Unraveling the core microorganisms and metabolic pathways related to off-flavor compounds formation during *Jiang*-flavor *Baijiu* fermentation

Jiaxin Gong [a,b], Qiancheng Zuo [c], Zhaoqing Wu [c], Cong Zhao [c], Junlin Wei [a,b], Yongguang Huang [a,b,*]

[a] *College of Liquor and Food Engineering, Key Laboratory of Fermentation Engineering and Biological Pharmacy of Guizhou Province, Guizhou University, Guiyang, Guizhou, 550025, China*
[b] *Key Laboratory of Fermentation Engineering and Biological Pharmacy of Guizhou Province, China*
[c] *Guizhou Hanko Junfeng Liquor Industry Co. Ltd., Renhuai, China*

ABSTRACT

Off-flavor is one of the most frequent and serious causes for the aroma deterioration in *Jiang*-flavor *Baijiu*. However, the key compounds and their formation mechanism responsible for off-flavor are still unclear. This study identified 271 volatile compounds from 1 normal and 5 types of off-flavor fermented grains (putrid, rancidity, mud, musty, and burnt) by headspace solid-phase microextraction combined with gas chromatography–mass spectrometry. Using VIP and OAV analysis, 47 key flavor compounds including indole, phenol, isoamyl alcohol, diacetyl, acetic acid, isobutyric acid, and isovaleric acid were found to distinguish normal and off-flavor fermented grains. Furthermore, 40 microbial genera (mainly *Monascus*, *Enterococcus*, *Dyadobacter*, *Ottowia*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Pseudomonas*, and *Xanthomonas*) were significantly ($p < 0.05$, Pearson correlation) related to these 47 compounds. Finally, metabolic pathways for off-flavor compounds formation were constructed. This study provides comprehensive information on the off-flavor compounds and their potential formation mechanism during *Jiang*-flavor *Baijiu* fermentation.

1. Introduction

As one of the basic aroma types of Chinese liquor, *Jiang*-flavor *Baijiu* has a pivotal role within the distilled spirits consumption market. The brewing process of *Jiang*-flavor *Baijiu* is based on solid-state fermentation with pure mixed bacteria. During solid-state fermentation, there are complex solid-liquid and gas-liquid interfaces within the fermented grains (Gong et al., 2023). The multi-facial effect strongly influences the diversity of microorganisms and their metabolism within the fermented grains and cellar, resulting in the brewing of a variety of rich flavor compounds in *Baijiu*. However, as the brewing operation *Jiang*-flavor *Baijiu* is carried out in a semi-open environment, the flavor compounds are easily affected by a variety of factors such as the type of raw materials and fermentation process. The presence of off-flavor compounds is largely indicative of abnormal fermentation of the fermented grains. This includes the disruption of the normal brewing micro-ecological balance and the decline of the brewing function. This is ultimately reflected in a decline in the quality of the liquor, showing a variety of off-flavors (Wei, Lu, et al., 2023) (Fig. 1).

The main flavor components of *Baijiu* include esters, alcohols, aldehydes, and acids (Qiao et al., 2023), and the contents and proportion of these compounds ultimately determine the quality of various types of *Baijiu*. When some flavor compounds exceed the standard contents or the ratio is imbalanced, *Baijiu* presents off-flavor. In recent years, many studies have conducted the traceability analysis of the key compounds leading to the off-flavor in different types of *Baijiu*. For example, 3-methylindole is characterized as a source of mud odor in strong-flavored *Baijiu* (Dong et al., 2018). 3-Methylbutyric acid is considered to be an off-flavor acid in *Baijiu* (Niu et al., 2020). 2-Methyl-3-furyl disulfide and 2-furyl mercaptan were identified as kimchi off-flavor by aroma recombination and mission (Wang et al., 2020; Zhu et al., 2023). Phenols present an unpleasant smoky and pungent flavor in strong-flavored *Baijiu* (Zhang et al., 2014). 2-Hydroxymethyl-3 and 6-diethyl-5-methylpyrazine cause a burnt off-flavor (Zhao et al., 2018). However, these studies mainly focused on describing the key compounds responsible for the off-flavor sensory in *Baijiu*, the production and formation mechanisms of these off-flavor compounds have not been elucidated, making it difficult to effectively control the production of off-flavor compounds.

The presence of off-flavor compounds is inextricably linked to microbial metabolism during *Baijiu* fermentation (Du et al., 2021). For example, geosmin, causing the earthy off-flavor in light-flavor *Baijiu*, is produced by *Streptomyces* in *Daqu* (Du & Xu, 2012). The bitter-smelling higher alcohols and kimchi-flavored sulfur compounds in *Baijiu* are generated by *Saccharomyces* and *Lactobacillus* (Liu et al., 2017; Serjak et al., 1954). In addition, certain non-brewing microorganisms can also generate off-flavor compounds, resulting in abnormal fermentation. For example, *p*-Cresol, known for its carcinogenic properties and unpleasant taste, has been observed to be primarily produced by *Dorea*, *Sporanaerobacter*, and *Clostridium* (Ji et al., 2020). Therefore, exploring the potential relationship between microbial community and off-flavor compounds in abnormal fermentation is conducive to revealing the formation patterns of these off-flavor compounds and guiding efforts towards regulating microbial community and enhancing the flavor profile of *Baijiu*. This study focused on the 1–7 rounds of *Jiang*-flavor *Baijiu* production process, examining two types of fermented grains: normal and abnormal fermentation (exhibiting putrid, rancidity, mud, musty, and burnt off-flavors) to reveal the potential compounds contributing to the presence of off-flavors and their generation by microbial community. For that, the sensory profiles and volatile compounds of two types of fermented grains were identified by combining sensory evaluation with headspace solid-phase microextraction combined with gas chromatography–mass spectrometry (HS-SPME-GC–MS). Metagenomic sequencing was used to investigate the microbial communities of two types of fermented grains. Furthermore, the relationships between off-flavor compounds and key microorganisms were revealed and the potential metabolic pathways of key off-flavor compounds were illustrated. This study can provide guidance for understanding off-flavor compounds and their generation in *Jiang*-flavor *Baijiu* and improving *Baijiu* quality.

2. Materials and methods

2.1. Sample collection

The samples were collected from a typical *Jiang*-flavor *Baijiu* distillery in Guizhou Province, China. The normal fermented grains were collected during 1–7 rounds of stacking and cellar fermentation (upper, middle, and lower) process from 6 workshops from February to October 2023. Randomly collecting the fermented grains that have been identified as off-flavor fermented grains (mainly including putrid, rancidity, mud, musty, and burnt) after sensory evaluation in stacking and cellar fermentation. Finally, a total of 420 small samples were collected from 1 to 7 rounds of stacking and cellar fermentation, resulting in 124 mixed samples. The samples were categorized and processed, for physico-chemical indicators and flavor determination are usually stored at 4 °C and samples for DNA extraction at −80 °C.

Fig. 1. Off-flavor wheel in the process of *Jiang*-flavor *Baijiu*.

2.2. Sensory evaluation

Sensory evaluation was performed in accordance with GB/T 10345–2007 "Methods for the Analysis of *Baijiu*" and the method described by He, Liu, Qian et al. (He et al., 2020) with slight modifications. The sensory tasting panel comprised 13 sommeliers (including 2 national *Baijiu* judges, 6 Chinese provincial *Baijiu* judges, 4 sommeliers of grade 2 or above, and 5 postgraduate sommeliers) aged 21–45, each possessing over 2 years of professional training and experience in *Baijiu* sensory evaluation. All participants were informed of all the details of the sensory experiment and their consent was obtained before the experiment began. All participants provided written informed consent prior to study participation, and participation was completely voluntary. After the training, normal and abnormal fermented grains were randomly coded, and the olfaction sensory indicators were described using a 6-point (1 = extremely low, 3 = moderate intensity, 6 = extremely high) sensory score value (SV). The sensory modules with a lot of descriptors in the flavor wheel in Fig. 1 were used as radar charts to visualize the sensory flavors of different *Baijiu*.

