genera during the first stage were predominantly yeasts and *Aspergillus*, consistent with previous findings³. In the second stage, functional *Daqu* was dominated by *Aspergillus* and *Thermomyces*, while traditional *Daqu* also showed high abundances of *Saccharomycopsis* and *Pichia*. During the storage period, both types predominantly featured *Aspergillus* and *Thermomyces* as the main fungal genera. In summary, the introduction of functional agents caused a bioturbation effect in the microbial community of the MHT *Daqu* fermentation system, likely promoting the growth of *Bacillus* while inhibiting *Pantoea* and *Dipodascaceae*. Relevant report also suggests a positive correlation between *Aspergillus* and *Bacillus*, while its relationship with *Pantoea* is relatively weak (Zhu et al., 2022). This may be due to enzymes secreted by *Aspergillus*, such as proteases and amylases (Chen, Xu, & Qian, 2018; Li et al., 2015), which improve the growth environment for *Bacillus* by degrading complex organic compounds.

3.4. Effect of functional agents on microbial co-occurrence patterns and temporal distribution

Microbial co-occurrence network analysis elucidates the impact of functional agent inoculation on the co-occurrence patterns and functional differentiation of microorganisms in environmental samples. This analysis evaluates the regulatory effects on microbial communities and identify key core microbial groups and their interactions that maintain the stability of the micro-ecosystem (Ma et al., 2016). Microorganisms within the same module often share similar or complementary functions, while different modules may serve distinct ecological roles. Modularization allows the microbial communities in the *Daqu* fermentation

system to be categorized into different modules, simplifying functions analysis (Ma et al., 2016). In this study, the microbial ecological network of traditional *Daqu* consisted of 72 nodes and 371 edges (Fig. 5A), clustered into four modules: Module 0 (37.5 %), Module 1 (29.17 %), Module 3 (19.44 %), and other modules (13.89 %). In contrast, the functional *Daqu* showed reduced nodes and edges in its microbial co-occurrence network (Fig. 5B), with six modules: Module 0 (30.43 %), Module 4 (27.54 %), Module 3 (15.94 %), and other modules (26.09 %). The microorganisms in the top three modules likely contribute most to maintaining overall ecosystem stability (Tang et al., 2023). Furthermore, the topological parameters of the functional *Daqu* network, including average degree, modularity, average clustering coefficient, and average path length, were slightly lower than those in traditional *Daqu*. This reduction reflects decreased microbial interactions, weakened local interactions, and changes in overall network compactness in functional *Daqu*. It suggests that the introduced functional agents may compete with native microorganisms in specific environments, subtly altering the original microbial community structure and functions, thereby simplifying the overall ecological network. To our knowledge, in complex microbial ecosystems, an excess of microbial nodes and interactions can introduce variability, complicating the control of fermentation conditions and leading to irregularities. By simplifying these interactions, functional agents enable the fermentation process to be primarily governed by specific functional microorganisms, ensuring more stable and predictable parameters. Furthermore, this purification process helps to clarify and stabilize the metabolic pathways of functional microorganisms, leading to consistent production of metabolites and ensuring product stability.

