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Investigating the mechanism of the flavor formation in Sichuan sun vinegar based on flavor-orientation and metagenomics

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ABSTRACT

Fermentation and aging are the key stages of flavor formation in Sichuan sun vinegar (SSV), but the generation mechanisms of the flavor produced by these processes are unknown. However, complex microbial metabolism is critical to the flavor development of SSV. In this study, we analyzed the key flavor compounds present in SSV. Combined with odor activity value (OAV), the main aroma components of SSV were screened, and the relationship between microorganisms and key flavor formation was predicted using metagenomic sequencing technology. The results revealed 38 key flavor compounds in SSV. *Lactobacillus*, *Weissella*, *Acetobacter*, *Lichtheimia*, *Pediococcus*, *Oenococcus*, *Brettanomyces*, *Kazachstania*, *Pichia*, *Xanthomonas*, *Lenconostoc* are widely involved in the production of key flavor compounds such as 2,3-butanediol, 2-Furanmethanol, phenylethanol, 3-(Methylthio)-1-propanol, acetic acid, lactic acid, butyric acid, isovaleric acid and other organic acids. Among them, *Lichtheimia* and *Lactobacillus* are important genera for the degradation of starch, arabinoxylan and cellulose. The acetaldehyde,4-ethyl-2-methoxy-phenol and 2-methoxy-4-methyl-phenol production pathway may be related to *Lactobacillus*, *Acetobacter* and *Brettanomyces*. This study provides a new understanding of the key flavor-formation stage and flavor compound generation mechanism of SSV and provides a reference for the screening and isolation of functional strains and the reconstruction of microbial communities.

1. Introduction

Vinegar has been produced for more than 3000 years in China, and Shanxi aged vinegar (SAV), Sichuan Baoning vinegar (SBV), Fujian Yongchun vinegar, and Zhenjiang aromatic vinegar (ZAV) are the most famous (Budak et al., 2014; Liang et al., 2016; Liu et al., 2004). Traditional Chinese vinegars are mostly brewed through multi-strain mixed fermentation in an open environment using cereals as raw material. However, different vinegars have distinct characteristics depending on the various flavor substances present (Nie et al., 2019), and because of their different raw materials, climates, regions, and microbial community structures (Al-Dalali et al., 2019; Zhang et al., 2019). Furthermore, some vinegars must be brewed in a multi-step process, and the various steps have a significant impact on flavor (Li et al., 2022). Sichuan sun vinegar (SSV), a unique Sichuan bran vinegar, is made with wheat bran as the main raw material and the Chinese herbal medicine koji as the starter (*Qu*). The SSV production process differs from that of other types of vinegar. Vinegar grains (*Cupei*) are fermented using a unique trilateral co-fermentation process, during which saccharification, alcohol fermentation, and acetic acid fermentation occur simultaneously (Fu et al., 2022; Zhang et al., 2022a), and subsequently are aged in the cylinder for 1–5 years. It is currently unclear how the characteristic flavor components of SSV are formed, because the process is so complex. Moreover, the mechanism of the development of characteristic flavor components in SSV is still unknown.

Flavor-orientation was developed while investigating the Chinese liquor *Baijiu*. Using modern flavor chemistry and analytical chemistry theory, flavor substance contributions to the liquor were identified from thousands of trace components, and the chemical essences of key flavor and odor substances were excavated and confirmed. The formation mechanism, and pathway of key flavor and odor substances were studied, through the orientation of flavor compounds, functional microorganism screening, and flavor compound fermentation control. Moreover, flavor optimization recombination technologies have been developed (Xu, 2015). This research has facilitated investigation into various flavors of *Baijiu*. For instance, the primary flavor components of *Maotai*-flavor *Baijiu* include ethyl butyrate, ethyl 2-methyl propionate, ethyl valerate, and ethyl octanoate, and functional microorganisms that

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significantly contribute to the primary flavor have been screened (Fan and Qian, 2006; Wu et al., 2012). The flavor-orientation of flavor compounds in vinegar brewing process was also investigated. For example, the primary flavor components of sherry vinegar were identified using a combination of gas chromatography-mass spectrometry (GC-MS) and odor activity value (OAV) analysis (Callejón et al., 2008). Furthermore, Xu et al. (2022) explored the characteristic flavor compounds of fresh and aged coconut water vinegar and found 17 key metabolic pathways and 3 key metabolic substrates; namely, aspartic, glutamic and pyruvic acid. Wu et al. (2021a) reconstructed the metabolic network of key organic acids with an OAV higher than 1 through the theory of flavor-orientation, and revealed the predominant genus (*Acetobacter*, *Lactobacillus*) for organic acid metabolism. This provided a preliminary understanding of the formation mechanism of vinegar flavor compounds. However, flavor-orientation is undoubtedly more efficient for SSV, which has a complex brewing process.

Microorganism metabolism is one of the mechanisms of vinegar flavor formation, for example, the succession of *Lactococcus, Oenococcus*, and *Acetobacter* in apple cider vinegar, which is closely related to the flavor formation of phenethyl acetate, acetic acid, and isoamyl acetate (Song et al., 2019). *Lactobacillus* and *Acetobacter* in ZAV may be involved in the formation of flavor compounds such as acetic acid, lactic acid, and phenylethanol (Fang et al., 2021). Some microorganisms participate in the synthesis of flavor precursors, for example, *Acetobacter pasteurianus, Lactobacillus buchneri*, *Lactobacillus reuteri*, *Lactobacillus fermentum*, and *Lactobacillus brevis* are acetoin-producing functional strains of ZAV (Lu et al., 2016). Therefore, studying the structure and succession of microbial communities can reveal the formation of flavor compounds in fermented foods.

