Volatile organic compounds (VOCs) are chemicals responsible for antagonistic activity between microorganisms. The impact of VOCs on microbial community succession of fermentation is not well understood. In this study, *Pichia* spp. were evaluated for VOC production as a part of antifungal activity during baijiu fermentation. The results showed that the abundance of *Pichia* in the defect group (agglomerated fermented grains) was lower than that in control group, and a negative interaction between *Pichia* and *Monascus* was determined (*P* < 0.05). In addition, the disruption of fungi was significantly related to the differences of metabolic profiles in fermented grains. To determine production of VOCs from *Pichia* and its effect on *Monascus purpureus*, a double-dish system was assessed, and the incidence of *M. purpureus* reduction was 39.22% after 7 days. As to antifungal volatile compounds, 2-phenylethanol was identified to have an antifungal effect on *M. purpureus* through contact and noncontact. To further confirm the antifungal activity of 2-phenylethanol, scanning electron microscopy showed that 2-phenylethanol widely and significantly inhibited conidium germination and mycelial growth of filamentous fungi. Metatranscriptomic analysis revealed that the Ehrlich pathway is the metabolic path of 2-phenylethanol in *Pichia* and identified potential antifungal mechanisms, including protein synthesis and DNA damage. This study demonstrated the role of volatile compound-mediated microbial interaction in microbiome assembly and discovered a plausible scenario in which *Pichia* antagonized fungal blooms. The results may improve the niche establishment and growth of the functional yeast that enhances the flavor of baijiu.

**IMPORTANCE** Fermentation of food occurs within communities of interacting species. The importance of microbial interactions in shaping microbial structure and metabolic performance to optimize the traditional fermentation process has long been emphasized, but the interaction mechanisms remain unclear. This study applied metabolome analysis and amplicon sequencing along with metatranscriptomic analysis to examine the volatile organic compound-mediated antifungal activity of *Pichia* and its effect on the metabolism of ethanol during baijiu fermentation, potentially enhancing the establishment of the fermentation niche and improving ethanol metabolism.

**KEYWORDS** biocontrol, volatile organic compounds, yeasts, metatranscriptomic analysis, 2-phenylethanol

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**Editor** Ning-Yi Zhou, Shanghai Jiao Tong University

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Address correspondence to Hai Du, dihuai88@126.com, or Yan Xu, yxu@jiangnan.edu.cn.

Received 12 December 2020

Accepted 12 February 2021

Accepted manuscript posted online 19 February 2021

Published 13 April 2021
boiling point, and a lipophilic moiety (4). These characteristics provide VOCs with significant antimicrobial properties, even at low concentrations (4, 5). The mobility of VOCs facilitates evaporation and diffusion through solid-, liquid-, and gas-filled pores in the environment, and because direct contact between microorganisms is not required for them to function, VOCs are considered ideal antimicrobials (5, 6). The importance of VOCs has been studied in the rhizosphere microbial community (7) and among plant-pathogenic soil fungi (8). For many fermented products, VOCs provide vital contributions to the quality and commercial value of the product (9). Although it is well understood that significant differences in flavor may be driven by different microbial structures, the ecological functions of VOCs in the fermentation process remain largely elusive.

Chinese liquor, or baijiu, is a valuable product produced by traditional spontaneous solid-state fermentation (10). Filamentous fungi are important saccharifying microorganisms (11, 12), but due to their physiological characteristics, they may have both positive and negative effects during baijiu fermentation. For the positive effects, filamentous fungi secrete quantities of extracellular enzymes (α-amylase, β-amylase, and glucoamylase) and participate in starch and complex polymer decomposition (13–15). Other enzymes, including lipase and 3-keto acid decarboxylase, excreted by filamentous fungi are important for the creation of flavors (16, 17). Filamentous fungi are diverse (11, 18), but the total population must be maintained at a low level (maximum, \(1.28 \times 10^4\) CFU/g) during fermentation. Blooms of filamentous fungi during the baijiu fermentation process can have negative effects that include significant alcohol yield declines and flavor defects, known as “stuck fermentation” (diaopai in Chinese) (Fig. 1A). Previous studies have shown that filamentous fungi reduced the alcohol yield by producing polyols, including mannitol and erythritol (19), and may also generate mycotoxins (20–22), such as ochratoxin A (21) and citrinin (23), that pose a threat to food safety. Therefore, it is of great importance to control the development of filamentous fungi in the fermentation process.