Based on the stage divisions derived from microbial community similarities (Fig. 4B), we performed ternary phase diagram analysis on the microbial groups within the major modules of the co-occurrence network. This analysis aimed to reveal the specific fermentation stages during which these microbes perform certain functions, helping us infer their potential roles at different stages (Ma et al., 2016) and enhancing our understanding of the *Daqu* maturation mechanism. A comparison of the microbial group composition in the major modules of both *Daqu* types (Fig. 5) indicates that the inoculation of functional agents altered the ecological niches of several microbes, including bacteria such as *Weissella*, *Lactococcus*, *Kroppenstedtia*, *Pseudomonas*, *Enterobacter*, and *Enterococcus*, along with fungi like *Saccharomycopsis*, *Pichia*, and *Thermomyces*. Functional agents also modified the temporal distribution of certain microorganisms (Fig. 5 and Tables S2-S3). In traditional *Daqu*, *Bacillus*, *Virgibacillus*, *Thermoactinomyces*, *Thermoascus*, and *Aspergillus* were predominantly present in the storage period, indicating that thermophilic microbes remain active during storage. Conversely, *Lactobacillus*, *Saccharomyces*, and *Pichia* were concentrated in the first stage, suggesting that yeasts primarily populate the early fermentation phase, consistent with previous findings (Zhu et al., 2022). However, post-bio-augmentation, *Bacillus* and *Thermoascus* shifted their activity from the third to the second stage. *Saccharomycopsis*, *Pediococcus*, *Lactococcus*, *Weissella*, and *Leuconostoc* were concentrated in the first stage, further confirming that yeasts and lactic acid bacteria dominate the early fermentation stage. Notably, thermophilic microbes such as *Thermoactinomyces*, *Thermomyces*, *Kroppenstedtia*, and *Virgibacillus* were mainly concentrated during the *Daqu* storage period, resembling the temporal distribution in traditional *Daqu*. Although the distribution stage of *Aspergillus*, the functional agent used, remained unchanged, its primary function in functional *Daqu* may have shifted compared to traditional *Daqu*. We hypothesize that these changes in microbial co-occurrence patterns after bio-augmentation could contribute to the production of aromatic compounds, a hypothesis that will be further explored in section 3.5, which examines the metabolic function response of microbiomes.



Microbial community succession in *Daqu* is influenced by both microbial ecological relationships and abiotic factors such as environmental and physicochemical conditions, which serve as major driving forces for controlled fermentation (Ma et al., 2022). Microbes within the same module often exhibit similar or complementary functions, potentially occupying similar ecological niches, and responding similarly to environmental factors (Ma et al., 2016). To further elucidate the functional differentiation of the major microbial modules throughout the MHT *Daqu* fermentation process and their responses to these factors, we conducted a Mantel test to analyze the relationships between the modules, environmental variables, enzymatic properties, and related volatile flavors (Fig. 6A and B).

For traditional *Daqu* (Fig. 6A), Module 0 showed significant positive correlations with temperature, moisture, and PE, along with a highly significant positive correlation with EA, esters, and EP. This largest module comprises 27 genera, including 22 bacteria and 5 fungi (Fig. 5A). Key members of Module 0 include *unclassified-f_Lactobacillaceae*, *Bacillus*, *Thermoactinomyces*, *Virgibacillus* (bacteria), as well as *Thermoascus*, *Aspergillus*, and *Pichia* (fungi). These thermophilic microbes are well-adapted to the high temperature of *Daqu* fermentation (He et al., 2019; Ma et al., 2022), explaining the correlation with temperature. Additionally, moisture and temperature drive the succession of *Aspergillus*, *Pichia*, and *Saccharomyces* (Ban et al., 2022). Importantly, *Bacillus*, *Virgibacillus*, *Lactobacillus*, yeasts, and *Aspergillus* in Module 0 all possess ester-producing capabilities (Huang et al., 2014; Song et al., 2021; Su et al., 2020). Module 1, which is positively correlated with PE and EP, includes key groups like *Saccharomycopsis* and