High-throughput amplicon sequencing has been used to investigate the formation of microbial community structures during vinegar fermentation (Kou et al., 2022). It enabled more accurate prediction of the correlation between microorganisms and flavor substances. However, the production of characteristic flavor requires a large enrichment of related microorganisms, and therefore, the prediction of flavor formation by microorganisms is skewed. Moreover, flavor development is frequently delayed, and correlation analysis is not precise (Jia et al., 2021b). The principle of amplicon sequencing is that bacteria and fungi are counted separately, consequently, there is a limited overview of the entire microbial community (Jia et al., 2021a). In addition, recently developed metagenomic technology has played an important role in exploring the structure, function, and metabolic potential of microbial communities in fermented food. It can filter out actively expressed genes in complex microbial communities and simultaneously identify the temporal and spatial changes in related microorganisms and their functions (Mota-Gutierrez et al., 2021). This method can avoid erroneously linking microbial communities and flavor, which assists in clarifying the microorganisms and related enzyme genes related to the characteristic flavor in fermented foods. Moreover it reveals the relationship between individual species, flavor metabolic pathways, and flavor compounds during fermentation. Therefore, the combination of metagenomic technology and flavor-oriented approaches seems to be more attractive. The flavor-oriented approach enables the rapid determination of the main stages of flavor formation in multi-process fermented foods, while metagenomic exploration of microbial community changes and expression of related enzymes at the main stages, improve the accuracy and efficiency of data analysis.

During the course of this study investigating SSV and *Cupei*, amino acids and organic acids were determined by high-performance liquid chromatography (HPLC), volatile flavor compounds were identified by headspace solid-phase microextraction gas chromatography-mass (HS-HPME-GC-MS), and critical aroma compounds during the fermentation process of SSV were explored in combination with OAV to clarify their production stages. Metagenomic sequencing was used to determine the formation mechanisms and pathways of flavor compounds during SSV fermentation and to explore the main microorganism flora related to flavor metabolism. It provides a reference for the screening and isolation of functional strains and reconstruction of microbial communities.

2. Materials and methods

The workflow of this study is shown in Fig. 1.

2.1. Chemicals and reagents

Chromatographically pure oxalic-, acetic-, lactic-, pyroglutamic-, succinic-, tartaric-, pyruvic-, α-ketoglutaric-, citric-, fumaric- and butyric acid, was purchased from Tianjin fine chemical (Tianjin, China). Serine, alanine, glycine, threonine, aspartic acid, glutamic acid, arginine, histidine, tyrosine, leucine, valine, methionine, isoleucine, phenylalanine, lysine, proline, and chromatographically pure methyl octanoate was obtained from Shanghai Maclean Biochemical Co, Ltd (Shanghai, China), while acetonitrile was obtained from Merck (Darmstadt, Germany), and sodium chloride, zinc sulfate, sodium acetate anhydrous, phenyl isothiocyanate, triethylamine, potassium ferrocyanide were purchased from Tianjin Kemiou Chemical Reagent Co, Ltd (Tianjin, China).

2.2. Sample collection

Cupei samples were collected from Sichuan Taiyuanjing sun vinegar Co. Ltd (Zigong, China). at a depth of less than 30 cm from the surface of the *Cupei* and stored in sterile bottles at −20 °C. Based on our previous study, samples varied from unfermented (0d), 3d, 11d, and 17d of fermentation, and aged *Cupei* samples from one, three, and five years for freezing, marked as H0, H3, H11, H17, 1y, 3y, 5y, respectively. Vinegar samples, SAV, ZAV, SBV, and SSV, were all purchased from supermarkets. All samples had been aged for three years and had an acidity of at least 6°. Samples were stored at 4 °C until analysis.

2.3. Sample pretreatment

Roughly 30 g of *Cupei* and 90 mL distilled water were added to a 250 mL glass beaker, incubated for 2h at room-temperature (approximately 21 °C) and then filtered with medium-speed filter paper. Subsequently, 25 mL filtrate and 5 mL vinegar were added to a 100 mL volumetric flask. Then, 5 mL zinc sulfate (300 g/L) and potassium ferricyanide (106 g/L) was also added and the flask was filled to 100 mL with distilled water and mixed well. After the mixture was incubated for 30 min at approximately 21 °C,it was then filtered with double medium-speed filter paper. Finally, a 0.22 μm microporous membrane filtered the filtrate for organic acid or amino acid analysis.

2.4. Organic acid determination

Organic acids were determined by LC-2030C 3DPLus (Shimadzu, Japan) equipped with a C18 column (ZORBAX SB-Aq, 4.6 mm x 250mm, 5 μM particle size) and a UV detector. The measurements were acquired according to the method described by Huang et al. (2017) with minor modifications. The mobile phase was 2% methanol and 98% 20 mmol/L NaH_2PO_4 (pH 2.7, adjusted with H_3PO_4), and the column temperature was set at 35 °C with a flow rate of 0.4 mL/min, injection volume 10 μL, and the wavelength of UV detector was set as 210 nm.