Filamentous fungal blooms are complex and have long-term effects. The many possible physical, chemical, and biological causes often make them difficult to control. Previous attempts to explain these patterns of problematic fermentation have focused on the abiotic environment (24), but the application of traditional measures (e.g., temperature or acidity adjustment and novel starter cultures) has not always solved the problem. Despite decades of research, the cause of this pattern has remained elusive because the spontaneous fermentation of baijiu happens within communities of interacting species (10). Therefore, biologically based technologies, such as biological control, could be a more promising approach to solve the urgent requirements in fermentation management (3). Because of the antagonistic action toward fungi, yeasts have been developed as biocontrol agents of food spoilage (25). Pichia organisms have recently been proven to have antagonistic interactions with filamentous fungi in baijiu fermentation (26). A more comprehensive understanding of the inhibitory compounds produced by Pichia, and an evaluation of the effects of antagonism interactions on metabolic differences, needs further study.

In this study, we used the sauce flavor baijiu fermentation system as a model to identify the antifungal of antagonism interaction mechanism of Pichia and its effect on the metabolic difference in baijiu fermentation. By combining microbial community succession from in situ communities and in vitro reconstructions of experimental communities, we revealed that the microbial structural and metabolic performances of baijiu fermentation are driven by different interactions. A novel perspective is presented on how to use modulation of interactions as a means to alter the product characteristics of baijiu.

**RESULTS**

**Microbial succession during sauce flavor baijiu fermentation.** In the practical production process, sauce flavor baijiu fermentation is divided into two stages: heap
fermentation and pit fermentation. The two batches of baijiu fermentation in our study used the same raw materials (sorghum) and operation technology. Mature daqu powder was mixed with the sorghum steamed before. Then the mixture was heaped as a cone on the ground for natural fermentation. The endpoint of heap fermentation was assessed by experienced workers. At the end of heap fermentation, the group with agglomerated fermented grains was defined as the defect group and the other as the control group (Fig. 1A). The control and defect groups took 6 and 8 days, respectively, for heap fermentation.

To characterize the microbial community structures of different fermentation groups, high-throughput sequencing was used. Sequencing coverage was satisfactory for all samples (see Table S1 in the supplemental material). After quality control, 993,850 high-quality reads of bacteria and 855,294 fungal sequences were obtained from 24 samples of the different groups. The operational taxonomic units (OTUs) were clustered at a 97% similarity level of sequences. Phylogenetic analysis established that the sequence reads corresponded to 14 different bacterial phyla (mainly Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes) and 5 different fungal phyla (mainly Ascomycota, Basidiomycota, Mucoromycota, and Streptophyta). The microbial diversity

**FIG 1** Microbial communities in fermented grains. (A) The status of fermented grains in the control group and defect group. (B) Microbial community diversity of fermented grains. *, P < 0.05 (Tukey’s test). (C) Succession of bacteria communities during heap (H) and pit (F) fermentation. (D) Succession of fungal communities during heap (H) and pit (F) fermentation. (E) Nonmetric multidimensional scaling (NMDS) plot of fungal communities. (F) Relationships among fungal communities. A connection represents a significant (P < 0.05) and strong (Spearman’s |ρ| > 0.5) correlation. The color of the edges corresponds to a positive (red) or negative (blue) relationship.
was significantly higher \( (P < 0.05) \) in the defect group according to the observed species index (Fig. 1B).

The relative abundance of microorganisms was determined at the genus level to determine the significant contributors in different fermentation groups. As shown in Fig. 1C, we found that for bacteria, the largest groups of genera were *Kroppenstedtia, Bacillus*, and *Virgibacillus* during heap fermentation, while *Lactobacillus* (relative abundance potential > 80%) dominated during the pit fermentation. For the fungal genus, *Picha* had a higher relative abundance in the control group (potential of > 64% [Fig. 1D and Fig. S1]). However, *Monascus* was abundant in the defect group but rare in the control group (3% in the control group).

More specifically, metastaististical analysis revealed that 77 fungal OTUs were significantly different \( (P < 0.05; q < 0.5) \) between the two groups, and 15 OTUs among them were found with an average abundance of >0.1% (Table S2). OTU207 (*Picha kudriavzevii*), OTU208 (*P. kudriavzevii*), OTU25 (*Saccharomyces paradoxus*), OTU26 (*S. paradoxus*) and OTU157 (*Byssochlamyx spectabilis*) were more abundant in the control group than in the defect group. However, OTU11 (*Zygosaccharomyces bailii*), OTU209 (*Leiothecium ellipsoidaeum*), OTU239 (*Toxicocladosporium irritans*), OTU241 (*Cladosporium phaeocoma*), OUT262 (*Picha membranifaciens*), OTU278 (*Hyphopichia burtonii*), OTU309 (*Geotrichum silvicola*), OTU53 (*Rhizopus azygosporus*), OTU93 (*Monascus pilosus*), and OTU97 (*Monascus purpureus*) showed the opposite trend.