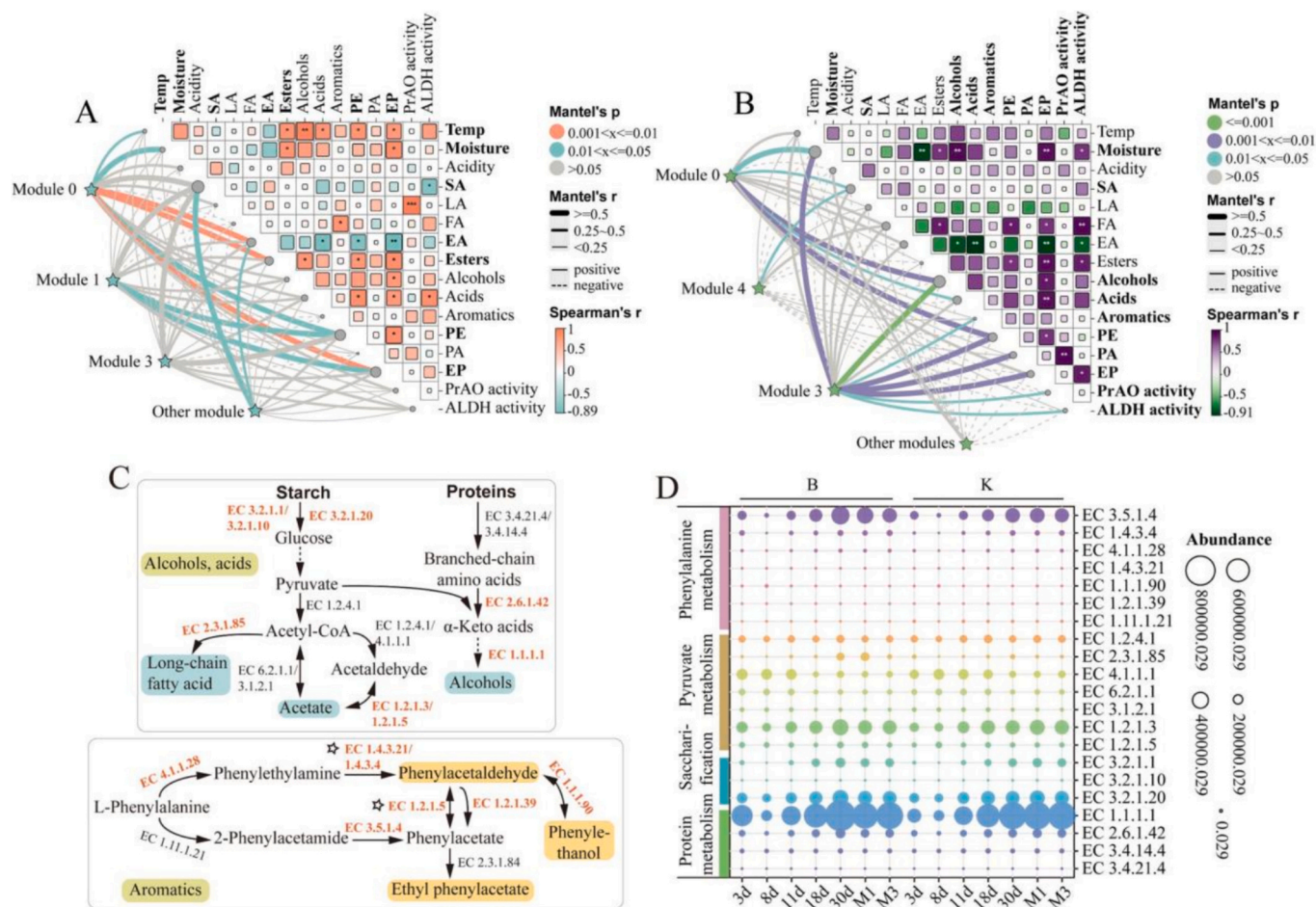


Fig. 6. Environmental response and metabolic function of microbiomes. Mantel test of modules and related variables for traditional *Daqu* (A) and functional *Daqu* (B). Metabolic pathways of alcohols, acids, and aromatic compounds (C), and the abundance variation of expressed enzymes (D) during MHT-*Daqu* production. In the mantel test, edges colour indicate statistical significance, edge widths represent the correlation strength, and solid/dashed edges denote positive/negative correlations, respectively. The r -value is derived from Spearman's correlation coefficient (* $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$). Temp, temperature; SA, saccharifying ability; LA, liquefying ability; FA, fermenting ability; EA, esterifying ability; PE, phenylethanol; PA, phenylacetaldehyde; EP, ethyl phenylacetate; PrAO, primary amine oxidase; ALDH, aldehyde dehydrogenase. In the metabolic pathways, enzymes with increased expression after biofortification are shown in red font; blue backgrounds indicate alcohols and acids, while yellow backgrounds indicate aromatic compounds. The pentagram indicates PrAO and ALDH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Wickerhamomyces, potentially linked to aromatic compound production (Zha et al., 2018). Post-bio-augmentation, changes in microbial functions were reflected in altered response patterns to various factors. As shown in Fig. 6B, Module 0 lost sensitivity to temperature and instead exhibited a significant positive correlation with alcohols and acids, consistent with earlier findings (Fig. 3C). This shift may be due to the concentrated distribution of lactic acid bacteria within specific modules and stages (Fig. 5B), promoting synergistic acid production. Additionally, the bio-perturbation effect led to Modules 0 and 4 gaining saccharification capabilities, potentially linked to ecological shifts involving *Monascus*, *Saccharomyces*, *Weissella*, and *Rhizopus* (Zhao et al., 2021). Notably, the introduction of functional agents endowed Module 3 with enhanced functions, particularly in aromatic compounds