2.5. Free amino acid determination

The free amino acids were analyzed using HPLC L-3000 (Hmc co ltd., Suzhou, China) equipped with a C18 column (ZORBAX SB-Aq, 4.6 mm x 250mm, 5pμm), according to the method described by Xie et al. (2016) with some modifications. Pre-column derivatization using phenyl isothiocyanate (PITC). The gradient solvent system was mobile phase A (1.64 g of anhydrous sodium acetate dissolved and added to 0.5 mL of

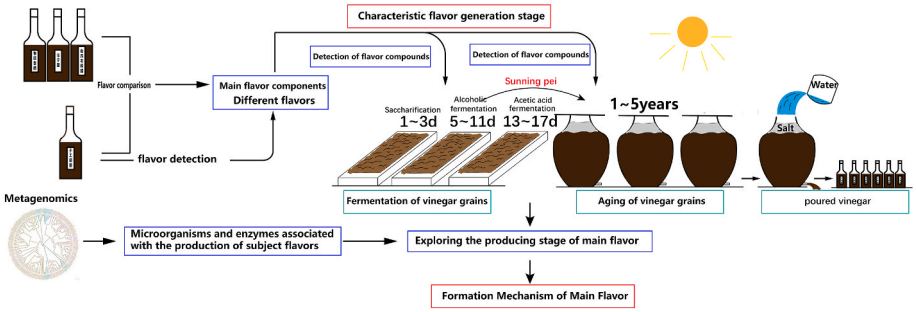

Fig. 1. Research process.

triethylamine, fixed to 1 L with water, 20% acetic acid adjusted to pH 6.2) and mobile phase B (80% acetonitrile, 20% water). Elution gradient 0–2 min, 92% A: 2–10 min, 90% A: 10-10 min, 81% A: 12–19 min, 74% A: 19–21 min, 65% A: 21–31 min, 54% A: 31–33 min, 0% A: 33–36 min, 92% A. The flow rate was 1.0 mL/min, the column temperature was 40 °C, and the injection volume was 10 μL. The free amino acids were detected at a wavelength of 254 nm.

2.6. Volatile flavor determination

The volatile compounds were analyzed using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC/MS) 7890 A (Agilent Technologies Inc., California, USA), according to Yang et al. (2017), with some modifications. A vinegar sample and *Cupei* were extracted using HS-SPME with 50/30 μm DVB/CAR/PDMS fiber (Supelco, Pennsylvania, USA). Methyl octanoate (0.8775 g/L) was used as an internal standard, and volatiles were analyzed using GC-MS, equipped with a DB-WAX column (0.25mm × 60 mm column , 0.25 μM film thickness, Agilent, Santa Clara, USA), with 2g NaCl to promote volatile aroma. Six replicates of each sample were preheated at 50 °C for 10 min, and the 50/30 μm DVB/CAR/PDMS SPME fiber head was inserted into the headspace vial and kept at 50 °C for 40 min for extraction. After the extraction was complete, the SPME fiber head was inserted into the GC inlet and desorbed at 230 °C for 5 min.

The GC -MS conditions were starting at 35 °C and holding for 1 min, then ramped at 5 °C/min to 130 °C and held for 1 min, then increased to 180 °C at 3 °C/min, and maintained for 1 min. Finally, the temperature was raised to 230 °C at 8 °C/min and held for 1 min. The ion source was at 230 °C, and the MS quadrupole at 150 °C, with a solvent delay of 3 min, in acquisition mode full scan.

2.7. Odor activity value (OAV)

The OAV was used to evaluate the contribution of individual compounds to the overall aroma compounds, with $0 \leq$ OAV <1 indicating that the compound made a small contribution to the overall aroma and had a modifying effect. An OAV ≥ 1 shows that a compound makes a significant contribution to the overall aroma; the higher the OAV value, the greater the contribution to the aroma compounds (Yin et al., 2022). The calculation is performed according to Xiao et al. (2021).

$$\mathrm{OAV} = \mathrm{C}/\mathrm{OT} \quad (1)$$

Where C is the concentration of the volatile compounds in the sample and OT indicates the odor threshold.

2.8. Metagenomic sequencing

Total genomic DNA samples were extracted using the FastDNA SPIN kit (MP Biomedicals Inc., Irvine, CA, USA) following the manufacturer's instructions and stored at −20 °C prior to further analysis. The purity and integrity of extracted DNA were checked by a Nanodrop 2000 (Nanodrop Technologies) and agarose gel electrophoresis, respectively. The DNA was randomly sheared using a Covaris Ultrasonic Processor into approximately 400 bp fragments. The PE library was constructed using the NEXTFLEXTM Rapid DNA-Seq Kit (BIOO Scientific Crop., Austin, TX, USA). Metagenomic sequencing was performed using the Illumina NovaSep 6000 platform (Illumina, USA).

