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Exploring major variable factors influencing flavor and microbial characteristics of upper jiupei

Shuangping Liu [a,c,d,e,1], Dongliang Ren [a,e,1], Hui Qin [b], Qianqian Yin [a], Yan Yang [b], Tiantian Liu [a], Suyi Zhang [b,*], Jian Mao [a,c,d,e,*]

[a] National Engineering Research Center of Cereal Fermentation and Food Biomanufacturing, State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China
[b] Luzhou Laojiao Group Co. Ltd, Luzhou 646000, China
[c] Shaoxing Key Laboratory of Traditional Fermentation Food and Human Health, Jiangnan University (Shaoxing) Industrial Technology Research Institute, Shaoxing, Zhejiang 312000, China
[d] National Engineering Research Center of Huangjiu, Zhejiang Guyuelongshan Shaoxing Wine Co., Ltd., Shaoxing, Zhejiang 312000, China
[e] Jiangsu Provincial Engineering Research Center for Bioactive Product Processing, Jiangnan University, Wuxi, Jiangsu 214122, China

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ABSTRACT

The flavor quality of *jiupei* gradually decreased with the increase of cellar height. In this study, high-throughput sequencing, metabolomics and HS-SPME-GC-MS techniques were used to explore the mechanism of flavor quality decline in upper *jiupei* in mud sealed cellars. The results showed the total content of flavor compounds increased from 1947.48 mg/L in top-site to 3855.51 mg/L in bottom of the cellar, and 19 differential flavor compounds were identified based on OPLS-DA, mainly including 12 esters such as ethyl hexanoate, ethyl butyrate, propyl hexanoate, hexyl caproate and 5 other substances such as caprylic acid, decanal and non-aldehyde. *Lactobacillus*, *Prevotella* and *Methanobacterium* were dominant genus of bacteria in all of cellars, while *Thermomyces*, *Aspergillus*, *Pichia*, *Trichosporon* and *Rhizopus* were the dominant genera of fungi. Oxygen was the key factor causing the quality heterogeneity of flavor substances and microbial communities in *jiupei* at different depths. Anaerobic micro-pressure sealed cellars (AMSC) method was developed and applied to *jiupei* fermentation, the difference in oxygen content between top site (5.90 ± 0.62 %) and bottom of the cellar (4.17 ± 0.75 %) in AMSC was smaller than that in mud sealed cellars, there were no significant differences in flavor substances content between top site and bottom of the cellar, and microbial communities showed no significant differences of the four-layer *jiupei*. This study provides a theoretical support for improving the flavor quality of upper *jiupei*.

1. Introduction

The solid-state fermentation of strong-flavor baijiu (SFB) is fermented from mixture of grains (sorghum, corn, rice and wheat) through a unique anaerobic fermentation process (Jin, Zhu, & Xu, 2017), and usually carried out in a mud pit (normally with a volume of 6–8 m^3) (Fig. S1). Natural microorganisms involved in SFB fermentation mainly originated from daqu, *jiupei*, pit mud and fermentation environments (Cai W. C., Wang, Ni, Liu, Liu, Zhong, et al., 2021). However, there always exists differences in the types due to different geographical climate and production process (Wang L., Huang, Hu, & Li, 2021; Zou, Zhao, & Luo, 2018). Strong-flavor *baijiu* (SFB) is deeply loved by consumers due to its unique flavor, and its production and sales account for >70 % of the total *baijiu* in China (Wang X. S., Du, Zhang, & Xu, 2018). Most of the microorganisms inhabited in *jiupei* and pit mud are strictly anaerobic or facultative anaerobic (Li X. R., Ma, Yan, Meng, Du, Zhang, et al., 2011; Wang H. Y., Zhang, Zhao, & Xu, 2008), which also plays an essential role in the formation and quality of SFB (Cai W. C., Xue, Tang, Wang, Yang, Liu, et al., 2022; Jin, Zhu, & Xu, 2017). Microorganisms metabolize nutrients in raw materials to produce acids, alcohols and other flavor compounds and enzymes in an anaerobic or microaerobic environment (Peng, Zheng, Meng, Yu, Xie, Zhang, et al., 2022; Peng, Zheng, Meng, Zhu, Zhu, Zhu, et al., 2022; Qian, Lu, Chai, Zhang, Li, Wang et al., 2021; Wang C. D., Chen, Wang, Li, Leng, Li, et al., 2014). The characteristic

* Corresponding authors at: National Engineering Research Center of Cereal Fermentation and Food Biomanufacturing, Jiangnan University, Wuxi, Jiangsu 214122, China (J. Mao).
E-mail addresses: zhangsy@lzlj.com (S. Zhang), maojian@jiangnan.edu.cn (J. Mao).
[1] The authors contributed equally to this article.

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flavor compounds of SFB include hexanoic, butyric, acetic and lactic acids, and their corresponding ethyl esters (Fang, Du, Jia, & Xu, 2019). In factory production, the cellar is often sealed to create an anaerobic environment for brewing microorganisms and prevent the infiltration of microorganisms from the external environment (Bokulich, Bergsveinson, Ziola, & Mills, 2015). The change of cellar fermentation environment will affect the composition of microorganisms in different spatial locations, and further affect the content of flavor compounds such as acids and alcohols in *jiupei* (Zhang, Wang, Tan, Wang, Yang, Chen, et al., 2021), and eventually decreasing yield and quality of liquor. In the traditional brewing process of SFB, yellow mud is often used to seal the cellar, it directly contacts with *jiupei*, which will affect the sealing of the cellar and further influence the quality of raw liquor. However, there have been no reports on the impact of the sealing method on the differences in raw liquor quality among different *jiupei* layers in the brewing of strong-flavor *baijiu*. Production practice has proven that there are differences in the quality of raw liquor in different layers of the cellar (Rhee, Lee, & Lee, 2011). The deeper the cellar, the higher the quality of the raw liquor produced. Congruently, the quality of the raw liquor from upper *jiupei* is the lowest. This severely limits improving production of high-quality *baijiu*. However, the reason for this phenomenon is unclear and the relevant mechanisms require urgent analysis.

We investigate the cause of the decline in flavor quality of upper *jiupei* in mud sealed cellars. We hypothesized that (hypothesis 1) the types and contents of metabolites in upper *jiupei* are different from lower *jiupei*, causing the decline of flavor quality of upper liquor; (hypothesis 2) external environmental conditions outside yellow mud might disrupt the *jiupei* microecosystem, resulting quality decline of upper *jiupei*.