2.3. Determination of physicochemical indices

The moisture content of fermented grains was measured using the gravimetric method, drying the samples at 105 °C until constant weight. Total acidity was determined by sodium hydroxide titration (0.1 mol/L) (Wang et al., 2021). The reducing sugar content and starch content were determined by referring to the DB34/T 2264 standard. To be specific, the fermented grains (10 g) were soaked in water (100 mL) for 30 min, filtered and the filtrate was used to calculate the content of reducing sugars. Starch was extracted from fermented grains by acid hydrolysis (20% HCl, *v/v*) for 30 min. The pH of the hydrolysate was adjusted to 7.0 with 20% (*w/v*) NaOH, and then the reducing sugar consumption is measured to estimate the starch content.

2.4. Determination of volatile compounds

Volatile compounds were determined by HS-SPME-GC–MS according to the method previously described by Li et al.(Li, Chen, et al., 2020). The sample (2 g) was mixed with Milli-Q water (8 mL) in triplicate and ultrasonic treatment for 30 min. The sample solution was centrifuged at 10,000 rpm at 4 °C for 20 min. Supernatant of centrifuged samples were added to a 20 mL headspace vial, with 20 μL of 2-octanol. A rotor was placed in the extraction vial, and the extraction head (Supelco Co., Bellefonte, Palo Alto, CA, USA) was inserted into the air above the liquid level of the extraction vial. The extraction was taken at 45 °C for 45 min, with a rotational speed of 250 r/min, and an extraction time of 45 min. The extraction head was inserted into the inlet of the GC–MS (Fuli-Chromatec Crystal 9000) and resolved at 250 °C for 5 min. The chromatographic and mass spectrometric conditions were described in the literature (Li, Fan, et al., 2020). The characterization of the substances was carried out with reference to the NIST library and retention indices. The quantification of the compounds was performed using 2-octanol as the internal standard. The content of each compound was calculated by comparing its peak area to an internal standard. Compounds with standards are calculated according to the method of the standard curve.

2.5. DNA extraction and metagenome sequencing

Sample DNA was extracted according to a previous report (Wang et al., 2021), and the amplicon was sequenced and analyzed. Bacteria V3–V4 region of 16S rRNA genes were amplified by special primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGAC-TACHVGGGTWTCTAAT-3′). Fungal PCR amplification was conducted with the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′). Purified amplifiers were sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA).

2.6. Identification of functional genes, and metabolic pathways

Quality-checked reads were assembled into alleles using MEGAHIT (https://github.com/voutcn/megahit), non-redundant gene sets with 95% identity and 90% contrast coverage were constructed using default parameters, and Prodigal (https://github.com/ hyattpd/Prodigal) for gene prediction for each allele and scaffold (Yao et al., 2023). Functional annotation was performed using HUMAnN3 (v3.6), and annotation information and relative abundance tables were obtained from KEGG according to the Uniref 90 IDs and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.7. Statistical analysis

All samples were repeated three times. Data are presented as the mean ± standard deviation (SD). One-way analysis of (ANOVA) and Duncan's multiple range test were analyzed by IBM SPSS Statistics (version 25.0; IBM Co., New York, USA). IBM SPSS Statistics also performed Spearman's correlation analysis. Network graphs were created by Gephi (v 0.9.2) to organize and visualize correlations between microbial genera and flavor compounds. STAMP(https://beikolab.cs.dal. ca/software/STAMP)was used to analyze differences in metabolic pathways directly across samples. Principal component analysis (PCA) and partial least squares (PLS) analysis were performed using SIMCA software (v.1.0.1, Umetrics AB, Umea, Sweden). Other statistical analyses and the generation of graphs were performed in Origin 2021 and R (v3.4.3). P value < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Comparison of physicochemical indices and sensory characteristics between normal and abnormal fermented grains

The collected fermented grains were subjected to sensory evaluation and clustered into six categories: 1 normal (Control) and 5 abnormal (Putrid, Rancidity, Mud, Musty, and Burnt off-flavors) (Fig. 2a).

Normal fermented grains had a prominent *jiang* flavor (SV:3.50), while off-flavor fermented grains had sensory characteristics such as acid (SV:2.11–3.01), musty (SV:1.29–4.51), grassy (SV:1.00–3.12), mud (SV:1.04–3.02), and burnt (SV:1.21–3.41) (Fig. 2f-g). Abnormal fermentation would lead to the change of physicochemical characteristics of fermented grains (Liang et al., 2024; H. Wang et al., 2023). Hence, we compared the reducing sugars, starch, moisture, and acidity in the normal and off-flavor fermented grains. Overall, the abnormal fermented grains were characterized by lower acidity (0.63–1.10 mmol/ 10 g), lower reducing sugar (0.18–0.69%), lower starch contents (14.93–28.90%), and higher moisture contents (49.57–55.66%), compared with those in the normal fermented grains (Fig. 2b-e). Acidity was significantly ($p < 0.05$) lower in the abnormal fermented grains (0.63–1.10 mmol/10 g), which was related to the change of oxygen during the fermentation process in the cellar. The poorly closed cellar led to the elevation of oxygen and the entry of other microorganisms into the cellar. Reduced acidity (0.63–1.10 mmol/10 g) due to inhibition of acid-producing microorganisms in anaerobic fermentation, and the accumulation of lactic acid and other acidic products was not conducive to pasteurization and saccharification of the starch of the fermented grains. Also weakened the pathway of starch conversion to ethanol, which resulted in abnormal wine starch content (14.93–28.90%), reducing sugar (0.18–0.69%) and low wine yield (Chen, Gao, et al., 2022).

3.2. Comparison of microbial communities between normal and abnormal fermented grains

In order to reveal the differences of the microbial communities in the normal and abnormal fermented grains, microbial structures were
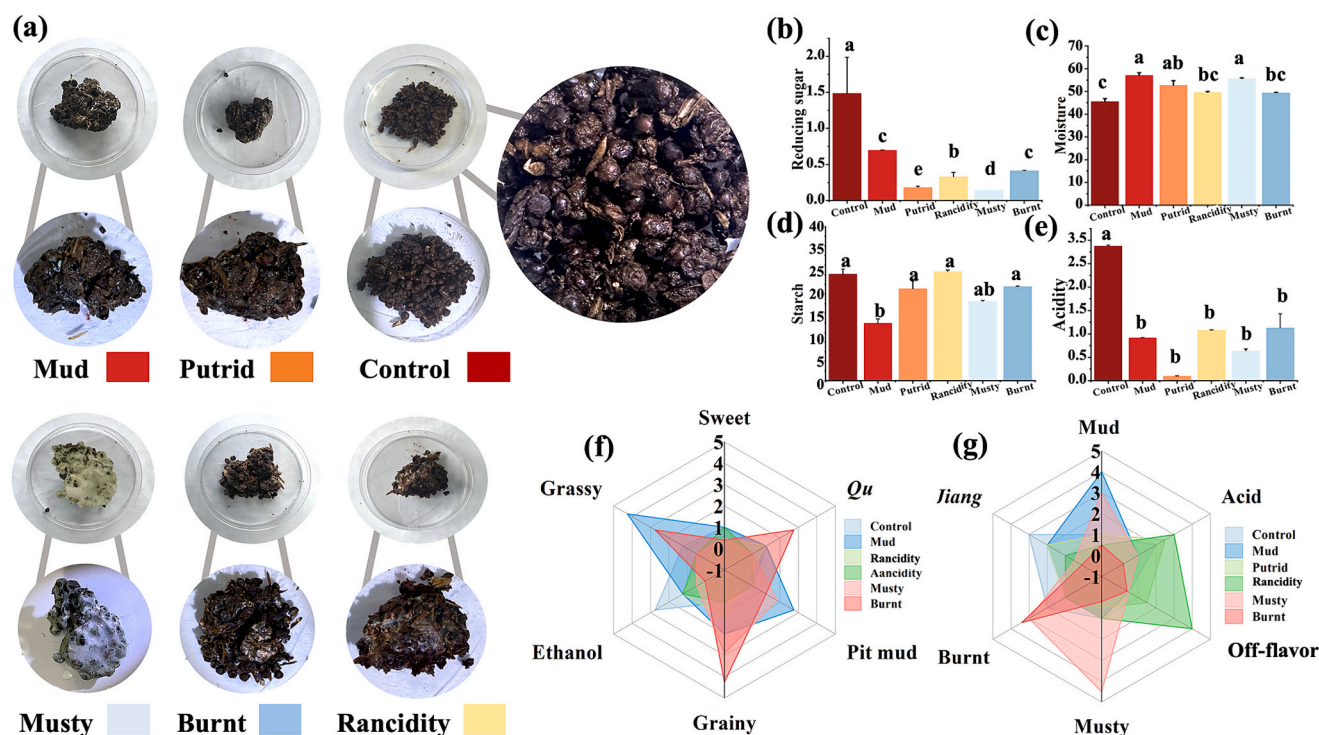
Fig. 2. Sensory characteristics and physicochemical indices in the normal and abnormal fermented grains (a-e of bar graphs indicate significant differences in SV within groups).