After sequencing, the original sequence data was controlled for quality. Low-quality sequences (length below 150bp, average Phred scores <20, mononucleotide repeats over 8 bp, and with ambiguous bases), and n-containing and contaminated reads were removed from the data, resulting in better quality assembled sequences. Open reading frame (ORF) prediction of contigs in the assembly results were obtained using MetaGene. Genes with nucleic acid length greater than or equal to 100 bp were selected and translated into amino acid sequences. Non-redundant gene sets were constructed by clustering using CD-HIT 4.5.6 software, and SOAPaligner 2.21 software, was used to compare the high-quality reads of each sample with the non-redundant gene set to calculate the abundance of the gene in the corresponding sample. Subsequently, BLASTP 2.6.0 alignment of non-redundant gene sets with Non-Redundancy (NR), Evolutionary genealogy of genes: Non-supervised Orthologous Groups (EggNOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases was performed using DIAMOND v 0.8.24.86 software. Species annotations from the taxonomic information database corresponding to the NR database were obtained, and statistical species at each taxonomic level, such as phylum, genus, and species were constructed. Bioinformatics analysis was completed by Shanghai Meiji Biological Co, Ltd, and metagenomic data were integrated to construct a network diagram of key microbial and flavor compounds using the KEGG database and species annotation abundance.

2.9. Statistical analysis

Unless otherwise stated, all tests were performed in triplicate, and the final results were expressed as the mean ± standard deviation of three replicates. Statistical analysis was performed with SPASS 22.0 software. Partial least squares discriminant analysis (PLS-DA), principal component analysis, heat map visualization, and Variable Importance in the Projection (VIP) analysis were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). One of the most commonly used techniques for developing classification models in food authentication is partial PLS-DA, which focuses on the differences among samples from different classes and operates by splitting the hyperspace of the variables, which is based on a comparison of the predicted response values from Y with a fixed scalar threshold, usually 0.5 (Jiménez-Carvelo et al., 2021). The VIP is a commonly used method for variable selection, where the basic idea is that the average VIP score of all variables is 1; therefore, any variable with a VIP score greater than 1 indicates its importance, and values less than 1 can be eliminated (Wang et al., 2022). OriginPro (Version 2021. OriginLab Corporation, Northampton, MA, USA. https://

www.originlab.com/) was used for the rest of the graphics.

3. Results and discussion

3.1. Analysis of non-volatile flavor compounds in SSV

The flavor of SSV is composed of main flavor and characteristic flavor. Analyzing the flavor differences between SSV and other vinegars brewed by solid-state fermentation facilitate exploring the characteristic flavor of SSV.

Analyzing the differences in organic acids present in four different vinegars (Fig. 2a), a total of 12 organic acids, including oxalic-, tartaric-, pyruvic-, lactic-, citric-, and pyroglutamic acid, were found. The highest concentrations of acetic acid, 7.0 ± 0.04g/100 mL, was found in SAV, whereas the highest concentrations of pyruvic-, lactic-, and fumaric acid were found in SBV. SSV contained higher concentrations of oxalic-, malic-, α-ketoglutaric-, citric-, tartaric-, succinic-, pyroglutamic-, and butyric acid than other vinegars.

The high concentration of organic acids in SSV is generally associated with the tricarboxylic acid cycle, which may be affiliated with active aerobic microbial metabolism during the fermentation stage. Furthermore, its butyric acid concentration was much higher than those of the other three vinegars, which may be attributed to anaerobic microbial metabolism during the fermentation or aging process.

Generally, the concentration of lactic acid is higher than that of acetic acid in bran vinegars, such as SBV and SSV (Liu et al., 2020). In our study, the concentration of acetic acid observed in SSV was 5.5 ± 0.02g/100 mL, in addition, the concentrations of lactic- and succinic acid were 5.97 ± 0.11g/100 mL and 4.7 ± 0.03g/100 mL, respectively. The relative contents of these three organic acids, which are the main organic acids in SSV, accounted for 29%, 31%, and 24% of the total organic acids, respectively. During the early and middle stages of fermentation, microbes have a strong ability to degrade starch. Microbial enzymes in the later stage convert pyruvic acid into intermediate metabolites, which in turn produce various flavor compounds in vinegar, especially acetic-, and lactic acid. However, lactic acid is difficult to volatilize, and the taste threshold is not as good as that of acetic acid, which makes acetic acid the main flavor organic acid in vinegar (Wu et al., 2021a). Acetic acid has a strong, pungent odor, whereas lactic- and other organic acids neutralize the pungent odor of acetic acid and soften its flavor (Krusong et al., 2020; Xu et al., 2022).

There are differences in free amino acid content among the four solid brewed vinegars (Fig. 2b). The glutamic acid, methionine, tyrosine, phenylalanine, arginine, and leucine concentrations of SBV were higher than those observed for the other vinegars, while the concentration of glycine was higher in ZAV than that in other vinegars. Furthermore, the concentrations of aspartic acid, lysine, serine, histidine, alanine, valine, and threonine in SSV were significantly higher than those observed in other kinds of vinegar. Amino acids mainly come from the hydrolysis of protein in raw materials by microbial groups (Liang et al., 2020). The difference in amino acid concentration may be attributed to the raw materials used in SSV fermentation. Diverse amino acids can offer different flavors (Duan et al., 2020). Bitter amino acids in SSV accounted for 64% of the total amino acids, proline, valine, histidine, and leucin, and their concentrations were 0.36 ± 0.02g/100 mL, 0.37 ± 0.04g/100 mL, 0.39 ± 0.01g/100 mL, and 0.45 ± 0.02g/100 mL, respectively, which collectively accounted for 50.7% of the total amino acids. They are followed by sweet and umami amino acids, which are similar to most other vinegars (Gong et al., 2020). The concentration of alanine in sweet amino acids was the highest, reaching 0.46 ± 0.01g/100 mL, accounting for 14.9% of the total amino acids. Alanine, proline, valine, histidine, and leucine were the main amino acids present in SSV. However, differences in amino acids may also be related to structural changes in the microbial community during fermentation. For example, the continuous proliferation of molds and yeasts may be positively correlated with proline and threonine content, and the increase in alanine and leucine content may be related to the presence of *Weissella* (Jiang et al., 2019). Organic acids and amino acids that were significantly different from other vinegars were screened to analyze their changes during brewing.