production. This is evidenced by significant correlations with target flavor compounds (phenylethanol, phenylacetaldehyde, ethyl phenylacetate) and key enzyme activities (PrAO and ALDH). After bio-augmentation, Module 3 primarily comprised *Aspergillus*, *Pichia*, *Millerozyma*, *Pseudomonas*, *Paenibacillus*, and *Rhizomucor*, confirming that shifts in microbial co-occurrence patterns facilitated the production of aromatic flavor compounds. Consequently, Module 3, identified as key group for aromatic flavor production, was mainly influenced by moisture as the driving factor. With changes in moisture content (Fig. 2B), the ecological

niches of microbes in *Daqu* shifted dynamically (Fig. 4C and D). Early in fermentation, *Daqu* blocks had high moisture, with yeasts such as *Pichia* and *Millerozyma* predominating, driving fermentation and substrate degradation. As moisture decreased, drought-tolerant microbes, including *Aspergillus*, *Pseudomonas*, *Paenibacillus*, and *Rhizomucor*, gradually took over, stabilizing the microbial community. Previous studies indicate that moisture significantly influences bacterial and fungal community dynamics during *Daqu* fermentation, serving as a major driver of microbial succession (Liu et al., 2023; Ma et al., 2022; Xu et al., 2024). Additionally, changes in moisture content further promote the production of various enzymes by microbes (Liu et al., 2023; Ma et al., 2022). Consequently, future efforts could focus on controlling moisture content during MHT *Daqu* fermentation to promote the accumulation of aromatic compounds and key enzymes.

The results indicate that functional microbial agents enhanced saccharification, alcohol, acid, and aromatic compound profiles by altering ecological interactions within the MHT *Daqu* microbial community. This improvement correlates closely with the expression of key functional enzymes in metabolic pathways. Therefore, we utilized PIC-RUST2 to predict variations in functional enzyme abundance during MHT *Daqu* preparation, and KEGG was used to annotate metabolic pathways associated with the formation of alcohols, acids, and aromatic