Furthermore, nonmetric multidimensional scaling analysis (NMDS) based on the Bray-Curtis distance matrix at the OTU level indicated that the microbial communities of bacteria were similar; however, the microbial communities of fungi were distinctly separate from other groups (Fig. 1E and Fig. S1). Further analysis of similarity (ANOSIM) also revealed significant differences \( (P < 0.001 \text{ [Fig. S1]}) \) in communities between the two groups. To explain the difference in the fungal community structure, the correlations of microbes (with an average abundance above 0.1%) were explored based on Spearman’s rank correlations \((|\rho| > 0.5 \text{ and } P < 0.05)\). The network of nine fungal genera was divided into two groups (Fig. 1F). The first group included *Saccharomyces* and four other fungi, among which *Saccharomyces* showed a significant negative correlation with other microorganisms. The second group included *Picha, Zygosaccharomyces, Monascus*, and *Aureobasidium*, among which *Monascus* was negatively correlated with *Picha*. These results indicated that the microbial interactions contributed to the assembly of fermentation-related microbial communities and that the disruption of their abundance may lead to a defect.

**Metabolic profiles and their relationships with microbial communities during fermentation.** Fermented grain samples were collected during fermentation from the control and defect groups (16 fermented grain samples total) to investigate the difference of volatile flavors in the two groups. A total of 50 metabolites (19 esters, 6 alcohols, 8 organic acids, 9 aromatics, and 8 others) were identified from the 16 samples. Principal-component analysis (PCA) was used to analyze the variability in the volatile flavors between the different groups. The PCA in Fig. 2A shows that the distribution of volatile flavors in stage I was clearly separated from stage II using the first principal component (PC1), whereas PC2 further differentiated the volatile flavors in the two different groups. Moreover, hierarchical cluster analysis (HCA) also revealed that the distance between the clusters tended to expand with fermentation time and groups (Fig. 2B). Combined with the analysis of the microbial structure, these results lead us to suggest that the difference in flavor may be related to the difference in fungal communities between the two groups.

Four distinct groups could be visualized in the PCA ordination space and hierarchical cluster analysis, and each group included more than three samples, so that further statistical tests could be performed. To sharpen the separation between samples of different groups during different stages, partial least squares discriminate analysis (PLS-DA) was performed (Fig. S2). As shown in Fig. S2, it was found that the categories and the content of volatile compounds increased with fermentation. The detailed distinctions of VOCs in different periods were revealed by the loading plot of PLS-DA (Fig. S2). Terms with variable
importance in the projection (VIP) of $>1.0$ are the most relevant for explaining the different clusters of different fermentation groups (Table S3). In stage I, eight VOCs (VIP $>1.0$) were significantly distinguished from the different groups. In stage II, there were nine volatile flavors with VIP of $>1.0$. The acids (acetic acid and propanoic acid), esters (ethyl lactate), and other compounds (trimethyl-pyrazine and tetramethyl-pyrazine) showed strong correlation with defect group. The esters (ethyl acetate and ethyl hexadecanoate) and alcohols ([2R,3R]-butanediol) (VIP $>1.0$) contributed to the specificity of the control group (Fig. 3C and D).

To detect the relationships between microbial communities and metabolites, Spearman’s correlation was performed to analyze the correlation between microbiota and the metabolites during fermentation. Combined with the significant differences in
fungal community structure, the relationship between fungi and different metabolites was analyzed. In stage I, eight different volatile flavors (VIP > 1.0) were significantly related to 12 fungi (Fig. 2C). Pichia was an important fungus that contributed to 2-phenylethanol and ethyl acetate. In stage II, nine different volatile flavors were significantly related to six fungi. Monascus and Zygosaccharomyces were the important fungi that contributed to propanoic acid and tetramethyl-pyrazine (Fig. 2D). These results demonstrated that microorganisms appear to underlie the production of different VOCs, but the influence of VOCs on microbial community structures needs further exploration.

Characteristics of volatile organic compounds produced by Pichia. To link in situ observations of species abundance, the volatile organic compounds produced by Pichia in vitro were demonstrated. Because the dominant OTU (OTU 208) of Pichia was closest to Pichia kudriavzevii and OTU 97 was closest to M. purpureus, these two microorganisms were isolated from the fermented samples. The potential antifungal effects of VOCs from P. kudriavzevii on the growth of M. purpureus were studied.

First, a double-dish system (DDS) of sorghum extract agar was used to determine if yeast could produce VOCs to affect M. purpureus growth (Fig. 3A). This antagonistic action was reflected by the significant reduction (P ≤ 0.05) of the mycelial growth cultured at 30°C (Fig. 3B). M. purpureus in the control batch grew at 11.26 ± 0.67 mm day⁻¹ on the sorghum extract agar plate; the addition of P. kudriavzevii significantly reduced the growth rate of M. purpureus (4.80 ± 1.07 mm day⁻¹). The reductions ranged from 33.90% (P < 0.01) in 5 days
to 39.22% in 7 days. These results provided a preliminary indication of the correspondence between the abundance patterns in experimental interactions observed in vitro and in situ.