3.2. Analysis of volatile flavor compounds in SSV

In total 72 volatile flavor compounds were identified by HS-HPME-GC-MS, including 9 esters, 15 alcohols, 8 aldehydes, 6 ketones, 10 acids, 7 phenols, 11 pyrazines, 2 lactones, 1 sulfide, and 3 other compounds (Fig. S1). The content of aldehyde, ester, alcohol, and pyrazines accounted for 26.27%, 5.44%, 10.42%, and 5.98%, respectively, of the total compounds. The acids had the highest concentration, reaching 46.02%. Esters are produced by the esterification of alcohols with acids by microorganisms with high glycolytic or lipolytic activity, and have fruity and floral aromas (Shen et al., 2021). Alcohol also has a scent-enhancing effect (Su et al., 2021). Aldehydes are mainly produced by amino acid catabolism or pyruvate acidification, and generally impart the flavor of champagne (H. Zhang et al., 2022b). Pyrazines are formed mainly by the degradation of amino acids, peptides, and reducing sugars through the Maillard reaction and impart a diverse variety of flavors, such as coffee, chocolate, and cocoa flavors (Picard et al., 2021). All these compounds impart a distinctive scent to SSV.

Combined with existing research, the OAV of 40 volatile compounds was calculated from the detected volatile flavor compounds (Table S1). There were 11 aroma compounds with an OAV <1. Among which was 5-methyl-2-furancarboxaldehyde and ethyl acetate. These compounds were highly concentrated, but with an OAV <1, contributed less to the overall aroma. Furthermore, 30 species of aroma compounds with an OAV ≥1, such as dihydro-5-pentyl-2(3h)-furanone, 2-methyl-propanal, 2,3-butanediol, 3-methyl-butanoic acid, butanoic acid, 4-methyl-

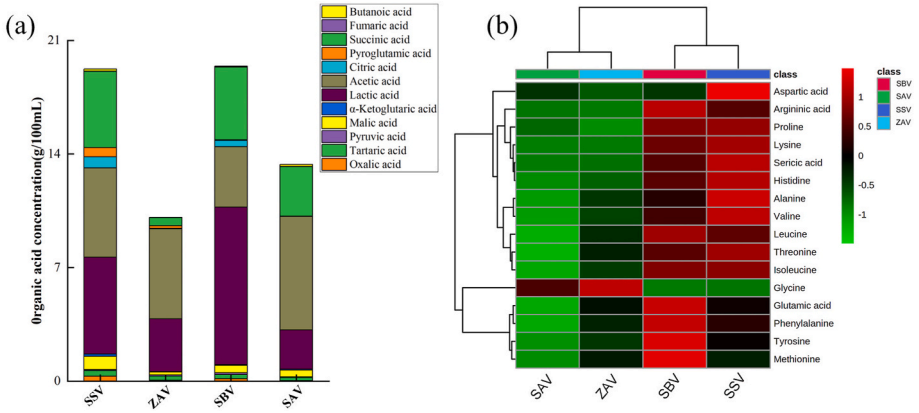

Fig. 2. The organic acid concentration (a) and amino acid concentration (b) of the four kinds of vinegar.

pentanoic acid, and 2,4-bis(1,1-dimethylethyl)-phenol (OAV >100), contributed significantly to the overall fragrance composition of SSV. In fact, 4-methyl-pentanoic acid, which has a characteristic cheese odor, was the most significant contributor to the SSV with an OAV of 20919, which was significantly higher than that of other aroma compounds. This was corroborated by results for SBV (Al-Dalali et al., 2020a), where 3-methyl-butanoic acid may be produced by the oxidation of 3-methyl--butanal or by the Strecker degradation of leucine or isoleucine during fermentation (Zhou et al., 2020). The Ehrlich pathway or Strecker degradation of α-amino acids produces 2-methyl-propanal by microbes (Nounah et al., 2020). The main metabolite of most polyols is 2,3-butanediol (Hu et al., 2019). In terms of concentration, acetic acid and furfural had the highest concentration, with a total concentration in excess of 50%, followed by 3-methyl-butanoic- (7.04%) and butyric acid (4.30%). There are also flavor compounds with relative concentrations over 0.5%, for which no threshold value can be determined, such as formic acid , 4-Ethyl-4-heptanol, 2,3-Dimethyl-5-ethylpyrazine, Propanol, methoxy-, acetate and aldehyde. These include primarily organic acids, pyrazines, alcohols, and aldehydes, which may also contribute to the formation of the main flavors of SSV. However, their threshold values require further investigation.