furtherly investigated. As shown in Fig. 3a and d, at the phylum level, the dominant bacterial phylum showed greater differences between normal and abnormal fermented grains than the fungal phylum. The dominant bacterial phyla in the normal fermented grains were Firmicutes (86.54%, the average relative abundance) and Proteobacteria (3.57%). In contrast, the dominant bacterial phyla in the abnormal fermented grains were Proteobacteria (29.62–49.02%) and Bacteroidetes (13.74–29.88%). The average relative abundance of Proteobacteria were 49.02% and 40.90% in the Putrid and Musty fermented grains, respectively. Whereas, Bacteroidetes was the highest (29.88%) in Mud fermented grains. The differences at the level of fungal phyla were smaller, for example, the relative abundance of Ascomycota was different in normal and abnormal fermented grains, with 0.55% and 3.12%, respectively. At the bacterial genus level, the relative abundance of *Lactobacillus* decreased significantly (1.12–28.46%) after the fermentation anomaly (Fig. 3d), while the relative abundance of other microorganisms increased, including *Stenotrophomonas* (14.39%), *Chryseobacterium* (7.19%), and *Acetobacter* (16.93%). In comparison with previous studies, it can be found that the above dominant microorganisms in abnormal fermented grains (Chryseobacterium and Stenotrophomonas) are not core microorganisms of the fermentation process (Pan et al., 2023). At the fungi genus level, the relative abundance of core functional yeast (*Schizosaccharomyces*, 0.39%) was reduced in the abnormal fermented grains, which produces ethanol and other flavor-related metabolites after abnormal fermentation (Du et al., 2021). Whereas some nondominant fungi, such as *Penicillium*, were found in Musty (0.73%), Rancidity (0.28%), and Putrid (0.14%) became dominant genera (Fig. 3b).

RDA demonstrated the effects of physicochemical factors on community structure during fermentation and showed that, with moisture positively affecting the composition of the bacteria to a greater extent than the fungi (Fig. S1). Bacterial communities in cellar fermentation have a greater impact on *Baijiu* quality than fungal communities (Wei, Shen, et al., 2023), Fungi can also have an impact on causing *Baijiu* fermentation. From the comparison of the bacteria and fungi, acidity content had a greater effect on the fungal structure (Fig. S1). Probably

due to less acid accumulation after abnormal fermentation, resulting in fewer acid metabolites promoting the growth of the dominant fungi (yeasts), which changed the abundance of the dominant fungi (Fig. S2) (Annalisa et al., 2015). Furthermore, moisture positively affected the changes in the fungi community in Rancidity, Musty, and Putrid fermented grains (Fig. S2).

3.3. Identification of differential microorganisms and metabolic pathways between normal and abnormal fermentation

Microorganisms (top 20) detected by metagenomic sequencing based on relative abundance >1% were analyzed, and 40 kinds of microorganisms were used as key compounds. Fold Change value (FC) and PCA were carried out and the results are shown in Fig. 4a-g.

It showed that microorganisms were significantly ($p < 0.05$) different between normal and abnormal fermented grains. The microorganisms that were significantly up-regulated included *Lactobacillus* (86.51%), *Saccharomyces* (0.11%), *Schizosaccharomyces* (0.34%), *Bacillus* (0.34%) in the normal fermented grains, and so on, which were similar to the previous study (Pan et al., 2023). These dominant genera contribute to the fermentation of *Baijiu* (Pang et al., 2021). The genera up-regulated during abnormal fermentation mainly included *Acetobacter* (2.15–16.92%), *Bacteroides* (0.11–2.15%), and *Penicillium* (0.01–0.75%) (Fig. 4a-e). Among them, the relative abundance of *Acetobacter* was up-regulated in Musty (10.66%) and Burnt (16.92%) fermented grains (Fig. 4a-b). *Bacteroides* was up-regulated in Rancidity (0.29%) and Mud fermented grains (2.10%) (Fig. 4c and d). *Penicillium* was able to produce ethanol and methylbutyl ketone. and its relative abundance was up-regulated in Musty (0.74%) and Burnt (0.37%) fermented grains (Fig. 4a and b).

In addition, some microorganisms up-regulated during abnormal fermentation (Fig. 4h). For example, *Flavobacterium* and *Stenotrophomonas* increased in relative abundance in the Putrid (14.39%) and Rancidity (8.83%) fermented grains. *Enterococcus* (0.90%) showed a significant increase in relative abundance in the Musty fermented grains. *Monascus* (7.46%), and *Zygosaccharomyces* (2.59%) showed an
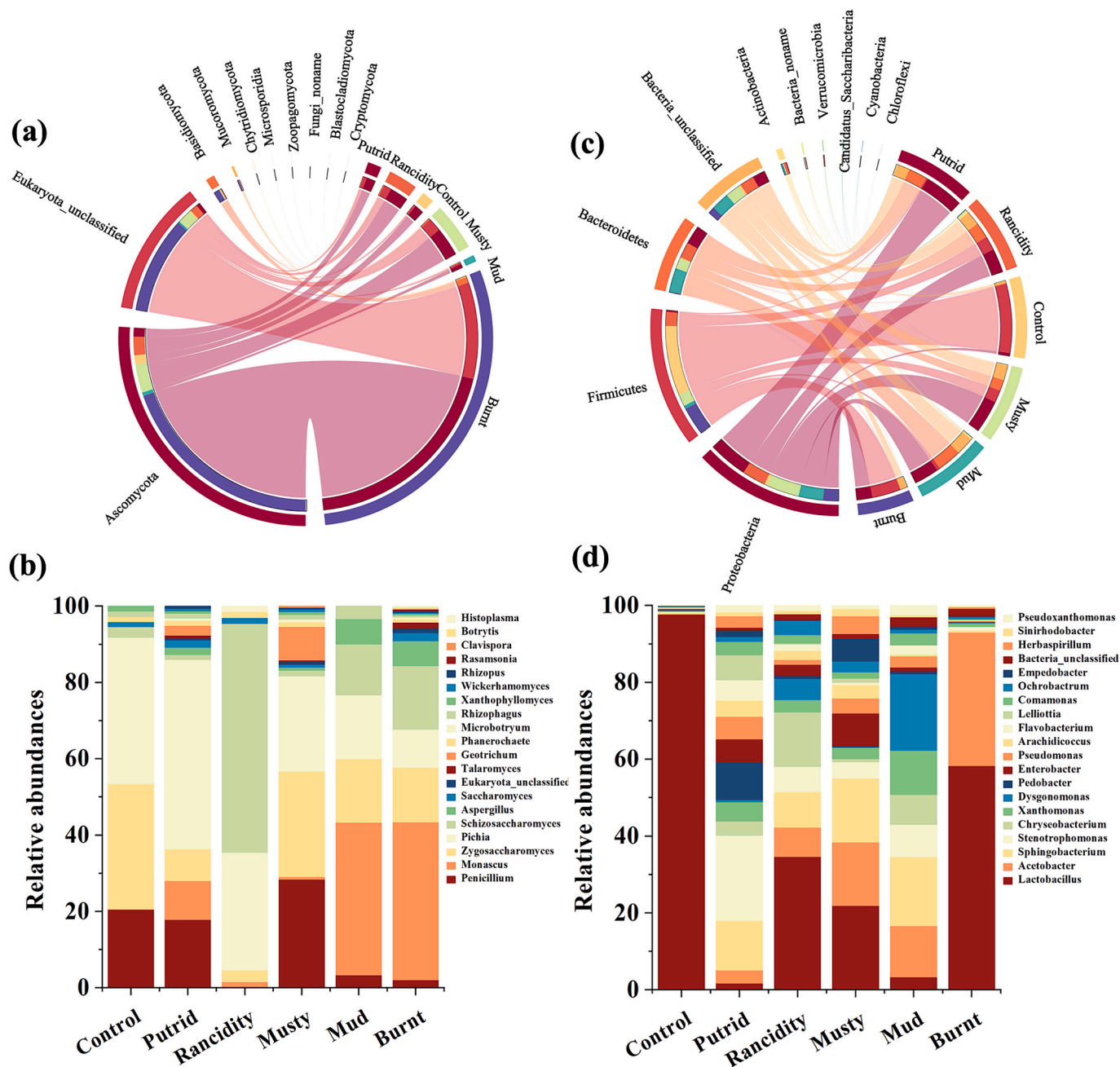
Fig. 3. Microbial structures in the normal and abnormal fermented grains. Community structure of bacteria (a) and fungi (c) at the phylum level in the normal and abnormal grains. Community structure of bacteria (b) and fungi (d) at the genus level in the normal and abnormal grains.