Second, to further identify antifungal volatile compounds, the fermentation experiments were performed with single strains and their combinations. The major volatile compounds in the fermented samples were extracted via headspace solid-phase microextraction (HS-SPME) and analyzed via gas chromatography-mass spectrometry (GC-MS). The heat map shown in Fig. 3C was used to visualize the details of the 25 volatile compounds, which were categorized as alcohols (9), acids (5), esters (4), aldehydes (2), ketones (2), and others (2). There were notable differences in the volatile compounds among P. kudriavzevii, M. purpureus, and their combinations. As shown in Fig. S3, when organisms were cultured alone, relatively low levels of the volatile compounds (3.7 ± 0.84 mg/liter) were detected in the fermentation of M. purpureus, and more compounds could be detected in the fermentation of P. kudriavzevii (21.83 ± 3.38 mg/liter). Although P. kudriavzevii significantly inhibited the growth of M. purpureus, the total content of volatile substances increased (attained 23.31 ± 1.98 mg/liter) when they were cocultured. The coculture mainly increased the production of esters and ketones (Fig. S3D and F). Moreover, the compounds produced by P. kudriavzevii in mono- and cocultures primarily included three alcohols (3-methyl-1-butanol, 2-methyl-1-butanol, and 2-phenylethanol) and two esters (ethyl acetate and 3-methylbutyl acetate). Among these, 2-phenylethanol has the highest content (Table 1).

Third, the antifungal activity of 2-phenylethanol was identified (Fig. 2C). As shown in Fig. 3D and E, 2-phenylethanol has effective antifungal influences on M. purpureus through both contact and noncontact. Under noncontact conditions, the suppression ratio of 2-phenylethanol against Monascus reached 31.24% (Fig. 3F). These results showed that Pichia inhibited Monascus by producing volatile compounds such as 2-phenylethanol.

To evaluate the effect of 2-phenylethanol on common fungi, the agar diffusion experiment and microscopic evaluation were conducted. The agar diffusion experiment showed that 2-phenylethanol could strongly inhibit the growth of all species of common fungi (Fig. 5A). Moreover, the effect of 2-phenylethanol on the mycelium morphological changes of common fungi were examined by scanning electron microscopy (SEM). As shown in Fig. 4F, untreated control hyphae had a uniform cell morphology and no visible damage, 2-phenylethanol significantly inhibited spore germination, and severely damaged mycelia with irregular morphology and flattened hyphae along with ruptured surfaces were observed. These results indicate that 2-phenylethanol has broad-spectrum antifungal properties against filamentous fungi.

**Antifungal mechanism of Pichia and effect on metabolic profiles.** To explore microbial function in different groups, functional gene expression in situ was determined by metatranscriptomic data analysis. A total of ~58.2 million Illumina HiSeq sequences were recovered as good-quality sequences from metatranscriptomic libraries. Using the de novo assembly program Trinity, a total of 169,290 contigs were assembled. For annotation, similarity searches were performed to annotate unigenes against different databases using BLASTX. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed and demonstrated 15,812 and 52,117 unigenes.

To understand the gene expression patterns in different groups, fragments per kilobase of transcript per million mapped reads (FPKM)s were calculated for each sample, and all the unigenes were annotated. First, sample correlation analysis based on

| TABLE 1 Main VOCs produced by Pichia and Monascus during incubation at 30°C for 5 days |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Organism(s) | Higher alcohol (mg/liter) | Ester (mg/liter) | Total |
| P. kudriavzevii | 2-Methyl-1-butanol | 1.23 ± 0.13 | 6.30 ± 1.22 | 9.16 |
| M. purpureus | 3-Methyl-1-butanol | 1.63 ± 0.18 | 0.10 ± 0.03 | 1.31 |
| P. kudriavzevii + M. purpureus | 2-Phenylethanol | 1.74 ± 0.53 | 0.01 ± 0.00 | 1.07 |
| | Total | 5.45 | 0.14 | 5.59 |
Pearson correlation indicated that the samples from different groups were significantly different ($r = 0.045$ [Fig. S5]). Second, differential miRNA expression analysis of the two libraries was performed using the DEGseq R package (27) based on read count. The significant differentially expressed genes (DEGs) were evaluated against a $|\log_2$ (fold change)$| \leq 2$ and false-discovery rate (FDR) of $<0.1$. The results showed that 323 genes were significantly differentially expressed, including 63 upregulated genes and 260 downregulated genes. Based on KEGG pathway annotation, the main downregulation expression pathways in the defect group were distributed in the citric acid cycle (tricarboxylic acid [TCA] cycle), 2-oxocarboxylic acid metabolism, RNA transport, carbon metabolism, biosynthesis of amino acids, biosynthesis of antibiotics, biosynthesis of secondary metabolites, and metabolic pathways, of which most are related to yeast. The clearly enriched pathways in the defect group were lysine biosynthesis, lysine degradation, starch and sucrose metabolism, oxidative phosphorylation, mitogen-activated protein kinase (MAPK) signaling pathway (yeast), biosynthesis of amino acids, ribosome, biosynthesis of antibiotics, metabolic pathways, and biosynthesis of
secondary metabolites, of which most were related to filamentous fungi. These results indicate that there are significant functional differences between the control and defect groups.