The volatile flavor compounds of the four vinegar types were analyzed using multivariate statistical analysis. The results of PLS-DA (Fig. 3a) show $R^2 = 0.83$ and $Q^2 = 0.81$ in the PLS-DA model. The four groups of samples were well clustered and separated significantly. Furthermore, which proved that the model was stable and effective. This also indicated that there were significant differences in the flavor compounds of the four vinegars. To further determine the differential volatile compounds of the four types of vinegar, a VIP analysis of the PLS-DA results showed differences in volatile flavors between SBV, ZAV, SSV, and SAV (Fig. 3b). Compounds with VIP >1 had a significant contribution to the separation of components, with 3-methyl-butanoic acid, butanoic acid, tetramethyl pyrazine and formic acid significantly higher in SSV than in the other three types of vinegar. These components contribute to the potential differences in the volatile flavors of the SSV. The aroma components that contribute significantly to the flavor of SSV and distinguish it from other types of vinegar were screened and used in subsequent experiments to analyze their changes during the brewing process.

3.3. Changes of non-volatile flavor compounds in different process stages

The key flavors of SSV originate from the different stages of the brewing process in general and the change in the organic acid concentration at different processing stages of SSV in particular (Table 1). All organic acids increased considerably during fermentation, but the concentration decreased after 1.5 years of aging and then stabilized. The lactic acid content increased first and then decreased with fermentation time, with the highest content being 11.4 ± 0.03g/100g dry weight at 11 days of fermentation, it may be that the continuous proliferation of lactic acid bacteria during fermentation leads to the increase of the lactic acid concentration (Lee et al., 2012; Verce et al., 2021). The lactic acid concentration decreased from 7.15 ± 0.03g/100g to 6.33 ± 0.03g/100g dry weight during the aging stage. The esterification reaction occurred during the aging stage, which may be the reason for the decrease in the lactic acid concentration (Mehta et al., 2020). The acetic acid content first increased and then decreased with fermentation duration, and reached 8.69 ± 0.07g/100g dry weight on the 11th day. It may be that ethanol dehydrogenase and acetaldehyde dehydrogenase secreted by *Acetobacter* oxidize ethanol to acetic acid (Qi et al., 2013) With the progress of fermentation, the content of acetic acid decreased, reaching 7.14 ± 0.01g/100g dry weight at the end of the fermentation. The decrease in acetic acid may be due to the conversion of esters, or may also be due to the consumption of acetic acid by *Acetobacter* or *Lactobacillus*. After 1.5 years of aging, the concentration of acetic acid decreased to 2.81 ± 0.02g/100g dry weight, and then the content continued to decrease slightly. The concentration of butyric acid was the highest on the 3rd day of fermentation, reaching 0.26 ± 0.02g/100g dry weight and then gradually decreasing to 0.02 ± 0.00g/100g dry weight after 1.5 years of aging, no significant change in concentration with increasing aging time. The remaining organic acids are generally intermediate metabolites of the tricarboxylic acid cycle. The concentration of organic acids in the aging stage was generally lower than that in the fermentation stage and was stable. This may be the initial stage of aging vinegar grains from an aerobic to an anaerobic environment. A large decline in aerobic microbes causes the tricarboxylic acid cycle to gradually decay and finally stabilize. For example, succinic acid is produced by *yeast* metabolism. The succinic acid concentration reached 4.98 ± 0.06g/100g dry weight at the early fermentation stage (H3) and decreased to 2.13 ± 0.03g/100g dry weight at the later stage (H17). Some of the malic acids may be metabolized by *yeast* to succinic acid in the early fermentation stage and oxidized to fumaric acid in the later stages (Mendes Ferreira and Mendes-Faia, 2020; Jin et al., 2021). The concentration of citric acid did not significantly change over 11 days of fermentation but increased abruptly to 0.7 ± 0.02g/100g dry weight in 17 days. The concentration of citric acid decreased significantly during aging, and reached 0.44 ± 0.02g/100g after 3 years, which was significantly lower than that at 17 days of fermentation. Malic acid reached 1.25 ± 0.03g/100g dry weight before fermentation and reached its highest level of 3.03 ± 0.02g/100g dry weight during 3 days of fermentation. With the fermentation, the concentration decreased continuously and reached 0.15 ± 0.02g/100g dry weight for 17 days. The concentration of malic acid in the aging stage was significantly lower than in the fermentation stage, and the highest concentration was 0.45 ± 0.01g/100g dry weight after aging for 5 years. Malic acid is an intermediate in the tricarboxylic acid cycle, and the malic-lactic acid pathway may be one of the reasons for the decreased malic acid content (Qi et al., 2013). Therefore, organic acids are mainly produced in the fermentation stage and are closely related to microbial metabolism.

Free amino acid concentration in the SSV varies during the different process stages (Table 2). The histidine concentration increased slowly during the fermentation stage, and the concentration was the highest at

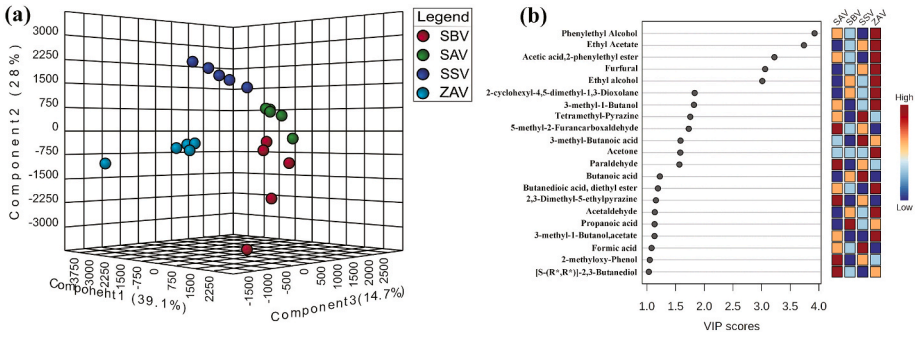

Fig. 3. PLS-DA of four kinds of vinegar aroma compounds (a) VIP score of four kinds of vinegar aroma compounds (b).