increase in Burnt fermented grains (Fig. 4b). *Monascus* (7.46%) can help to drive metabolism of *Bacillus* (C. Zhang et al., 2021). *Dyadobacter* (0.79%), *Ottowia* (1.04%), and *Pseudoxanthomonas* (1.34%) had higher abundance in the Mud fermented grains (Fig. 4d). *Pedopader* (3.83%) had a higher abundance in the Putrid fermented grains (Fig. 4e), which can utilize amino acids to produce unpleasant sulfides, esters, and amines (Casaburi et al., 2015). The relative abundance of *Lelliottia* (4.25%) increased only in Putrid fermented grains (Fig. 4e) and *Lelliottia* is associated with the metabolism of threonine (Zhuo et al., 2021). We found that most of the microorganisms with increased relative abundance after fermentation anomalies had specialized aerobic microorganisms with oxygen as the final electron acceptor (Zheng et al., 2018). In addition, some microorganisms were only present in certain types of fermented grains, such as *Candidatus_Koribacter* in Rancidity fermented grains and *Ilyobacter* in Mud fermented grains.

Moreover, the microorganisms had a reciprocal relationship (Fig. 4g) and the correlation between the dominant genera and the other genera together determined the outcome of the fermentation. For example, *Lactobacillus* was positively correlated with *Schizosaccharomyces* and *Pichia* and significantly positively correlated with acidity ($p < 0.01$), and *Lactobacillus* was significantly ($p < 0.05$) negatively correlated with most of the aerobic bacteria, such as *Pseudoxanthomonas* and *Stenotrophomonas*, which was related to the fact that *Lactobacillus* secreted bacteriocins with bactericidal or bacteriostatic effects.

KEGG functional annotation showed that amino acid and carbohydrate metabolism dominated the fermentation (Fig. S3). The density plot showed that there were distinct metabolic pathways between different types of fermented grains (Fig. S4). Pyruvate metabolism, glycolysis/gluconeogenesis, amino sugar and nucleotide sugar metabolism, pentose phosphate pathway, and lysine biosynthesis were up-regulated, with
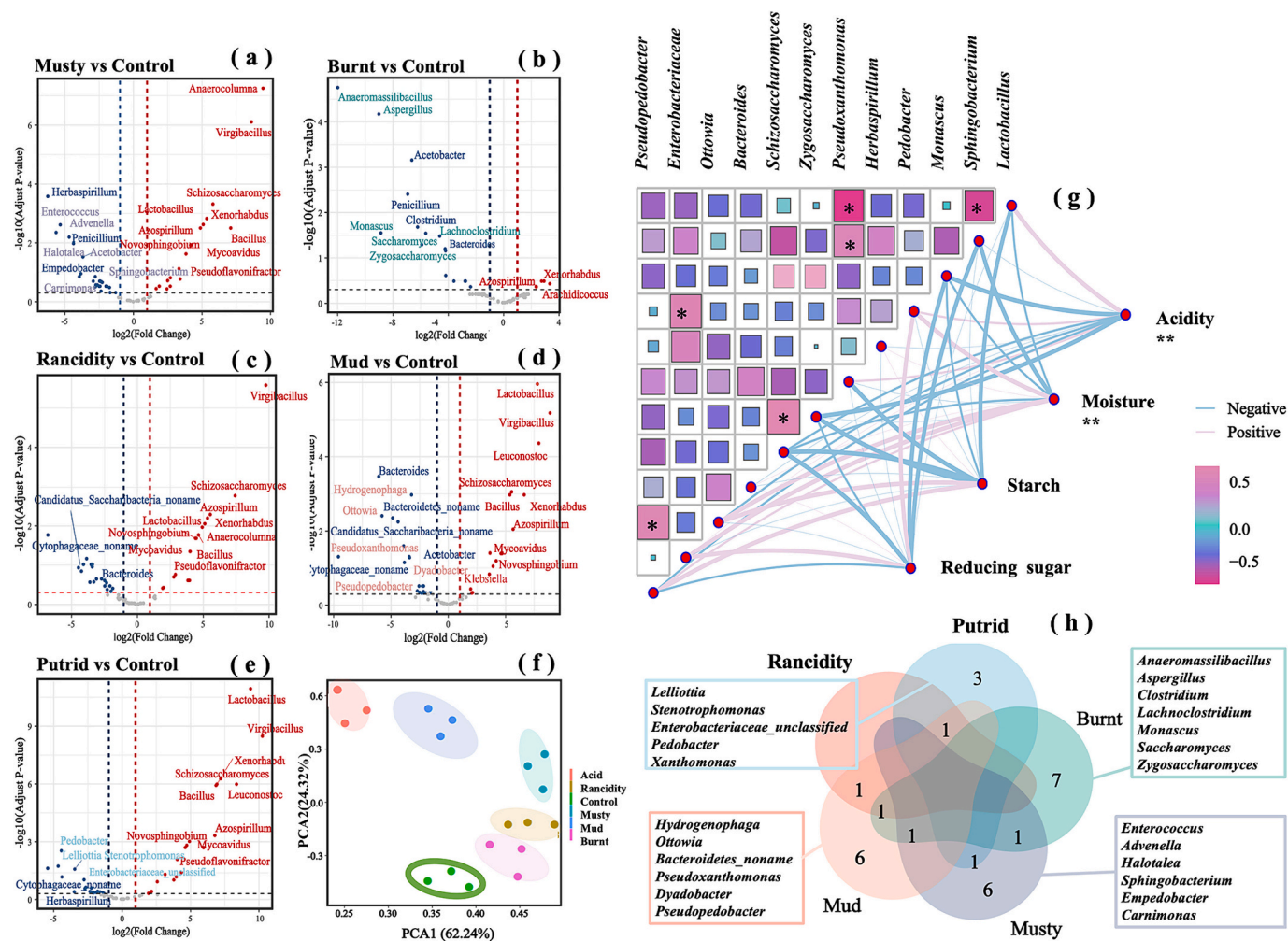
Fig. 4. Core microorganisms are responsible for the differences between normal and abnormal fermented grains. (a-e) Up-regulation (Red dots are normal fermented grains upregulated, blue are abnormal fermented grains.) of microbial abundance in normal and off-flavor fermented grains. (f) PCA of core microorganisms in different fermented grains. (g) Analysis of microbial interactions with significant differences in relative abundance and correlation of physicochemical indices. (h) Microorganisms are specific to different off-flavor fermented grains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fold changes of 1.28, 1.04, 1.19, 1.94, and 1.91, respectively in the normal fermented grains (Fig. 5f). During abnormal fermentation, the metabolic pathways of fermented grains were up-regulated by ascorbate and aldarate metabolism (FC: 18.29), inositol phosphate metabolism (FC: 11.38), c5-branched dibasic acid metabolism (FC: 18.32), citrate cycle (TCA cycle) (FC: 9.12), valine, leucine and isoleucine degradation (FC: 6.56) and Glyoxylate and dicarboxylate metabolism (FC: 1.90) (Fig. 5a-e). The microbial metabolism in the citrate cycle (FC: 9.12) and glyoxylate and dicarboxylate metabolism (FC: 1.90) pathways were generally up-regulated in the case of abnormal fermented grains, whereas that of pyruvate metabolism (FC: 0.78) and glycolysis/gluconeogenesis (FC: 0.95) pathways was down-regulated, suggesting that glucose containment effect of the abnormal fermented grains was lowered in the cellar during the fermentation process by the influence of the environmental factors. This leads to stronger aerobic metabolism than glucose metabolism, thus inhibiting pyruvate and glycolysis metabolism.