Luzhou is a representative area of SFB (Li Y. L., Liu, Zhang, Liu, Qin, Shen, et al., 2022), therefore, we selected Luzhoulaojiao factory as the research subject in our study. The aim of this research was to analyze the flavor compounds, microorganisms, and metabolites in mud sealed cellars based on 16S rRNA and internal transcribed spacer (ITS) gene amplicon full-length sequencing, metabolomics and HS-SPME-GC–MS techniques, further to explore the mechanisms of decline of flavor quality in upper *jiupei* and propose an improved cellar sealing method based on the research results.

2. Materials and methods

2.1. Experimental design and sample collection

The *jiupei* used in this experiment was taken from a normal production cellar in the brewing base of Luzhou Laojiao Group in the Sichuan Province. *jiupei* was sampled from the mud sealed cellar after fermentation. As shown in Fig. S1, each cellar was assigned four positions, T-A, T-B, T-C and T-D, each layer used the five-point sampling method of retrieving 50 g of *jiupei* and thoroughly mixing them as a single sample. Four-layers of *jiupei* samples were taken from five cellars as parallels, stored in sterile sampling bags, and stored at −80 °C. Equal amounts of T-C and T-D were mixed evenly as a sample, named T-E. Metabolomics analysis of T-A, T-B and T-E samples was performed. Similarly, the *jiupei* in the anaerobic micro-pressure sealed cellars was sampled and denoted as P-A, P-B, P-C, P-D and P-E respectively. Raw liquor: the raw liquor used in this experiment was taken from the unaged liquor distilled from the above-mentioned *jiupei* in the workshop, and three samples were collected as parallels and stored at 4 °C to determine the content of flavor substances. The raw liquor samples in the mud-sealed cellar were recorded as JT-A, JT-B, JT-C and JT-D, and the raw liquor samples in the anaerobic micro-pressure sealed cellar were recorded as JP-A, JP-B, JP- C and JP-D.

2.2. Jiupei physicochemical properties and raw liquor flavor compounds content

Changes in oxygen content in the *jiupei* were monitored using a microsensor monometer with a CY-12C portable oxygen meter (Lu, Liu, Ma, Liu, Wu, Zeng, et al., 2016). Moisture content was analyzed immediately post sampling by drying *jiupei* samples to a set weight in an oven at 105 °C for 4 h. The pH was determined in a 1:10 *jiupei* /water (wt/vol) slurry using a pH meter (PB10; Sartorius, Gottingen, Germany). The content of total starch and sugar were monitored by the method described by Bravo et al. (Bravo, Siddhuraju, & Saura-Calixto, 1998) and Miller et al. (Miller, 1959). Total acidity was determined by sodium hydroxide titration according to the methods of Chai, Lu, et al. (Chai, Lu, Zhang, Ma, Xu, Qian, et al., 2019). The concentration of the organic acids caproic acid, butyric acid, and acetic acid in the *jiupei* were measured by GC–MS (Gao J., Ren, Liu, Xiangyang, Wang, Fangping, et al., 2019). The flavor compounds of *jiupei* were extracted using HS-SPME and analyzed using GC–MS (Tan, Zhong, Zhao, Du, & Xu, 2019).

2.3. DNA extraction, 16S rRNA gene, and ITS amplicon sequencing

The DNA of *jiupei* samples was extracted by FastDNA Spin Kit for Soil. PCR amplification was performed on a PacBio Sequel II instrument with reference to the method of Li et. al (Li et al., 2011). All raw data were processed by QIIME (v1.9.1) using Uclust to cluster high-quality sequences into operational taxonomic units (OTUs) with 97 % sequence similarity, the bacterial 16S sequence and fungal ITS sequence alignment databases were Silva database (v13.2) and UNITE database (v12.11) respectively. ACE, Coverage, Chao1 abundance, and Shannon diversity indices were calculated using QIIME.

2.4. Metabolomic analysis of jiupei

Untargeted metabolomic analysis of *jiupei* samples was performed using GC–MS and LC-MS. GC–MS analysis was performed using a 7890A gas chromatograph (Agilent) coupled with a PEGASUS HT mass spectrometer (LECO) (Kind, Wohlgemuth, Lee, Lu, Palazoglu, Shahbaz, et al., 2009). LC-MS analysis was performed on a Vanquish UPLC system (Thermo Fisher Scientific) coupled with a Q Exactive HFX mass spectrometer (Thermo Fisher Scientific) (Cai Y., Weng, Guo, Peng, & Zhu, 2015).

GC–MS raw data analysis (Dunn, David, Paul, Eva, Sue, Nadine, et al., 2018), including peak extraction, baseline adjustment, deconvolution, alignment, and integration, was finished with Chroma TOF (V 4.3x, LECO) software and the LECO-Fiehn Rtx5 database was used for metabolite identification by matching the mass spectrum and retention index. Finally, the peaks detected in less than half of the QC samples or RSD > 30 % in QC samples were removed. LC-MS raw data were converted to mzXML format using ProteoWizard and processed with an in-house program, which was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration. An in-house MS2 database (BiotreeDB) was then applied in metabolite annotation (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006). The cutoff for annotation was set at 0.3.

2.5. Statistical analysis

Origin 2017 64 Bit and SPSS 21 software were used for data processing and analysis. Principal component analysis was calculated by SIMCA (13.0.0.0), the cluster analysis was conducted with the unweighted-pair group method using average linkages (UPGMA) based on the Bray-Curtis distance in PAST (2.17). Nonmetric multidimensional scaling (NMDS) provides visualization of the microbial community composition. Similarity analysis (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were used to determine the differences in microbial communities. Redundancy analysis (RDA) was

performed using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY). The statistical significance of the difference between the means of samples was tested by one-way analysis of variance (ANOVA) with the Tukey post hoc test.

3. Results and discussion

3.1. The content and proportion of flavor substances are crucial factors determining the quality of baijiu

Headspace solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC–MS) was used to analyze the volatile aroma compounds of the raw liquor. The results showed (Fig. 1a) that 31 esters, 6 alcohols, 3 acids, 3 aldehydes, 2 phenols, and 2 esters were detected from the raw liquor. Comparatively, the proportion of esters is highest, followed by alcohols and acids.