Table 1
Variation in organic acid concentration at different process stages.

Compounds	Concentration (g/100g dry weight)						
	H0	H3	H11	H17	1.5y	3y	5y
Acetic acid	2.30 ± 0.02f	3.12 ± 0.05c	8.69 ± 0.07a	7.14 ± 0.01b	2.81 ± 0.02d	2.53 ± 0.03e	2.19 ± 0.02g
Lactic acid	1.10 ± 0.02g	5.20 ± 0.07f	11.4 ± 0.03a	6.80 ± 0.04c	7.15 ± 0.03b	6.70 ± 0.04d	6.33 ± 0.03e
Succinic acid	0.08 ± 0.01g	4.98 ± 0.06a	3.67 ± 0.04b	2.13 ± 0.03c	2.16 ± 0.03c	2.00 ± 0.01d	1.76 ± 0.02f
Oxalic acid	0d	0.001 ± 0.00d	0.003 ± 0.00d	2.50 ± 0.02a	0.10 ± 0.00c	0.12 ± 0.01b	0.12 ± 0.01b
Malic acid	1.25 ± 0.03b	3.03 ± 0.02a	3.00 ± 0.04a	0.15 ± 0.02e	0.33 ± 0.01d	0.44 ± 0.02c	0.45 ± 0.01c
α-ketoglutaric acid	0.04 ± 0.01f	0.16 ± 0.02b	0.23 ± 0.01a	0.09 ± 0.01c	0.04 ± 0.00ef	0.06 ± 0.01de	0.06 ± 0.01d
Citric acid	0.06 ± 0.01d	0.04 ± 0.01d	0.07 ± 0.01d	0.70 ± 0.02a	0.31 ± 0.04c	0.44 ± 0.02b	0.41 ± 0.03b
Tartaric acid	0.65 ± 0.06b	0.75 ± 0.05a	0.68 ± 0.05 ab	0.62 ± 0.02b	0.21 ± 0.03c	0.20 ± 0.04c	0.23 ± 0.02c
Pyroglutamic acid	0.42 ± 0.04c	0.28 ± 0.03d	0.50 ± 0.02b	0.70 ± 0.01a	0.02 ± 0.00e	0.04 ± 0.00e	0.02 ± 0.00e
Butyric Acid	0.18 ± 0.00b	0.26 ± 0.02a	0.16 ± 0.01b	0.12 ± 0.02c	0.02 ± 0.00d	0.02 ± 0.00d	0.02 ± 0.00d

Means by the different letters in the same row are a significant difference ($P < 0.05$).

Table 2
Changes of amino acid concentration in different process stages.

Compounds	Concentration (g/100g dry weight)						
	H0	H3	H11	H17	1.5y	3y	5y
Alanine acid	0.10 ± 0.01g	0.75 ± 0.03d	1.79 ± 0.04c	2.24 ± 0.01a	1.93 ± 0.03b	0.20 ± 0.02f	0.25 ± 0.02e
Histidine	0.01 ± 0.00d	0.01 ± 0.00d	0.02 ± 0.00d	0.02 ± 0.00d	0.92 ± 0.02b	1.15 ± 0.03a	0.66 ± 0.03c
Valine	0.05 ± 0.01g	0.37 ± 0.02f	0.66 ± 0.02e	0.88 ± 0.04d	0.99 ± 0.03c	1.58 ± 0.02b	1.84 ± 0.03a
Leucine	0.22 ± 0.02d	0.76 ± 0.04c	1.37 ± 0.05b	1.67 ± 0.03a	1.40 ± 0.04b	1.42 ± 0.03b	1.68 ± 0.02a
Proline	0.07 ± 0.01g	0.66 ± 0.02f	1.63 ± 0.04b	2.20 ± 0.03a	0.74 ± 0.02e	1.11 ± 0.01d	1.37 ± 0.02c
Lysine	0.01 ± 0.00e	0.06 ± 0.01c	0.02 ± 0.00e	0.04 ± 0.01d	0.21 ± 0.01a	0.18 ± 0.02b	0.20 ± 0.01a
Serine	0.02 ± 0.00f	0.15 ± 0.02e	0.29 ± 0.02d	0.35 ± 0.02c	0.58 ± 0.02a	0.53 ± 0.03b	0.01 ± 0.00f
Threonine	0.07 ± 0.01e	0.22 ± 0.03d	1.19 ± 0.04b	1.57 ± 0.03a	0.33 ± 0.02c	0.04 ± 0.01e	0.20 ± 0.01d
Aspartic acid	0.15 ± 0.02c	0.09 ± 0.02d	0.05 ± 0.00e	0.04 ± 0.01e	0.27 ± 0.01b	0.30 ± 0.01a	0.25 ± 0.02b

Means by the different letters in the same row are a significant difference ($P < 0.05$).