For a detailed understanding of the function of filamentous fungi in the two groups, the genes expressed in filamentous fungi, which were determined using the FPKM method, were compared. Clusters of orthologous groups (COGs) were mostly affiliated with translation, ribosomal structure, and biogenesis, followed by energy production and conversion (Fig. 4B). Several ribosomal component genes (83 genes) were significantly downregulated in the control group, including more than 26 protein genes (posttranslational modification and protein turnover). Furthermore, the groups of filamentous fungus genes were significantly downregulated in response to *Pichia* (Fig. 4C). In addition, protein phosphatase with serine/threonine phosphatase activity (PPP1C) was ubiquitously expressed in the control group, which influences the negative control of cell growth and division.

Metatranscriptomic analysis also provided further insight into the pathway of *Pichia* on the metabolic profiles of baijiu fermentation. First, a schematic diagram of the 2-phenylethanol metabolic pathway in *Pichia* was reconstructed (Fig. 5A). 2-Phenylethanol was mainly produced via the Ehrlich pathway and higher levels of gene expression in the control group (Fig. 5B). This is consistent with the significantly higher 2-phenylethanol in the control group. Second, ethanol is the main product of baijiu fermentation, and the core microorganisms and genes related to ethanol production are illustrated. As shown in Fig.

**FIG 5** KEGG expression genes of *Pichia* on the metabolic profiles during baijiu fermentation. (A) Ehrlich pathways to produce 2-phenylethanol. (B) FPKM of KEGG expression genes related to 2-phenylethanol metabolism. ****, false-discovery rate (FDR) < 0.001. (C) FPKM of KEGG expression genes related to metabolic pathway from acetaldehyde to ethanol. (D) Dynamics of ethanol content during baijiu fermentation.
In the control group, various yeasts (including *P. kudriavzevii*, *Z. bailii*, *Saccharomyces cerevisiae*, *Schizosaccharomyces cryophilus*, and *Brettanomyces bruxellensis*) were the dominant functional contributors to ethanol production (FPKM > 100 [Fig. 5C]). Meanwhile, in the defect group, *Z. bailii* and filamentous fungi (*Aspergillus fumigatus*, *Aspergillus calidoustus*, and *Penicillium expansum*) showed transcript abundance of the gene encoding ADH (FPKM > 100). ADH in different microorganisms displayed different metabolic capacities to form ethanol, which explained the variation in ethanol content between samples during production (Fig. 5D). These results indicated that the community driven by *Pichia* was beneficial to maintain the diversity of yeast and the ethanol metabolism. Our findings showed that the antagonism of *Pichia* plays an important role in the fermentation of sauce flavor baijiu.

**DISCUSSION**

Microbial interactions have been frequently proposed as potential drivers of microbiome diversity and thereby determine the performance of their communities; however, the mechanisms underlying this phenomenon are not well understood. In this study, we used the sauce flavor baijiu fermentation system as a model to identify the pairwise negative interactions between *Pichia* and filamentous fungi. By integrating observations from an *in situ* community and reconstructions of an *in vitro* community, we demonstrated that antifungal activity of *Pichia* can be achieved by the VOCs, mainly 2-phenylethanol (Fig. 6). Further, the positive effect of this antagonism interaction on the fermentation community was also determined. These results demonstrated that *Pichia* organisms secrete volatile metabolites regulating the community structure and flavor characteristics during sauce flavor baijiu fermentation.

It is of significant interest to determine the antagonistic potential of native microorganisms against the filamentous fungal blooms for controlling fermentation quality and mycotoxin contamination. Previous studies have highlighted the potential of microbial interaction in biocontrol methods and used the native *Bacillus* species in daqu to prevent growth of *Streptomyces* spp. and geosmin contamination in baijiu fermentation (3, 28). In this study, *Pichia* organisms were identified as antagonistic native microorganisms against filamentous fungal blooms (Fig. 1). This antagonism effect is consistent with previous reports in which *Pichia* species have been described as potential biocontrol agents and used to suppress the growth of plant pathogens and...
postharvest fungi (5, 6). This provides additional evidence that the non-Saccharomyces yeasts are an important source of biocontrol agents. There were rich non-Saccharomyces yeasts in the fermentation process of baijiu. Our research results are helpful for the development of microbial resources from the baijiu fermentation microbial community and their application as biological control agents in agriculture and food industries.