With an increase in the depth of the cellar, the total content of flavor compounds increased from 1947.48 mg/L in JT-A to 3855.51 mg/L in JT-D ($P < 0.05$), and the total content of acid, ester, and alcohol accounted for>90 % of the total content of flavor compounds in the raw liquor. In general, the content of four major esters increased significantly from 601.55 mg/L (JT-A) to 1369.18 mg/L (JT-D), and the total esters increased significantly from 1744.26 mg/L (JT-A) to 3657.96 mg/L (JT-D). The total acid content of JT-D reached 38.25 mg/L, which was significantly higher than those of the raw liquor in other three layers ($P < 0.05$), and caproic acid was the main organic acid (31.84 mg/L) in JT-D. The N-hexanol and n-octanol showed no significant difference in the raw liquor of different layers. The PCA results (Fig. 1b) showed that flavor compounds in JT-B and JT-C were clustered together, and JT-A and JT-D were outliers, indicating the content of flavor compounds in JT-B and JT-C were relatively similar, but in JT-A, JT-D and other layers of raw liquor were quite different. The upper *jiupei* is most susceptible to cellar opening, and the huangshui produced during fermentation causes microorganisms and metabolites to transfer to bottom of the cellar, leading to the difference between JT-A and JT-D.

To further investigate the characteristic flavor compounds that lead to the difference in the quality of raw liquor, the flavor compounds of JT-A and JT-D were selected for partial least squares discrimination analysis (OPLS-DA). Through the variable importance in the projection analysis of flavor compounds, 19 flavor compounds with VIP > 1.2 were selected as the essential characterizing flavor compounds of JT-A and JT-D. As can be seen in Fig. S2a, 12 esters such as ethyl hexanoate, ethyl butyrate, propyl hexanoate, and hexyl caproate, and 5 other substances such as caprylic acid, decanal, and nonaldehyde are concentrated in JT-D, indicating that the content of these flavor compounds is positively correlated with JT-D. The content of esters in JT-D is higher than that in JT-A, and the content of ethyl caproate, the characteristic flavor compound of Luzhou-flavor liquor (Yao, Yi, Shen, Tao, Liu, Lin, et al., 2015), is also higher than that of JT-A, which is consistent with the above results. In conclusion, esters are the key flavor substances influencing flavor quality in raw liquor with different layers.

3.2. Metabolomics analysis of metabolites in jiupei

Our study identified a total of 1051 metabolites in *jiupei*, including 151 metabolites identified by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and 900 metabolites identified by ultra-high-performance-liquid-chromatography q-exactivemass spectrometer (UHPLC-QE-MS). As shown in Fig. 1c, the metabolites in *jiupei* mainly comprised of organic acids and derivatives (21.98 %), lipids and lipid-like molecules (18.27 %), organic heterocyclic compounds (14.08 %), benzenoids (8.18 %), and organic oxygen compounds (7.80 %). Previous study showed that lipids and lipid-like molecules, organoheterocyclic compounds and organic acids and derivatives were the key metabolites in artificial pit mud (Liu, Tang, Guo, Zhao, Penttinen, Tian, et al., 2020), indicating that there was a potential metabolite exchange between pit mud and *jiupei*.

Metabolites displayed a clear separation of the layers in the (OPLS-

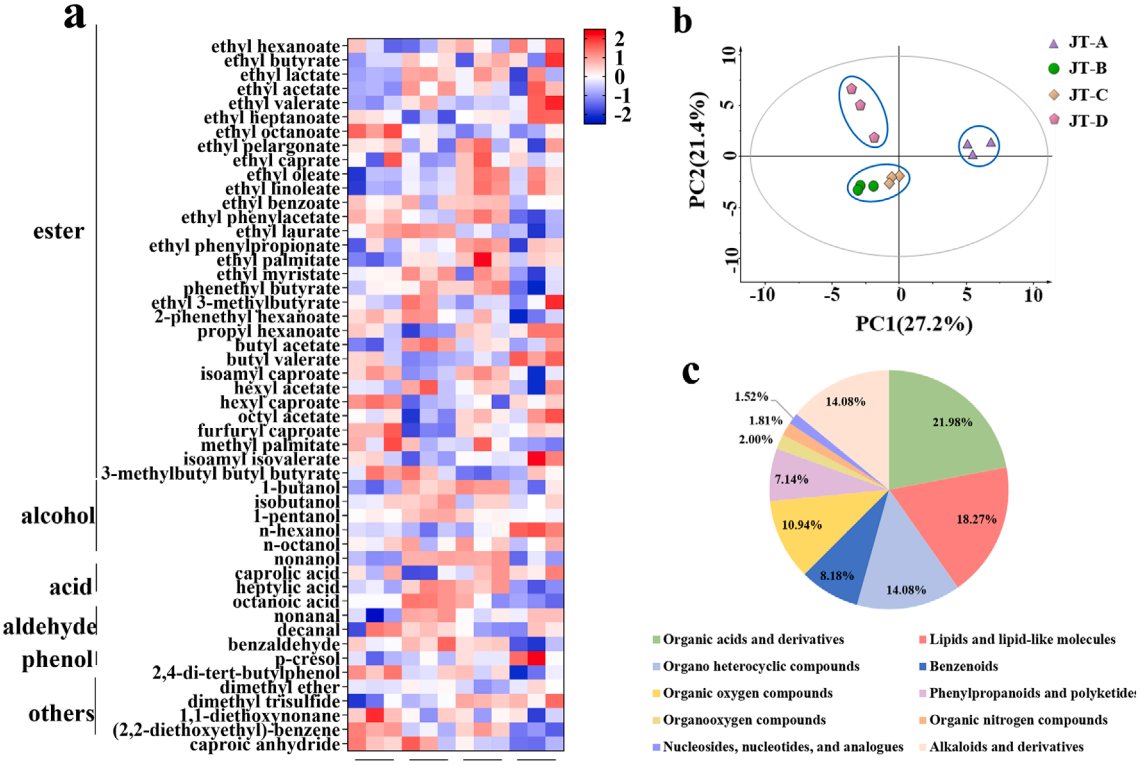

Fig. 1. Flavor compounds of raw liquor and metabolites of *jiupei* in mud sealed cellars. (a) Heat map of concentrations of flavor compounds (log2 scale) in raw liquor. (b) PCA of flavor compounds in raw liquor. (c) Classification of metabolites of *jiupei*.

DA) scatter plots (Fig. S2b). A total of 47 metabolites were identified as differential metabolites between T-A and T-B, while 70 were identified between T-B and T-E (variable importance in projection VIP > 1, P < 0.05). As shown in Fig. S2c, comparing T-A with T-B, the relative abundance of 37 and 10 metabolites increased and decreased, respectively. Likewise, comparing T-B with T-E, the relative abundance of 33 and 37 metabolites increased and decreased, respectively. Among these metabolites, the content of organic acids and their derivatives changed most significantly.