Meantime, valine, leucine, and isoleucine degradation (FC: 7.57) was significantly up-regulated in the Rancidity fermented grains (Fig. 5b). Valine, leucine and isoleucine biosynthesis (FC: 7.57) was significantly up-regulated in Putrid fermented grains, and this pathway is associated with the formation of diacetyl, which may be responsible for the acid flavor of fermented grains (Fig. 5a) (Casey et al., 2004). Musty

fermented grains showed a significant up-regulation in tyrosine metabolism (FC: 2.16), phenylalanine, tyrosine and tryptophan biosynthesis (FC: 0.57), and pyruvate metabolism (FC: 1.06). Among them, phenylalanine, tyrosine and tryptophan biosynthesis is the core metabolic pathway for aromatic compounds (Hassing et al., 2019) (Fig. 5d).

In addition, the results of the correlation analysis between the key differential microorganisms and the metabolic pathways were (Fig. S5). *Lelliottia*, *Bacteroides*, and *Pseudomonas* were positively correlated with the metabolism of Valine, leucine and isoleucine degradation, and the metabolism of *Schizosaccharomyces* and *Zygosaccharomyces* were significantly ($p < 0.05$) positive correlated with the Cysteine and methionine metabolism. Cysteine and methionine metabolism can be facilitated by the production of cofactor SAM to promote high enzyme activity required for efficient synthesis of natural products in *Saccharomyces* (Chen, Xiong, et al., 2022).

3.4. Identification of the key microorganisms related to the off-flavor compounds

To reveal the key compounds responsible for the presence of off-flavor fermented grains, we first detected the volatile compounds in both the normal and abnormal fermented grains and 271 volatile compounds were detected, including 55 esters, 42 alcohols, 19 acids, 46
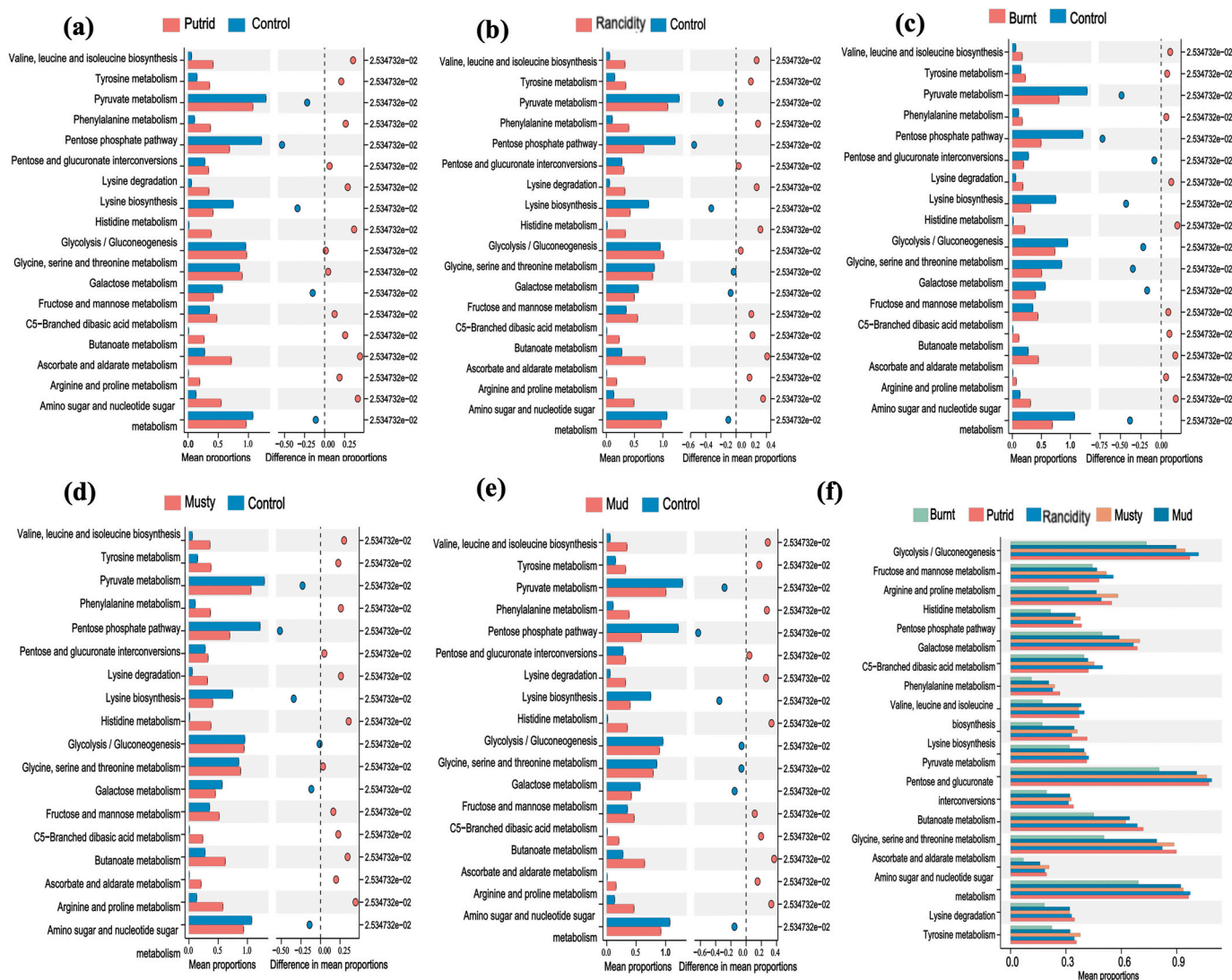
Fig. 5. Analysis of difference KEGG metabolic pathway between normal and abnormal fermented grains. (a-f) Stamp analysis of KEGG metabolic pathways in off-flavor fermented grains.

aromatics, 8 aldehydes and ketones, 5 furans, 30 alkanes and hydrocarbons, 4 pyrazines, 6 phenols, and 57 other compounds (Fig. 6a-b). Normal fermented grains had the highest contents of esters (70.33 ± 12.42 mg/kg) and acids (41.62 ± 0.03 mg/kg). The most prominent contents of ester were ethyl butyrate (0.15 ± 0.03 mg/kg), ethyl lactate (24.53 ± 2.84 mg/kg), and ethyl acetate (36.91 ± 1.02 mg/kg). The most prominent acid compound was acetic acid (24.73 ± 1.75 mg/kg) (Fig. 6a-b). Mud fermented grains had the highest contents of other compounds (33.25 ± 0.71 mg/kg). The highest contents of aromatic (21.88 ± 0.82 mg/kg) and pyrazine compounds (1.04 mg/kg) were found in the Burnt fermented grains, such as styrene (10.76 ± 2.11 mg/kg) and 2,3,5,6-tetramethylpyrazine (0.82 ± 1.1 mg/kg). The highest content of aldehydes and ketones was found in Musty fermented grains (27.15 ± 1.11 mg/kg). The highest content of alkanes was found in Putrid fermented grains (51.50 ± 14.03 mg/kg). Rancidity fermented grains contained the highest number of acids (38.11 ± 3.04 mg/kg), with isovaleric acid (2.19 ± 1.11 mg/kg) dominated.