The potential roles of VOCs as driving forces of community assembly in the food industry were clarified. More than a direct competition, the antagonists can also interact through space competition (Fig. 3). The most abundant VOC produced by Pichia was 2-phenylethanol, followed by ethyl acetate (Fig. 2 and 3). A similar finding was reported for biocontrol yeasts (5), and 2-phenylethanol was identified as the most abundant and active VOC against pathogenic molds (29). Recently, it has become increasingly evident that microbial volatile compounds can significantly contribute to long-distance interactions within microbial communities (30), acting as infochemicals affecting the population dynamics and gene expression in target microorganisms and as competition tools (4, 31). In agriculture, VOCs have been used in biological control strategies to prevent the growth of plant and postharvest pathogenic molds (32, 33). Our study demonstrated that VOCs can potentially be the driving force of community assembly in the food industry. The antibacterial activity of volatile compounds is a promising approach to control baijiu fermentation. In particular, the volatile organic substances can be transmitted in a solid-liquid-gas three-phase manner to inhibit mold, both for substrate and for aerial mycelium. Our results revealed that yeast community volatile compounds predominantly suppressed the development of fungal community composition, whereas development of bacteria was hardly affected, and suggest that manipulating VOCs is a novel strategy for improving fermentation processes.

The inhibitory role of 2-phenylethanol against filamentous fungi has also been investigated, which provided novel insights into the fermentation process of foods, which, in turn, impacts food spoilage research. A previous study had demonstrated the two antibacterial modes of 2-phenylethanol. First, 2-phenylethanol blocked DNA synthesis and affected permeability, thus inhibiting both Gram-negative and Gram-positive bacteria (34, 35). However, from the statistical results, there was no significant difference in the structure of bacterial community between the two groups (Fig. 1 and Fig. S1), and the abundance of Lactobacillus was gradually increased in both groups. Lactobacillus may underlie the observed effect. It produces lactic acid, antibacterial peptides, and bacteriocins (36, 37) and may play a major role in the distribution of bacteria, thus masking the effect of 2-phenylethanol. Second, 2-phenylethanol are lipophilic compounds with a high affinity for the plasma membrane, demonstrating a higher toxicity than ethanol (4). This means that 2-phenylethanol has higher antibacterial properties at low concentrations (Table 1). For example, the antimicrobial properties of 2-phenylethanol include inhibition of spore germination and colony growth of Aspergillus flavus, even at low concentrations (38). In addition, studies have also shown that phenylethanol suppressed the synthesis of plasma membrane proteins (39) and showed competitive and noncompetitive mixed inhibition on mushroom tyrosinase (34). In our study, 2-phenylethanol was able to affect the growth and gene expression of filamentous fungi by inhibiting protein synthesis and causing DNA damage. Such DNA damage resulted in the ubiquitous expression of serine/threonine phosphatase activity (PPP1C), which is involved in the negative control of cell growth and division (40, 41). Moreover, a previous study concluded that synergy between ethanol and the higher alcohols occurs to produce membrane-associated lesions with deleterious effects during fermentation (42, 43). This may accelerate the antifungal activity of 2-phenylethanol in the ethanol fermentation process. Hence, 2-phenylethanol may be of great ecological importance for the interactions between spatially distant fermentation organisms.

The antifungal activity of Pichia had a positive effect on the microbial community
function of baijiu fermentation. 2-Phenylethanol is a higher alcohol, formed by the anabolism or catabolism (Ehrlich pathway) of amino acids (44, 45). Previous studies have shown a significant correlation between Pichia and 2-phenylethanol during baijiu fermentation (46). In the control group, the biosynthesis of 2-phenylethanol was significantly upregulated (Fig. 5) and resulted in elevated levels of higher alcohols. In addition, as shown in Fig. S3, M. purpureus itself has low ester production ability (0.12 ± 0.03 mg/liter). However, M. purpureus has the ability to produce esterifying enzyme (47). During coculture, the antifungal property of P. kudriavzevii against M. purpureus increased the permeability of the cell wall and the extracellular release of the esterifying enzyme, thus catalyzing the acid and alcohol in the coculture system. This is consistent with the phenotype that the acid decreases but ester increases in the system. Previous studies have shown that higher alcohols and acyl coenzyme A (acyl-CoA) are the precursors of synthetic esters (44), and the abundant higher alcohol in heap fermentation (stage I) was consistent with the higher esters in pit fermentation (stage II). Regarding ethanol metabolism, Pichia helps maintain the diversity of yeasts in the community and significantly improves the community’s ability to produce ethanol.