3.3. Microbiota diversity was different in different layers of the cellar

With the increase in cellar depth, Chao1 and Shannon indices of bacteria both initially decreased and then increased (Tab.S1), indicating a corresponding pattern in bacterial diversity. This may be due to a simpler *jiupei* fermentation environment at greater depths as well as a reduction in influence of yellow mud and the external environment, resulting in the elimination of bacteria that are unable to adapt to the brewing environment. The bottom of the cellar is most affected by pit mud, which may explain the increased bacterial diversity in T-D. As shown in Fig. 2a, A total of 40 bacterial species were identified from the bacterial community structure of *jiupei*, including five dominant species: uncultured *Lactobacillus* sp (14.44–53.87 %), *Lactobacillus acetotolerans* (23.11–41.45 %), uncultured *Prevotella* sp (0.79–37.05 %), uncultured *Treponema* 2 (0.04–1.17 %), and uncultured *Methanobacterium* (0.12–0.9 %). Of these, *Lactobacillus acetotolerans* originated from pit mud, which was the dominant species in the late fermentation stage and played an important role in the fermentation of SFB (Sulaiman, Gan, Yin, & Chan, 2014). Tao et al (Tao, Wang, Li, Wei, Jin, Xu, et al., 2017) revealed that the microbiota owned metabolic potential for the caproic acid production with lactic acid as precursor substance, indicating that LAB bacteria play an important role in the fermentation process (Xie, Zheng, Qiu, Lin, Peng, Bealu, et al., 2021). *Methanobacterium* has high abundance in the late fermentation stage, which can promote the production of caproic acid through the synergistic effect with *Caproiciproducens* and *Clostridium sensu stricto* (Ding, Wu, Huang, & Zhou, 2015), further increasing the content of ethyl caproate in the late fermentation stage. PERMANOVA was performed on the bacterial community structure of *jiupei* at different depths based on community composition, apart from T-C and T-D, there were significant differences in bacterial community structure between any two spatial locations of *jiupei* (P < 0.05). The NMDS analysis (Fig. 2c) shown that bacterial communities of T-B, T-C and T-D were relatively similar, while the bacterial community of T-A was different from that of the other three layers of *jiupei* (P < 0.05). Microorganisms migrate into T-A, resulting in differences in microbial community structure between T-A and other layers of *jiupei*.

The ACE (1200–1400) and Chao1 index (1064–1180) of fungi had no significant differences at different depths in *jiupei*, indicating a corresponding similarity in fungal abundance of *jiupei*. However, the Shannon index of T-C was significantly lower than that of T-A and T-B (P < 0.05),

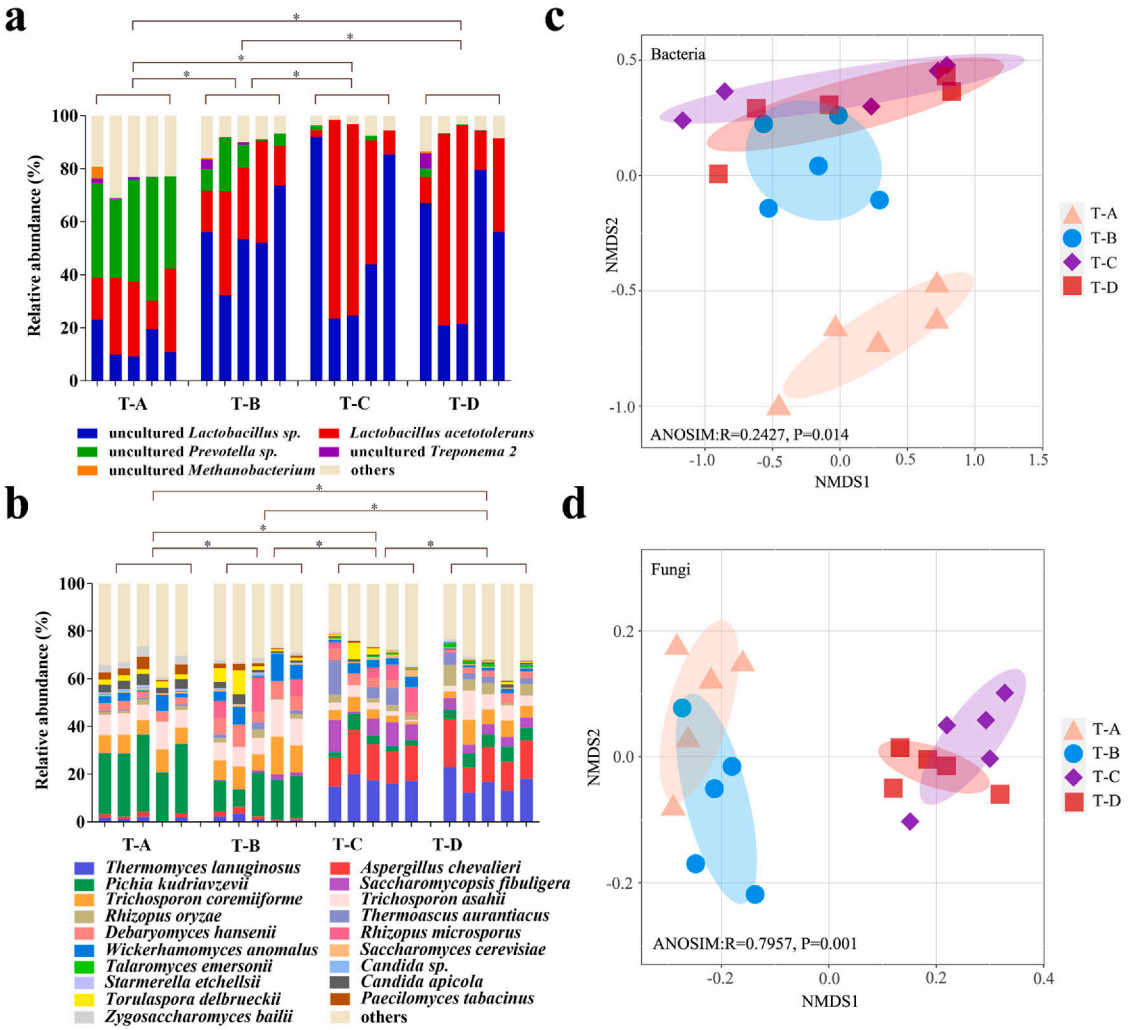

Fig. 2. Microbial community of *Jiupei* at different depths of the mud sealed cellars (a) Bacterial species level (b) Fungul species level (* refers to the microbial difference between the two *jiupei* groups with different layers. P < 0.05) (c) NMDS analysis of Bacteria (d) NMDS analysis of Fungi.