PCA analysis shows that the flavor compounds in the normal and Rancidity fermented grains were highly similar and were significantly different from the Putrid, Musty, and Mud fermented grains (Fig. 6c). In addition, significantly altered volatile compounds were further identified by PLS-DA analysis. A 100-permutation test showed that the PLS-DA

model was reliable, with a Q2 intercept of −0.874 (Fig. 6d). The expected variable importance (VIP) summarizes and explains the significance of the correlation between X and Y. The aroma activity value (OAV) gives an idea of the aroma contribution of the flavor substances. Then 47 key flavor compounds (VIP > 1 and OAV > 1) were identified (Fig. S6), mainly including isoamyl alcohol, 2-octanone, isobutyric acid, phenol, and 2,3,5-trimethylpyrazine. Among them, the compounds with higher OAV and contents in normal fermented grains included ethyl butyrate (banana flavor), acetic acid (sour flavor), 1-heptanol (grass flavor), 2,3,5-trimethylpyrazine (nut flavor). However, the key compounds in off-flavor fermented grains differed from those in normal fermented grains, key compounds in Putrid fermented grains include ethyl 2-methylbutyrate (OAV = 63,704), 2-octanone (OAV = 6.50), isovaleric acid (OAV = 1.49), and indole (OAV = 1.22). Key compounds in rancidity fermented grains include isobutyric acid (OAV = 39.22), isovaleric acid (OAV = 1.82), and Phenylacetic acid ethyl ester (OAV = 1.84). Key compounds in Musty fermented grains include isoamylol (OAV = 1374.06), 1-octen-3-ol (OAV = 142.93). Key compounds in Burnt fermented grains include isoamyl alcohol (OAV = 145.67), m-Xylene (OAV = 3.27), 2-Pentylfuran (OAV = 1.12), and 2,5-Dimethylpyrazine (OAV = 1.03). Key compounds in Mud fermented grains include indole (OAV = 406.67), isoamyl alcohol (OAV = 101.89),
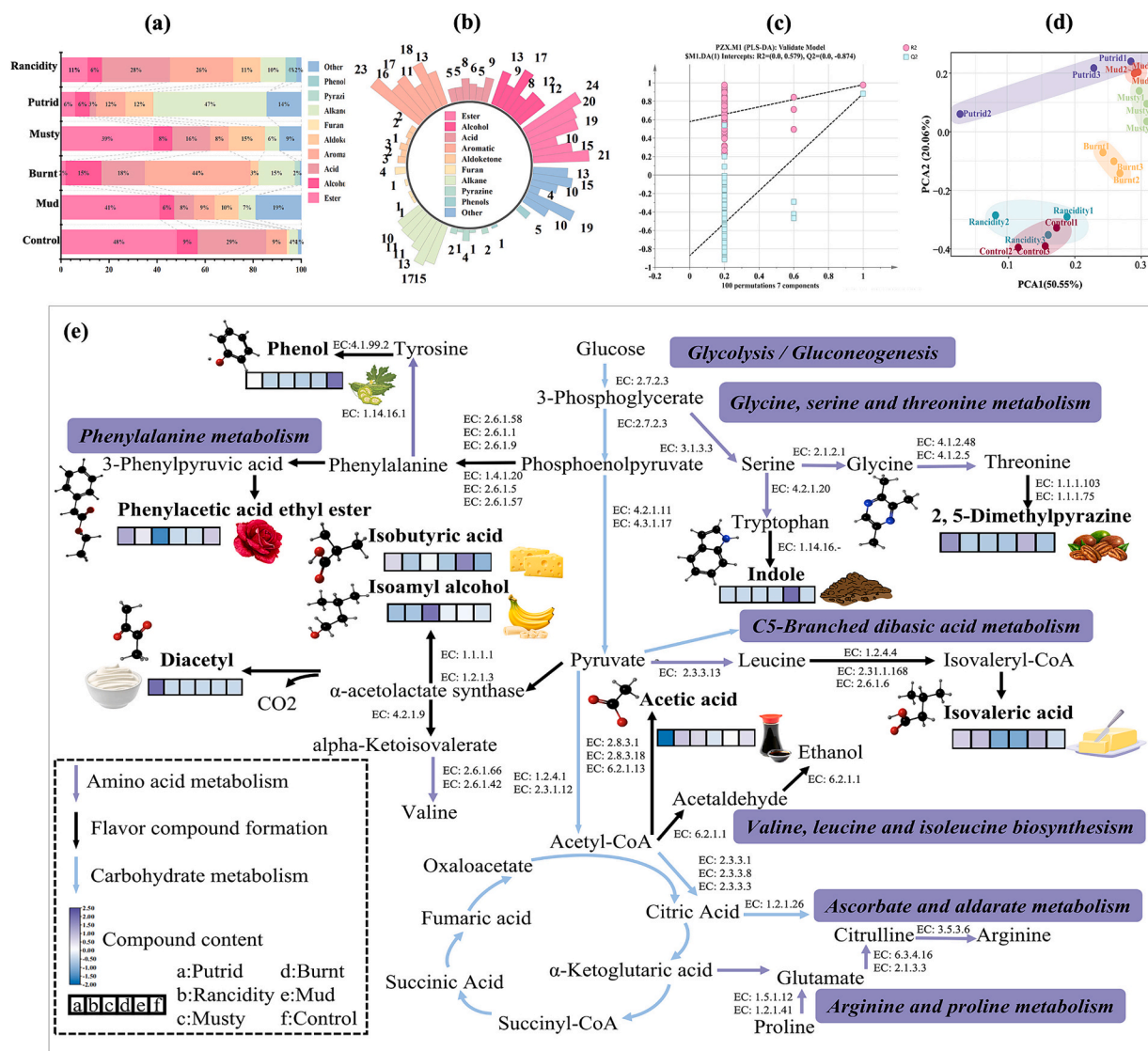
Fig. 6. Constitute characterization and metabolic pathway of flavor compounds in normal and abnormal fermented grains. Content (a) and type (b) of flavor compounds in normal and abnormal fermented grains. PLS-DA (c) and PCA (d) analysis of flavor compounds. (e) Metabolic pathway network prediction of off-flavor compounds.

isobutyric acid (OAV = 8.55). Correlations between microorganisms and 47 key flavor compounds were revealed by Pearson correlation analysis (Fig. S7). Genera with significant ($p < 0.05$) correlations with flavor compounds were considered as core functional genera, including *Pseudoflavonifractor, Sphingobacterium, Herbaspirillum, Lactobacillus, Acinetobacter, Monascus, Enterococcus, Dyadobacter, Ottowia, Stenotrophomonas, Pseudomonas, Xanthomonas* and other core functional genera. Among them, *Pseudoflavonifractor* was positively associated with four key compounds, such as ethyl laurate and isobutyric acid. *Enterobacter* showed a significant positive correlation with isobutanol and Monascus showed a significant positive ($p < 0.05$) correlation with phenol. *Chryseobacterium* showed a significant positive ($p < 0.05$) correlation with 2-phenylbutan-1-ol.