In conclusion, this work shows that VOCs are key drivers of community assembly in the food industry and that 2-phenylethanol represents a key component of volatile compound-mediated antifungal activity. Our study suggests that specific VOCs can be used to inhibit the growth of filamentous fungi, which might serve as a novel strategy to construct an ideal fermentation microbial community by using VOCs. Further work should focus on investigating the dosage effect and antifungal mechanisms of 2-phenylethanol.

MATERIALS AND METHODS

Experimental design and sample collection. Two independent fermentation batches were made in a distinguished distillery of sauce flavor liquor in Guizhou Province, China (28.14 N, 106.18 E). The fermentation used the same raw materials (sorghum) and technological parameters. The two batches of grains selected for fermentation were named the control and defect groups based on the condition of the grains. The sampling of the fermenting grains was done in two steps following a process described for a previous study (48). Heap fermentation samples were collected daily (H1, H4, H5, H6, and H8) to observe the microbial community structure succession and assembly process. Pit fermentation samples were collected on days 0, 5, 10, and 15 (F00, F05, F10, and F15) based on results of a previous study that showed only minor structural changes in the later stages of fermentation. Samples taken from different points in the same layer were mixed to form a single sample to reduce the volatility before extraction and analysis. We obtained a total of 16 heap and 8 pit fermentation samples.

Fermentation parameters for detection and analysis. Ethanol was analyzed via a high-performance liquid chromatograph (HPLC; 2695; Waters Corporation, Milford, MA) equipped with a refractive index detector (RID; 2414), based on the method described for previous studies (49, 50).

Total DNA extraction, amplification, and sequencing. All samples were treated with sterile phosphate-buffered saline (0.1 mol/liter) and then centrifuged at 300 × g for 10 min (4°C). The supernatant was then centrifuged again at 13,000 × g for 10 min (4°C). The precipitated cells were milled with liquid nitrogen (48), and genomic DNA was extracted using a sodium laurate buffer (sodium laurate, 10 g/liter; Tris-HCl, 0.1 mol/liter; NaCl, 0.1 mol/liter; EDTA, 0.02 mol/liter) containing phenol-chloroform-isooamyl alcohol (25:24:1, vol/vol/vol). For bacteria, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using the universal degenerate primers 338F (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 806R (5′-GAC TAC HVG GTG WTC TAA T-3′) (51). For fungi, the internal transcribed spacer 2 (ITS2) region was amplified with primers ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (52). PCR products were purified using a PCR purification kit, and the concentrations were assessed with a Thermo Scientific NanoDrop 8000 UV-visible (UV-Vis) spectrophotometer (NanoDrop Technologies, Wilmington, DE). The barcoded PCR products were sequenced on a MiSeq benchtop sequencer (Illumina, San Diego, CA) for 250-bp paired-end sequencing (2 × 250 bp) at Beijing Auwigene Tech, Ltd. (Beijing, China).

All the raw sequences generated were processed via QIIME v.1.9.1 (53) and R v.3.3.1 (http://www.r-project.org). The representative bacterial OTU sequences were annotated using the Silva 132_16S rRNA database with a QIIME-based wrapper of RDP-classifier (v.2.2). The representative fungal OTU sequences were compared using a BLAST search against the UNITE fungal ITS database (https://unite.ut.ee/).

Volatile compound analysis. The volatile compounds of fermented grains were analyzed using an HS-SPME–GC–MS (GC 6890N and MS 5975; Agilent Technologies, Santa Clara, CA) on a DB-Wax column (30 m by 0.25 mm [inside diameter], 0.25-m film thickness; J&W Scientific, Folsom, CA), based on the method described for a previous study (54). The volatile compound flavor analysis included four samples.
during both heap and pit fermentation. Flavor compounds fermented by single fungal and coculture were also determined by HS-SPME–GC–MS.

**Antifungal evaluation.** The strains used in this study had been isolated and identified previously (26). *P. kudriavzevii* was precultured in sorghum extract medium at 30°C overnight and shaken at 200 rpm to obtain the seed culture. The fungus was cultivated in PDA (20% potato extract, 2% dextrose, 2% agar) plates for 7 days at 30°C. Spores were collected by adding sterile distilled water to the culture surface and gently scrubbing with a sterile spatula. Spore suspensions were obtained by filtering through four layers of sterile cheesecloth to remove any hyphal fragments (55). As Fig. 3A illustrates, a double-dish system (DDS; sorghum extract medium with agar) was used to determine the production by *P. kudriavzevii* of VOCs with capacity to control *M. purpureus* growth as described previously (29). Two plates were used per each confrontation. In step I, 10 μl of spores (10⁶ conidia/ml) of *M. purpureus* was inoculated in the central point of the down plate. In step II, 100 μl of *P. kudriavzevii* cells (10⁶ cells/ml) was seeded with a Digralski spatula in the upper plate. In step III, one plate was inverted onto the other and sealed with Parafilm. Double dishes were incubated at 30°C for 7 days, and the diameter of the mycelia was measured daily. As a control, a double dish was performed without the inoculation of yeast. The results were expressed as differences between the control (CK, without *P. kudriavzevii*) and biological control (+PK, with *P. kudriavzevii*). The reduction in *M. purpureus* growth was calculated according to the following formula: percent reduction of growth = (Dck – Dck/Pck × 100, where D is the diameter (in millimeters) of the mycelia.