while the other *jiupei* showed no significant differences between each other, indicating that *jiupei* fungal diversity first decreased and then increased with the increase in cellar depth, which was consistent with bacterial diversity. As seen in Fig. 2b, a total of 103 fungal species were identified, with 20 dominant species, including *Thermomyces lanuginosus*, *Aspergillus chevalieri*, *Pichia kudriavzevii*, *Trichosporon coremiiforme*, *Trichosporon asahii*, and *Rhizopus oryzae*. The fungal communities of different layers of *jiupei* were analyzed by PERMANOVA. Fig. 2d shows that there were significant differences in the fungal community structure between any two layers of *jiupei*, indicating that with the increase in depth of the cellar, the fungal communities of *jiupei* changes significantly ($P < 0.05$).

3.4. Reasons for jiupei-layer differences in microbial communities and flavor composition in jiupei

This study compared the oxygen content of T-A and T-D after seven days of fermentation, as well as the physical and chemical indices and organic acid contents of different layers of *jiupei* after fermentation (Tab. S1). The results showed that the oxygen content of T-A was significantly higher than that of T-D ($P < 0.05$), which may be due to the effect of gravity, the change in porosity of *jiupei* with depth, or the huangshui produced in the fermentation process of *jiupei* that collects at the bottom of the cellar, leading to an anaerobic environment. With the increase in cellar depth, starch content and acidity did not change significantly, however the pH of T-A was significantly higher than the other three layers ($P < 0.05$), and the reducing sugar content of T-D was

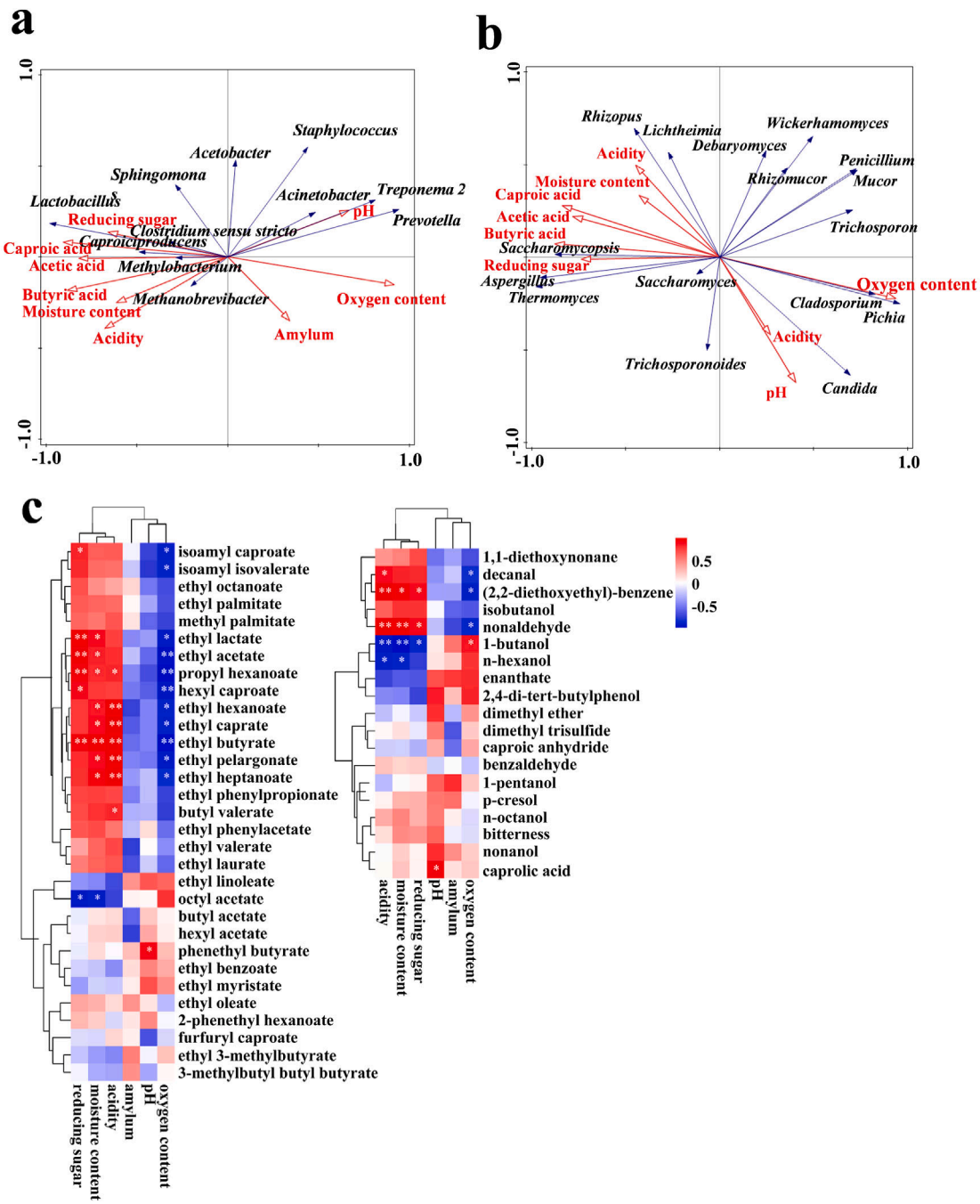

Fig. 3. RDA analysis of factors and community composition of bacteria (a) and fungi (b) in *Jiupei*. (c) Spearman correlation between factors of *Jiupei* and key flavor compounds of raw liquor. (*) $P < 0.05$, (**) $P < 0.01$.

significantly higher than the other three layers ($P < 0.05$). This may be that microorganisms and their metabolites (sugars, acids) from the middle and upper layers of fermented grains flow into T-D, resulting in a higher reducing sugar content (Gao Z. Z., Wu, & Zhang, 2020). The concentrations of caproic acid, butyric acid, and acetic acid all showed an increasing trend with the depth of the cellar. The content of caproic acid in T-D was 2.12 ± 0.46 g/kg, which was approximately 10 times higher than that in T-A (0.22 ± 0.06 g/kg). The concentrations of butyric acid and acetic acid in T-D were also significantly higher than that in T-A ($P < 0.05$) as the contact area between *jiupei* and pit mud increased with the depth. Acetic acid is an end metabolite of many microbiota metabolism (Hamdi, Ben Hania, Postec, Bouallagui, Hamdi, Bonin, et al., 2015), the level of acetic acid content can reflect the strength of microbial metabolism to a certain extent. Pit mud contains a large number of caproic acid–producing bacteria, there is a decrease in dissolved oxygen coefficients with an increase in pit depth, which is conducive to the metabolism of anaerobic bacteria such as caproic acid-producing bacteria to produce organic acids such as caproic acid and butyric acid.