3.5. Metabolic pathways related to the formation of key off-flavor compounds

The metabolic pathways of the key flavor compounds formation were predicted (Fig. 6d-m). Species annotation of enzyme-encoding genes in possible flavor-generation pathways was performed, and the enzymes and microorganisms associated with flavor formation are shown in Table S1. Acetic acid is found in normal spirits, and this acid metabolite is produced by Lactobacillus to inhibit the growth of acid-intolerant microorganisms (Li, Fan, et al., 2020). an enzyme which exerts an important influence on the ability of lactic acid bacteria to metabolize acetic acid. Meanwhile, the high level of isovaleric acid (2.19 mg/kg) in rancidity fermented grains was associated with a significant up-regulation of the c5-branched dibasic acid metabolism (FC: 18.32), valine, leucine and isoleucine degradation (FC: 7.57) (Fig. 5e), which led to the metabolism of more isovaleric acid (Casey et al., 2004). The pathway of valine, leucine and isoleucine degradation contains key enzymes for the synthesis of short-chain fatty acids. The first step in isovaleric acid synthesis is the catalytic production of α-keto acids from three amino acids by branched-chain amino acid aminotransferase (EC 2.6.1.42), thereafter the conversion of α-keto acids to acylated short-chain fatty acids by alpha-keto acid decarboxylase (EC 1.2.4.4) and dihydrolipoyl transferase (EC 2.3.1.168), and the final step is the release of free short-chain fatty acids using chitinase (EC 3.2.1.14) to release free short-chain fatty acids, and the genera annotated by these enzymes include *Stenotrophomonas, Pseudomonas, Flavobacterium* and others (Table S1). Mud fermented grains was high in indole. Indole is known to have fecal flavor among wine and dairy products (Dong et al., 2018),

and the production of indole is associated with tryptophanase (EC: 4.1.99.1), an enzyme annotated by *Weeksellaceae, Eubacteriales, Clostridium*, and *Ottowia* (Table S1), with higher abundance of *Ottowia* in Mud fermented grains. 2,5-Dimethylpyrazine exists in Burnt fermented grains (Fig. 5d). During fermentation, microorganisms can use threonine as a substrate, and catalyzed by the enzyme threonine dehydrogenase (EC: 4.3.1.19) they can produce L-2-amino-acetoacetic acid, which, because of the inability to stably exist, can generate aminoacetone. Aminoacetone is condensed and oxidized to form 2,5-dimethylpyrazine (H. Zhang et al., 2020). The enzyme is annotated to *Enterobacter, Pichia*. Characteristic compounds in Musty fermented grains include 1-octen-3-ol and isopentanol. 1-octen-3-ol is mainly derived from the oxidation of fatty acids (C. Zhao et al., 2024). Among them, isopentanol is metabolically synthesized mainly through valine, leucine and isoleucine biosynthesis, which is converted to isopentanol via α-keto isocaproic acid. α-Keto isocaproic acid was co-annotated to the genera *Enterobacter, Klebsiella, Acetobacter, Alcaligenes*, and *Clostridium*. Diacetyl is only present in Putrid fermented grains. Valine and leucine are synthesized by acetolactate synthase (EC: 2.2.1.6), which catalyzes the formation of 2-acetyl lactate from pyruvate, which is naturally decarboxylated to form diacetyl (Costa et al., 2023). Acetolactate synthase (EC: 2.2.1.6) that are annotated include including *Pedobacter, Sphingobium, Acetobacter, Rhizobium, Dysgonomonas, Xanthomonas*, and *Xanthomonas*. Xanthomonas was more abundant in Putrid fermented grains.

4. Conclusion

This study provides a comprehensive perspective of the relationship between the microbiota and flavor compounds of normal and abnormal fermented grain. We found that the physicochemical characteristics of abnormal fermented grains were characterized by low acidity, low reducing sugar, and higher water content, further leading to the observation of off-flavors such as musty, rancidity, mud, musty, and burnt flavor. A total of 271 flavor compounds were identified and 47 key compounds by normal and abnormal fermented grain included indole, phenol, isoamyl alcohol, diacetyl, acetic acid, isobutyric acid, isovaleric acid, 2,5-dimethylpyrazine. Furthermore, 40 microbial genera were identified as the core microorganisms by their correlations ($p < 0.05$) with key flavor compounds, mainly including *Lactobacillus, Monascus, Enterococcus, Dyadobacter, Ottowia, Pseudoxanthomonas, Stenotrophomonas, Pseudomonas*, and *Xanthomonas*. By constructing the metabolic prediction maps of key off-flavor, it can be seen that most of the production of off-flavor compounds originates from some acid-intolerant and aerobic off-flavor microorganisms. Therefore, the control of the metabolism of off-flavor microorganisms can be achieved by appropriately adjusting the fermentation parameters in *Baijiu* to inhibit the production of off-flavor compounds. This study provides a new idea and scientific reference for the production process and mechanism of off-flavor compounds in *Baijiu* and lays the foundation for the quality control of *Baijiu*.

Ethics declarations

Our study did not require further ethics committee approval as it did not involve animal or human clinical trials and was not unethical. In accordance with the ethical principles outlined in the Declaration of Helsinki, all participants provided informed consent before participating in the study. The anonymity and confidentiality of the participants are guaranteed, and participation was completely voluntary.

CRediT authorship contribution statement

Jiaxin Gong: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Qiancheng Zuo:** Methodology, Investigation. **Zhaoqin Wu:** Funding acquisition. **Chong Zhao:** Formal analysis.

Junlin Wei: Writing – original draft. **Yongguang Huang:** 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.

Data availability

The authors do not have permission to share data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101660.

References

Annalisa, C., Paola, P., George-John, N., Francesco, V., & Danilo, E. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiology, 45*, 83–102. https://doi.org/10.1016/j.fm.2014.02.002

Casaburi, A., Piombino, P., Nychas, G. J., Villani, F., & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiology, 45*(Pt A), 83–102. https://doi.org/10.1016/j.fm.2014.02.002

Casey, M. G., Bosset, J. O., Bütikofer, U., & Fröhlich-Wyder, M.-T. (2004). Effect of α-keto acids on the development of flavour in Swiss gruyere-type cheese. *LWT - Food Science and Technology, 37*(2), 269–273. https://doi.org/10.1016/j.lwt.2003.09.002

Chen, C., Xiong, Y., Xie, Y., Zhang, H., Jiang, K., Pang, X.-N., & Huang, M. (2022). Metabolic characteristics of lactic acid bacteria and interaction with yeast isolated from light-flavor baijiu fermentation. *Food Bioscience, 50*, Article 102102. https://doi.org/10.1016/j.fbio.2022.102102

Chen, R., Gao, J., Yu, W., Chen, X., Zhai, X., Chen, Y., & Zhou, Y. J. (2022). Engineering cofactor supply and recycling to drive phenolic acid biosynthesis in yeast. *Nature Chemical Biology, 18*(5), 520–529. https://doi.org/10.1038/s41589-022-01014-6

Costa, G. P., Queiroz, L. B., Manfroi, V., Rodrigues, R. C., & Hertz, P. F. (2023). Immobilization of alpha Acetolactate decarboxylase in hybrid gelatin/alginate support for application to reduce diacetyl off-flavor in beer. *Catalysts, 13*.

Dong, W., Shi, K., Liu, M., Shen, C., Li, A., Sun, X., & Huang, M. (2018). Characterization of 3-Methylindole as a source of a "mud"-like off-odor in strong-aroma types of base baijiu. *Journal of Agricultural and Food Chemistry, 66*(48), 12765–12772. https://doi.org/10.1021/acs.jafc.8b04734

Du, H., Song, Z., Zhang, M., Nie, Y., & Xu, Y. (2021). The deletion of Schizosaccharomyces pombe decreased the production of flavor-related metabolites during traditional baijiu fermentation. *Food Research International, 140*, Article 109872. https://doi.org/10.1016/j.foodres.2020.109872

Du, H., & Xu, Y. (2012). Determination of the microbial origin of Geosmin in Chinese liquor. *Journal of Agricultural and Food Chemistry, 60*(9), 2288–2292. https://doi.org/10.1021/jf204648e

Gong, J., Ma, Y., Li, L., Cheng, Y., & Huang, Y. (2023). Comparative characterization and contribution of key aroma compounds in the typical base liquor of Jiang-flavor baijiu from different distributions in the Chinese Chishui River basin. *Food Chemistry: X, 20*, Article 100932. https://doi.org/10.1016/j.fochx.2023.100932

Hassing, E.-J., de Groot, P. A., Marquenie, V. R., Pronk, J. T., & Daran, J.-M. G. (2019). Connecting central carbon and aromatic amino acid metabolisms to improve de novo 2-phenylethanol production in Saccharomyces cerevisiae. *Metabolic Engineering, 56*, 165–180. https://doi.org/10.1016/j.ymben.2019.09.011

He, Y., Liu, Z., Qian, M., Yu, X., Xu, Y., & Chen, S. (2020). Unraveling the chemosensory characteristics of strong-aroma type Baijiu from different regions using comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry and descriptive sensory analysis. *Food Chemistry, 331*, 127335. https://doi.org/10.1016/j.foodchem.2020.127335