For the volatile compound analysis, *P. kudriavzevii* and *M. purpureus* were also inoculated into the liquid medium of sorghum extract at a cell density of 10⁶ CFU/ml. Cocultures were conducted with a *P. kudriavzevii* and *M. purpureus* at a ratio of 10⁷:10⁵. Fermentation was performed at 30°C with shaking at 200 rpm. Samples were taken after 5 days to quantify the concentration of volatile compounds. The relevant methods are described in “Volatile compound analysis” above.

**Antifungal activity of single VOCs.** After the identification of the VOCs produced by *P. kudriavzevii*, the objective was to identify the main compounds with antifungal activity and understand how they work. The 2-phenylethanol used during the assay was purchased from Sigma-Aldrich (Germany). Relationships between the 2-phenylethanol and filamentous fungi were tested by an agar diffusion assay. Briefly, a 5-mm sterile filter paper was placed in the middle of a plate (sorghum extract medium with agar) which had been covered uniformly with 100 μl of filamentous fungi (10⁶ CFU/ml). To measure the antifungal activity, 10 μl of the 2-phenylethanol was spotted onto the filter paper. Sterile filter paper with 10 μl of sterile water was used to determine the negative control. The strong inhibition of 2-phenyl- ethanol was verified after incubation at 30°C for 5 days. Field-emission SEM images were collected for ultrastructural analysis (SU8010 instrument) at 20kV.

**Total RNA extraction and metatranscriptomic sequencing.** Microbial cell collection was performed in the step outlined for DNA extraction in “Total DNA extraction, amplification, and sequencing” above. The precipitated cells were collected according to the method of Song et al. (48). After the sample was qualified, the rRNA was removed with the Ribo-Zero kit (Epicentre, San Diego, CA). Metatranscriptomic libraries were constructed according to the protocol of the NEBNext Ultra RNA library prep kit (Illumina; New England Biolabs, Ipswich, MA) and were then sequenced on an Illumina HiSeqTM2500/4000 platform at Beijing Auwigen Tech, Ltd. (Beijing, China).

Raw metatranscriptomic data were processed by removing the rRNA sequences and low-quality reads (Q < 0.02). Trinity was employed for de novo transcriptome assemblies (56). Prodigal (57) software was used to conduct open reading frame (ORF) prediction on the assembled contig sequence, and CD-HIT (58) software was used to remove redundant genes from the predicted gene sequence with a similarity of 0.95 to obtain a nonredundant gene set. Subsequent analysis of gene expression levels was based on nonredundant gene sequences. Finally, databases such as NR, Pfam, KOG/COG (clusters of orthologous groups of proteins [https://www.ncbi.nlm.nih.gov/research/cog-project/]), Swiss-Prot, KEGG, GO, CAZyme, eggNOG, and CARD were used to obtain functional annotation information.

**Statistical analysis.** Histogram, bubble, and line charts were drawn in OriginPro2020 (OriginLab Corporation, Northampton, MA). Statistical differences in this study were obtained by OriginPro2020 using one-way analysis of variance (ANOVA) followed by Tukey’s test. A P value of <0.05 was used as the significance threshold. NMDS was conducted using Canoco software. Nonparametric analyses (e.g., ANOSIM) were conducted to check the significance of clusters (59). The relationships among microbial communities were calculated using Spearman’s rank correlations. The abundant genera (relative abundance in the top 10 is presented; total relative abundance >80%) and only significant correlations (P < 0.05, with false-discovery rate correction) were considered valid correlations. Heat maps were constructed using R (v.3.6.1) via the pheatmap package (v.1.0.12). The multivariate statistical analyses of flavor were performed with SIMCA-14.1 software (Umetrics, Sweden). For hierarchical cluster analysis (HCA), the distance between observations was calculated using Ward’s method based on the concentration of volatile organic compounds. Partial least squares discriminate analysis (PLS-DA) was employed to summarize volatile organic compound data by grouping variables. A VIP score of >1.0 from the PLS-DA model contributed to determination of the specificity of different groups.

**Data availability.** The amplicon and transcriptomic databases were submitted to the NCBI Sequence Read Archive (SRA) and are available under accession number PRJNA700798.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1** PDF file, 2.3 MB.
REFERENCES