Redundancy analysis showed that oxygen content had the most significant influence on the microbial communities, which could explain 98.7 % and 94.1 % of the bacterial (Fig. 3a) and fungal (Fig. 3b) community composition, respectively ($P < 0.01$). The relationship between the physicochemical indices of the *jiupei* and the flavor compounds of the raw liquor was further expounded based on the Spearman analysis. As shown in the Fig. 3c, the flavor compounds were most significantly related to oxygen content, among which 11 esters such as ethyl hexanoate, ethyl butyrate, and ethyl acetate, as well as nonanal, decanal, and (2,2-diethoxyethyl)-benzene were significantly negatively correlated with oxygen content ($P < 0.05$). Conversely, 1-butanol was significantly positively correlated with oxygen content ($P < 0.05$). Except for ethyl acetate, 13 components that negatively correlated with oxygen content were the main differential flavor compounds of T-A and T-D in mud sealed cellars. Spearman correlation analysis was used to expound the relationship between the *jiupei* microorganisms and flavor compounds, and the results are shown in Fig. S3a and b. The microorganisms significantly related to the flavor compounds of the raw liquor are essentially the differential microorganisms of T-A and T-D in the mud sealed cellars.

In summary, the difference in oxygen content in the early stage of fermentation is the key factor that causes the difference in the microbial community structure of *jiupei* at different depths, and these microorganisms are closely related to the formation of flavor substances (Sulaiman, Gan, Yin, & Chan, 2014; Walsh, Crispie, Kilcawley, O'Sullivan, O'Sullivan, Claesson, et al., 2016). Therefore, the oxygen content is the key factor causing flavor quality decline of upper *jiupei*.

3.5. The jiupei-layer differences of physicochemical characteristics were reduced by an AMSC

Based on the above analysis, the oxygen content was the key factor that caused the difference in flavor quality of *jiupei* between layers. During production, dry cracking and bulging of the yellow mud occurs and destroys the anaerobic environment of the cellar. Therefore, it is advisable to use cellar sealing equipment with better sealing performance to reduce the difference in the oxygen content of *jiupei* at different depths. Therefore, a new type of cellar sealing method, anaerobic micro-pressure sealed cellar (AMSC) technology was developed (Fig. S1). This process uses special equipment to replace the yellow mud, and the cellar is sealed using a stainless-steel cellar cover and water tank, and a liquid seal is used to form micro-pressure (0–0.5 KPa) with good sealing performance. When the pressure in the pit is high, the water in the tank will be washed. This solved the problem of an aerobic environment being created in the cellar by dry cracking and bulging of the sealing mud, and metabolic heterogeneous fermentation. The physicochemical factors of *jiupei* at different depths in the AMSC were measured and the results are shown in Table 1. The oxygen content of P-A was 5.90 ± 0.62 % at seven days of fermentation, which was significantly higher than that of P-D (4.17 ± 0.75 %) ($P < 0.05$), but the difference in oxygen content between P-A and P-D in AMSC was smaller than that in mud sealed cellars. The oxygen content in P-A was significantly lower than that in T-A ($P < 0.05$), indicating that AMSC are more tightly sealed, reducing the difference in oxygen content in the different depths of *jiupei* in the early stage of fermentation. The starch content, reducing sugar content, pH, and acidity of *jiupei* did not change significantly with the depth of the cellar. The content of caproic acid increased from 0.31 ± 0.12 g/kg in P-A to 1.59 ± 0.47 g/kg in P-D. The difference was smaller compared with the mud-sealed pit. The butyric acid content increased from 0.22 ± 0.11 g/kg in P-A to 0.63 ± 0.05 g/kg in P-D ($P < 0.05$). The acetic acid content did not change significantly with the depth of the cellar. When compared with the mud sealed cellars, the physicochemical properties of *jiupei* at different depths in the AMSC cellars are less disparate.

3.6. The jiupei-layer differences of microbial communities were reduced by AMSC

The dominant microorganisms of *jiupei* in AMSC were the same as those in mud sealed cellar, but their relative abundance varied with pit depth. At the genus level (Fig. S4a), *Lactobacillus* was dominant in AMSC, and showed little difference in four-layer *jiupei*. However, the relative abundance of *Gluconacetobacter* (6.04 %) and *Acinetobacter* (1.61 %) in P-A were significantly higher than those in the other three layers (<0.1 %, $P < 0.05$). At the species level (Fig. 4a), uncultured *Lactobacillus* sp. and *Lactobacillus acetotolerans* were dominant. However, the dominant species uncultured *Prevotella* sp. in *jiupei* in the mud sealed cellars had a relative abundance of 5.03 % in P-C, and was almost zero in P-A, P-B, and P-D. The NMDS (Fig. 4c) analysis showed no significant differences in the bacterial communities of the four-layer *jiupei*.

As shown in Fig. 4 and Fig. S4, the dominant fungi genus (Fig. S4d) and species (Fig. 4b) of *jiupei* in AMSC were the same as those in the mud sealed cellars (Fig. S4b), however the relative abundance showed different trends with the change in the depth of the cellar. At the genus level (Fig. S4b, d), *Aspergillus* (21.68 %) and *Thermomyces* (23.45 %) have the highest abundance, and showed no significant differences in the four-layer depth. However, the abundance of *Pichia* and *Trichosporon* in T-A in the mud sealed cellars was lower than in the AMSC (<1 %). At the species level (Fig. 4c), the relative abundance of *Thermomyces lanuginosus*, *Aspergillus chevalieri*, *Pichia kudriavzevii*, *Saccharomycopsis fibuligera* in AMSC did not change significantly with the increase of cellar depth. Among them, *Thermomyces lanuginosus* (22.98 %) and *Aspergillus chevalieri* (20.43 %) had the highest relative abundance. The PERMANOVA and NMDS analysis (Fig. 4d) showed no significant differences in the fungal communities of the four-layer *jiupei*.