Ji, M., Du, H., & Xu, Y. (2020). Structural and metabolic performance of p-cresol producing microbiota in different carbon sources. *Food Research International, 132*, Article 109049. https://doi.org/10.1016/j.foodres.2020.109049

Li, K., Chen, Y., Liu, T., Deng, M., Xu, Z., Fu, G., & Zheng, F. (2020). Analysis of spatial distribution of bacterial community associated with accumulation of volatile compounds in Jiupei during the brewing of special-flavor liquor. *LWT, 130*, Article 109620. https://doi.org/10.1016/j.lwt.2020.109620

Li, W., Fan, G., Fu, Z., Wang, W., Xu, Y., Teng, C., & Li, X. (2020). Effects of fortification of Daqu with various yeasts on microbial community structure and flavor metabolism. *Food Research International, 129*, Article 108837. https://doi.org/10.1016/j.foodres.2019.108837

Liang, Z., Zhang, P., Ma, W., Zeng, X., & Fang, Z. (2024). Physicochemical properties, antioxidant activities and comprehensive phenolic profiles of tea-macerated chardonnay wine and model wine. *Food Chemistry, 436*, Article 137748. https://doi.org/10.1016/j.foodchem.2023.137748

Liu, J., Wu, Q., Wang, P., Lin, J., Huang, L., & Xu, Y. (2017). Synergistic effect in Core microbiota associated with sulfur metabolism in spontaneous Chinese liquor fermentation. *Applied and Environmental Microbiology, 83*(24). https://doi.org/10.1128/aem.01475-17

Niu, Y., Zhang, J., Xiao, Z., & Zhu, J. (2020). Evaluation of the perceptual interactions between higher alcohols and off-odor acids in Laimao baijiu by σ–τ plot and partition coefficient. *Journal of Agricultural and Food Chemistry, 68*(50), 14938–14949. https://doi.org/10.1021/acs.jafc.0c05676

Pan, F., Qiu, S., Lv, Y., & Li, D. (2023). Exploring the controllability of the baijiu fermentation process with microbiota orientation. *Food Research International, 173*, Article 113249. https://doi.org/10.1016/j.foodres.2023.113249

Pang, X., Chen, C., Huang, X., Yan, Y., Chen, J., & Han, B. (2021). Influence of indigenous lactic acid bacteria on the volatile flavor profile of light-flavor baijiu. *LWT, 147*, Article 111540. https://doi.org/10.1016/j.lwt.2021.111540

Qiao, L., Wang, J., Wang, R., Zhang, N., & Zheng, F. (2023). A review on flavor of baijiu and other world-renowned distilled liquors. *Food Chemistry: X, 20*, Article 100870. https://doi.org/10.1016/j.fochx.2023.100870

Serjak, W. C., Day, W. H., Van Lanen, J. M., & Boruff, C. S. (1954). Acrolein production by bacteria found in distillery grain mashes. *Applied Microbiology, 2*(1), 14–20. https://doi.org/10.1128/am.2.1.14-20.1954

Wang, H., Huang, Y., & Huang, Y. (2021). Microbiome diversity and evolution in stacking fermentation during different rounds of Jiang-flavoured baijiu brewing. *LWT, 143*, Article 111119. https://doi.org/10.1016/j.lwt.2021.111119

Wang, H., Sun, C., Yang, S., Ruan, Y., Lyu, L., Guo, X., & Chen, Y. (2023). Exploring the impact of initial moisture content on microbial community and flavor generation in Xiaoqu baijiu fermentation. *Food Chemistry: X, 20*, Article 100981. https://doi.org/10.1016/j.fochx.2023.100981

Wang, L., Fan, S., Yan, Y., Yang, L., Chen, S., & Xu, Y. (2020). Characterization of potent odorants causing a pickle-like off-odor in Moutai-aroma type baijiu by comparative aroma extract dilution analysis, quantitative measurements, aroma addition, and omission studies. *Journal of Agricultural and Food Chemistry, 68*(6), 1666–1677. https://doi.org/10.1021/acs.jafc.9b07238

Wei, J., Lu, J., Nie, Y., Li, C., Du, H., & Xu, Y. (2023). Amino acids drive the deterministic assembly process of fungal community and affect the flavor metabolites in baijiu fermentation. *Microbiol Spectr, 11*(2), Article e0264022. https://doi.org/10.1128/spectrum.02640-22

Wei, W., Shen, Y., Cheng, W., & Zhang, W. (2023). Analysis of microbial diversity and volatile metabolites across different types of pit mud in sauce-flavored baijiu based on nanopore sequencing and metabolomics. *LWT, 188*, Article 115465. https://doi.org/10.1016/j.lwt.2023.115465

Yao, L., Zhang, J., Lu, J., Chen, D., Song, S., Wang, H., & Feng, T. (2023). Revealing the influence of microbiota on the flavor of kombucha during natural fermentation process by metagenomic and GC-MS analysis. *Food Research International, 169*, Article 112909. https://doi.org/10.1016/j.foodres.2023.112909

Zhang, C., Zhang, F., Wang, Y., Shi, X., Fan, R., & Ni, L. (2021). Ultrasonic and enzymatic pretreatments of Monascus fermentation byproduct for a sustainable production of Bacillus subtilis. *Journal of the Science of Food and Agriculture, 101*(9), 3836–3842. https://doi.org/10.1002/jsfa.11018

Zhang, H., Zhang, L., Yu, X., & Xu, Y. (2020). The biosynthesis mechanism involving 2,3-Pentanedione and Aminoacetone describes the production of 2-Ethyl-3,5-dimethyl-pyrazine and 2-Ethyl-3,6-dimethylpyrazine by Bacillus subtilis. *Journal of Agricultural and Food Chemistry, 68*(11), 3558–3567. https://doi.org/10.1021/acs.jafc.9b07809

Zhang, R., Wu, Q., & Xu, Y. (2014). Lichenysin, a Cyclooctapeptide occurring in Chinese liquor Jiannanchun reduced the headspace concentration of phenolic off-flavors via hydrogen-bond interactions. *Journal of Agricultural and Food Chemistry, 62*(33), 8302–8307. https://doi.org/10.1021/jf502053g

Zhao, C., Lin, J., Zhang, Y., Wu, H., Li, W., Lin, W., & Luo, L. (2024). Comprehensive analysis of flavor formation mechanisms in the mechanized preparation Cantonese soy sauce koji using absolute quantitative metabolomics and microbiomics approaches. *Food Research International, 180*, Article 114079. https://doi.org/10.1016/j.foodres.2024.114079

Zhao, T., Chen, S., Li, H., & Xu, Y. (2018). Identification of 2-Hydroxymethyl-3,6-diethyl-5-methylpyrazine as a key Retronasal burnt flavor compound in soy sauce aroma type baijiu using sensory-guided isolation assisted by multivariate data analysis. *Journal of Agricultural and Food Chemistry, 66*(40), 10496–10505. https://doi.org/10.1021/acs.jafc.8b03980

Zheng, J., Feng, J.-Q., Zhou, L., Mbadinga, S. M., Gu, J.-D., & Mu, B.-Z. (2018). Characterization of bacterial composition and diversity in a long-term petroleum contaminated soil and isolation of high-efficiency alkane-degrading strains using an improved medium. *World Journal of Microbiology and Biotechnology, 34*(2), 34. https://doi.org/10.1007/s11274-018-2417-8

Zhu, L., Song, X., Li, X., Geng, X., Zheng, F., Li, H., & Sun, B. (2023). Interactions between kafirin and pickle-like odorants in soy sauce flavor baijiu: Aroma profile change and binding mechanism. *Food Chemistry, 400*, Article 133854. https://doi.org/10.1016/j.foodchem.2022.133854

Zhuo, C., Xuefeng, W., Dongdong, M., Ying, H., Mingzhu, Z., Jing, C., … Jin, L. (2021). Determination and correlation analysis of flavor components with bacterial Community in Post-fermented red Sufu. *Food Science, 42*(6), 118–125. https://doi.org/10.7506/spkx1002-6630-20191203-043