compounds. As shown in Fig. 6C, starch is converted into glucose by alpha-amylase (EC 3.2.1.1), oligo-1,6-glucosidase (EC 3.2.1.10), and alpha-glucosidase (EC 3.2.1.20). The high expression levels of these enzymes in functional *Daqu* (Fig. 6D) account for the improved saccharification ability. Pyruvate, generated through glycolysis, can indirectly contribute to acid formation via acetyl-CoA or serve as an amino group acceptor in synthesizing higher alcohols. The increased abundance of fatty-acid synthase (EC 2.3.1.85) and aldehyde dehydrogenase (EC 1.2.1.3/1.2.1.5) (Fig. 6D) facilitates the production of long-chain fatty acids and acetic acid, which explains the rise in acid levels after biofortification (Fig. 3C). Additionally, branched-chain-amino-acid transaminase (EC 2.6.1.42) and alcohol dehydrogenase (EC 1.1.1.1) are crucial enzymes for branched-chain amino acids metabolism (Hazelwood Lucie et al., 2008; Liu et al., 2022), leading to higher alcohol production (Kłosowski et al., 2015). Their elevated expression in functional *Daqu* results in increased alcohol content (Fig. 3C). Furthermore, the production of aromatic compounds originates from the metabolism of aromatic amino acids, particularly phenylalanine (Wang, Tang, et al., 2024). As depicted in Fig. 6C, phenylacetaldehyde, phenylethanol, and ethyl phenylacetate are synthesized from *L*-phenylalanine through two pathways, similar to those found in sauce-flavored *Daqu*⁵. One pathway produces phenylethylamine via aromatic-*L*-amino-acid decarboxylase (EC 4.1.1.28), which is then oxidized by amine oxidase (EC 1.4.3.21/1.4.3.4) into phenylacetaldehyde. This compound can be reduced to phenylethanol or oxidized to phenylacetate by ALDH (EC 1.2.1.5), with the latter converted to ethyl phenylacetate by alcohol acetyltransferase (EC 2.3.1.84). Phenylacetate may also arise from *L*-phenylalanine through conversion to 2-phenylacetamide. Notably, after biofortification, most enzymes involved in aromatic compound synthesis exhibited increased expression in MHT *Daqu*. These results indicate that functional agents not only successfully promoted the high expression of two key enzymes, PrAO (EC 1.4.3.21) and ALDH (EC 1.2.1.5), in MHT *Daqu* but also enhanced the expression levels of other related enzymes, creating favorable conditions for the increased production of aromatics in strong-aroma Baijiu.

4. Conclusion

This research methodologically combines physicochemical evaluations with microbiomics and flavoromics techniques to deliver a thorough analysis of how *Aspergillus* functional agents with high enzyme activities impacts the overall quality of MHT *Daqu*. Flavoromics and enzyme activity analyses demonstrated that functional microbial agents promoted flavor compounds production, particularly by enhancing the activity of PrAO and ALDH in mature *Daqu*. Microbial composition and co-occurrence network analyses further revealed that functional agents primarily regulated the *Daqu* micro-ecosystem by altering the ecological niches and temporal distribution of microbial communities, thereby contributing to a more controlled fermentation process. Variations in microbial response patterns to different factors and the abundance of related enzymes confirmed that these shifts in ecological relationships facilitated the production of target flavor compounds and enzymes during MHT *Daqu* preparation. Moisture was identified as an abiotic driving factor for key aromatics-producing groups consisting of *Aspergillus*, *Pichia*, *Milleromyces*, *Pseudomonas*, *Paenibacillus*, and *Rhizomucor*. This research contributes to the development of a controllable micro-ecosystem that supports the targeted accumulation of aromatic substances, enhancing the flavor and quality of strong-flavor Baijiu.

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CRedit authorship contribution statement

Min Zhu: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Data curation, Conceptualization. **Zhao Deng:** Writing – review & editing, Visualization, Methodology. **Mingyao Wang:** Writing – review & editing, Visualization, Validation. **Yu Tie:** Writing – review & editing, Validation, Methodology. **Wenxue Zhang:** Supervision, Project administration, Funding acquisition. **Zhengyun Wu:** Writing – review & editing, Project administration. **Zhengfu Pan:** Validation, Supervision. **Guorong Luo:** Resources, Investigation. **Renfu Wu:** Validation. **Jianliang Qin:** Validation, Investigation. **Katsuya Gomi:** Writing – review & editing.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2025.102304>.

Data availability

Data will be made available on request.

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