3.7. The jiupei-layer differences of flavor quality were reduced by AMSC

Based on OPLS-DA of the differential metabolites of *jiupei*, it can be seen that P-A, P-B and P-E can be well separated on PC1 (Fig. S5), indicating that there were differences in *jiupei* metabolites among different layers. Differential metabolites (VIP > 1, $P < 0.05$) of *jiupei* at different depths were shown in Fig. 5b. Based on the analysis of P-A and P-B, it can be concluded that the relative abundance of five metabolites in P-B increased, such as acetyl-homoserine, biochanin A, mesalazine. Correspondingly, the relative abundance of artonol E decreased. It was worth mentioning that none of the six differential metabolites comprised the main aroma substances in the liquor. The analysis of P-B and P-E showed that the relative abundance of 25 and 10 metabolites increased, and decreased in P-E, respectively. Metabolites with increased relative abundance mainly include acids, such as suberic acid, methyl succinic acid, and mesaconic acid. Metabolites with decreased relative abundance mainly include cellobiose, dihydrojasmonic acid, and epicatechin. In conclusion, there were no significant differences in the types and

Table 1
Factors of the *Jiupei* at different depths of the mud sealed cellars and anaerobic micro-pressure sealed cellars.

Factors	T-A	T-B	T-C	T-D	P-A	P-B	P-C	P-D
Oxygen content (%)	7.67 ± 0.61a	–	–	4.43 ± 0.57b	5.90 ± 0.62a	–	–	4.17 ± 0.75b
Moisture content (%)	62.89 ± 0.22	63.36 ± 0.66	63.47 ± 0.57	63.56 ± 0.16	62.75 ± 1.50	63.27 ± 0.77	63.89 ± 1.14	64.12 ± 0.83
Amylum (%)	10.36 ± 0.25	10.01 ± 0.45	9.84 ± 0.49	10.15 ± 0.25	11.10 ± 0.07	10.79 ± 0.51	10.70 ± 0.20	10.58 ± 0.42
Reducing sugar (%)	10.84 ± 1.94b	11.89 ± 0.42b	14.48 ± 3.10b	18.49 ± 0.53a	15.73 ± 2.96	16.02 ± 2.60	17.77 ± 0.87	18.37 ± 1.20
pH	3.62 ± 0.14a	3.42 ± 0.05b	3.44 ± 0.04b	3.50 ± 0.05b	3.64 ± 0.17	3.58 ± 0.15	3.53 ± 0.09	3.57 ± 0.01
Acidity (mmol/10 g)	2.54 ± 0.08	3.43 ± 0.93	3.41 ± 0.24	3.48 ± 0.39	3.35 ± 0.27	3.46 ± 0.42	3.77 ± 0.18	3.79 ± 0.15
Caproic acid (g/kg)	0.22 ± 0.06d	1.06 ± 0.15c	1.64 ± 0.11b	2.12 ± 0.46a	0.31 ± 0.12c	0.51 ± 0.05bc	0.87 ± 0.09b	1.59 ± 0.47a
Butyric acid (g/kg)	0.28 ± 0.06b	0.45 ± 0.19b	0.74 ± 0.12a	0.88 ± 0.04a	0.22 ± 0.11b	0.26 ± 0.02b	0.40 ± 0.17b	0.63 ± 0.05a
Acetic acid (g/kg)	0.52 ± 0.10c	0.81 ± 0.07b	1.00 ± 0.11b	1.34 ± 0.24a	0.73 ± 0.14	0.83 ± 0.12	0.76 ± 0.14	0.95 ± 0.10

Note: Different letters in the same column indicate significant differences ($P < 0.05$).

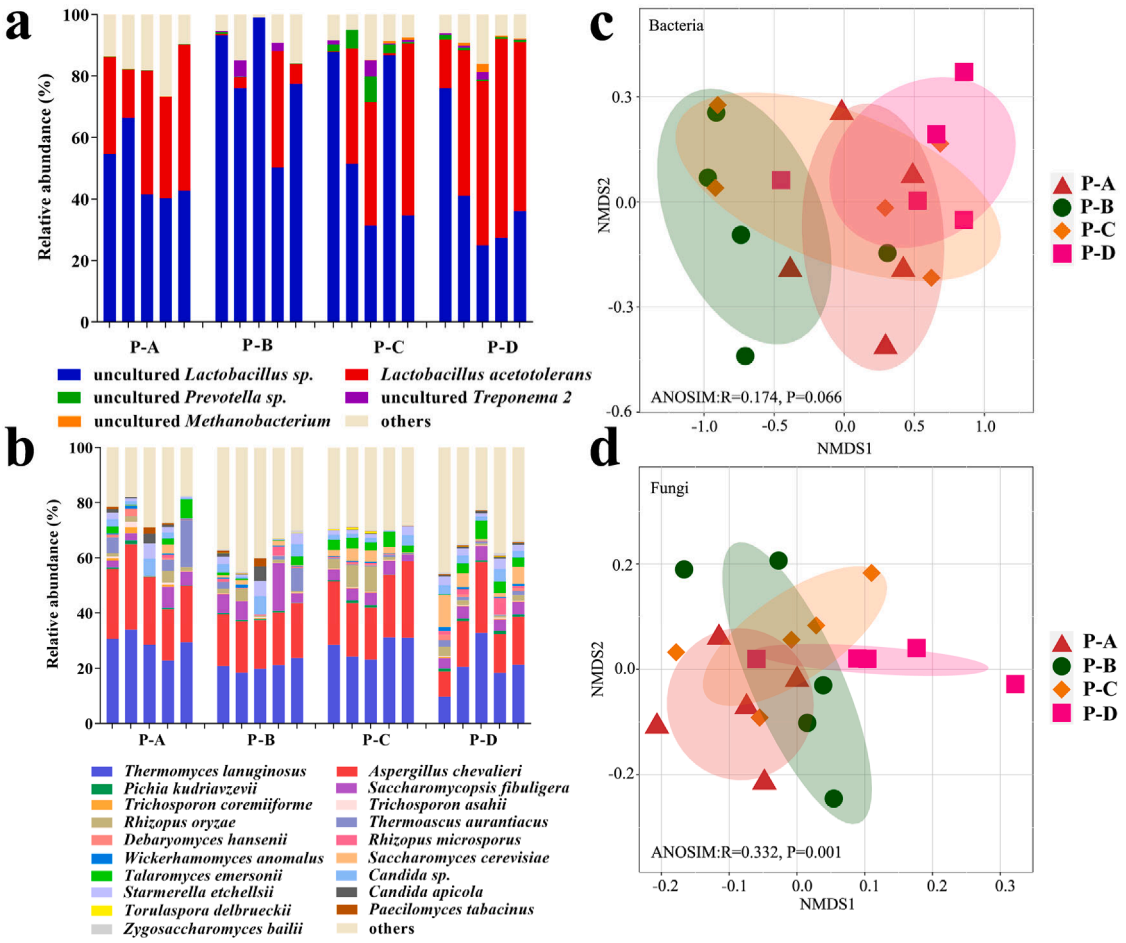

Fig. 4. Bacterial and fungal community of *jiupei* at different depths of the anaerobic micro-pressure sealed cellars (' others' means the collection of bacterial genus or species with average abundance<1 % in samples). (a, b) Species level, (c, d) NMDS analysis.

contents of P-A and P-B metabolites. Compared with P-B, the content of acids in P-E increased significantly ($P < 0.05$). As shown in Fig. 5a, PCA results were similar to raw liquor in the mud sealed cellars. The raw liquor of AMSC can be roughly divided into three parts (JP-A, JP-B and JP-C, JP-D respectively). However, unlike the mud sealed cellars, the JP-A is not separated from the other three-layer raw liquor on PC1. The specific performance is that on PC1, JP-D is located on the negative half axis, and the other three layers of raw liquor are on the positive half axis. On PC2, JP-A is far away from the other three-layer raw liquor. Fig. 5c showed the content of flavor compounds in different layers of raw liquor in AMSC. The total content of flavor compounds in JP-B (1706.21 ± 108.33 mg/L) was significantly lower than that in other three-layer raw liquor ($P < 0.05$), while other three-layer raw liquor showed no significant differences between each other. The total content of esters in JP-B (1599.14 ± 107.68 mg/L) was significantly lower than that in JP-D ($P < 0.05$), and there was no significant difference in the content of esters in other raw liquors. The total content of alcohols (36.10 ± 2.74 mg/L) in JP-D was significantly higher than that in other layers of raw liquors ($P < 0.05$). There was no significant difference in the acid content of the raw liquor. Compared with the mud sealed cellars, the content of esters and acids in different layers of the raw liquor in AMSC was similar. In conclusion, the quality of raw liquor at different depths in AMSC is more uniform, and to a certain extent, the problem of large differences in the quality of raw liquor at different depths in the mud sealed cellars is improved.

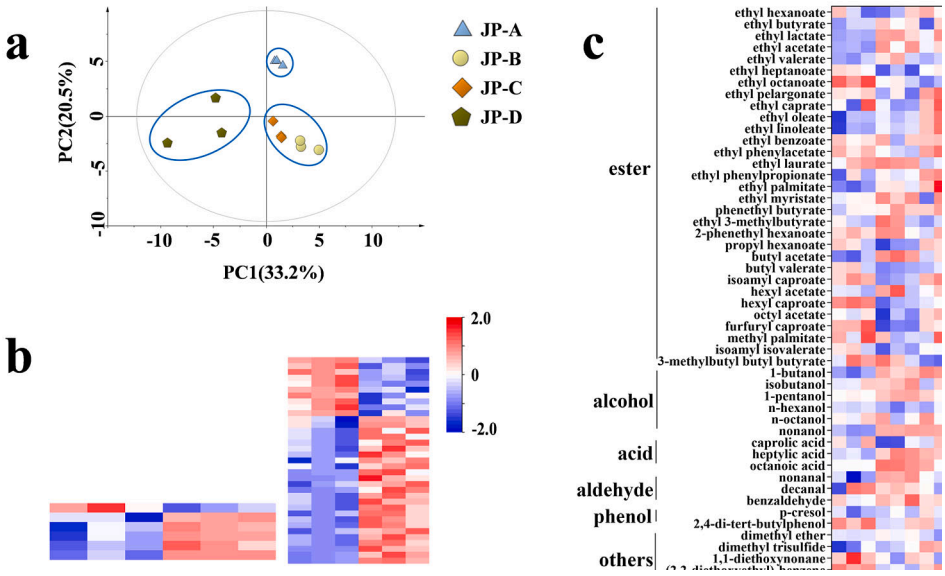

Fig. 5. Metabolites of jiupei and flavor compounds analysis in the anaerobic micro-pressure sealed cellars. (a) PCA analysis of flavor compounds in raw liquor. (b) Analysis of differential metabolites in jiupei. (c) Heat map of concentrations of flavor compounds (log2 scale) in raw liquor.

4. Conclusion

This study revealed the multidimensional flavor compounds, prokaryotic community, and metabolites of *jiupei* with different layers in mud sealed cellars and anaerobic micro-pressure sealed cellars (AMSC). The flavor compounds in mud sealed cellars showed compositional difference in different vertical depths, and 19 differential flavor compounds were identified based on OPLS-DA, mainly including 12 esters such as ethyl hexanoate, ethyl butyrate, propyl hexanoate and hexyl caproate. *Lactobacillus*, *Prevotella* and *Methanobacterium* were dominant genus of bacteria in all of cellars, while *Thermomyces*, *Aspergillus*, *Pichia*, *Trichosporon* and *Rhizopus* were the dominant genera of fungi. RDA analysis revealed oxygen was the key factor that causes the flavor quality decline in upper *jiupei* by traditional cellar sealing. Therefore, AMSC method with better sealing performance was developed and introduced. The difference in oxygen content between top site (5.90 ± 0.62 %) and bottom of the cellar (4.17 ± 0.75 %) in AMSC was smaller than that in mud sealed cellars. At the same time, the new method was narrowed the differences of microbial communities, flavor compounds and metabolites in *jiupei* with different layers. The results of this study provided a reference for the improvement in the production of Luzhou-flavor liquor and provided theoretical support for improving the flavor quality of upper *jiupei*.

CRediT authorship contribution statement

Shuangping Liu: Conceptualization, Methodology, Writing – original draft. **Dongliang Ren:** Writing – review & editing, Formal analysis, Investigation. **Hui Qin:** Software, Validation. **Qianqian Yin:** Resources, Data curation. **Yan Yang:** Investigation. **Tiantian Liu:** Software. **Suyi Zhang:** Writing – review & editing. **Jian Mao:** Funding acquisition.

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

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113057.

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