17 days of fermentation, reaching 0.02 ± 0.00g/100g dry weight, but this was much lower than the values observed during the aging stage which was 1.15 ± 0.03g/100g dry weight. This shows that histidine accumulated mainly during aging.

The concentration of aspartic acid decreased during fermentation but increased after 1.5 years of aging, after which the concentration slight decrease. Fermentation is the main stage for the formation of other amino acids; the concentration of serine and threonine positively correlated with the fermentation stage, reaching 0.35 ± 0.02g/100g and 1.57 ± 0.03g/100g dry weight on day 17 of fermentation, respectively. Proteases produced by microorganisms that hydrolyze proteins into amino acids also contribute to the increase in amino acid content (Wang et al., 2020). Furthermore, after 1.5 years of aging, the serine concentration reached 0.58 ± 0.02g/100g dry weight and then declined continuously. This can be caused by being broken down into amines, volatile acids, or as a byproduct of bacterial and enzyme breakdown (Anggo et al., 2015). The concentration of threonine fluctuated during the aging stage and was significantly lower than that during the fermentation stage. The concentration of lysine was the highest on the 3rd day of fermentation (0.06 ± 0.01g/100g dry weight), and the concentration reached 0.21 ± 0.01g/100g dry weight after 1.5 years of aging. The changes of threonine and lysine contents may be related to the metabolism of *Lactobacillus* and yeast during fermentation (Liu et al., 2022). The overall concentration of the remaining four amino acids increased in the fermentation stage. Microbial processes involved in the fermentation of raw materials produce proteinases that hydrolyze the proteins present in raw materials and produce amino acids. The decrease in amino acids during the aging stage may be due to the consumption of amino acids by the Maillard reaction.

3.4. Changes of volatile flavor compounds in different process stages

The characteristic aroma compounds of *Cupei* at different stages of aging and fermentation were noted (Table S2). Among the 35 aroma components, five compounds, including 2-methyl-propanal, 1-H-pyrrole-2-carboxaldehyde, 4-methyl-pentanoic acid, 2-methoxy-phenol, and formic acid, gradually increased during the aging process, while methoxy-propanol acetate, 2,6-dimethyl-Pyrazine, and 4-Ethyl-4-heptanol were produced after 1.5 years of aging. Compared to the fermentation stage, the concentrations of furfural and benzaldehyde increased significantly during the aging stage, with the highest concentration at 5 years of aging, indicating that the aging stage plays a crucial role in the formation of these aroma imparting components. The remaining aroma compounds were mainly produced during the fermentation stage, and their concentrations were significant; the concentration of ethyl alcohol reached 251.38 ± 1.21 mg/kg after 3 days of fermentation; 2-methyl-Propanoic acid were produced in the early stage of fermentation (3d), and the concentrations reached 76.82 ± 1.55 mg/kg. The concentrations of phenylethyl alcohol, acetic acid, 2-phenylmethyl ester, 2-methoxy-4-methyl-phenol, 2(3H)-furanone, dihydro-5-pentyl, butanoic acid and 3-methyl-1-butanol were peaked at 11 days after fermentation started. Benzeneacetic acid ethylester, 2,3-butanediol, 3-methyl- butanoic acid, 2,4-bis(1,1-dimethylethyl)-phenol, paraldehyde, and acetic acid reached their maximum concentrations after 17d of fermentation. The concentration of hexanoic acid increased significantly with the prolongation of fermentation time, and the concentration reached 19.08 ± 0.08 mg/kg at 17 days. The concentration increases slowly during aging. At 11 days of fermentation, 2-furanmethanol and 3-(methylthio)-1-propanol were produced, reaching 9.32 ± 0.07 mg/kg and 4.96 ± 0.04 mg/kg respectively, with a significant decrease in aroma compounds during aging compared to the fermentation stage. It can be seen that the fermentation stage influences the production of the majority of aroma compounds.

The aroma components produced at different stages of the process reflect the activity of microbes. The concentration of volatile phenol compounds (phenol, 2-methoxy-, phenol, 2-methoxy-4-methyl-, and phenol, 4-ethyl-2-methoxy-) may be related to *yeast* (du Plessis et al., 2021). Furthermore, 2,4-bis(1,1-dimethylethyl)-phenol is most likely the product of guaiacol deoxyalkylation (Yan et al., 2020). With the

exception of 4-methyl pyrazine, the remaining pyrazines, furans, and sulfur compounds may be formed by complex reactions, such as the Maillard reaction, Strecker degradation, and lipid oxidation (Jia et al., 2019; Yang et al., 2020). These compounds were mainly produced during the aging stage, indicating that most of the flavor compounds during the aging stage were generated by chemical changes and had no direct relationship with microbes. The enzymes produced by microorganisms may play an important role in the formation of precursors for these chemical reactions; however, further research is required to validate this hypothesis. Benzaldehyde is found in filamentous fungi or is formed by l-phenylalanine residues (Odriane Custodio Leite, 2020). Benzaldehyde accumulation in SSV occurs mainly during aging, which may be related to the enzymes produced by the fungus. However, these enzymes may also originate during fermentation. It is undeniable that the activity of microbial metabolism during the fermentation stage is much greater than that during the aging stage.

3.5. Evolution of microbial community structure during the fermentation of SSV

In this study, metagenomic sequencing was performed at various times during fermentation samples (H3, H11, H17), and the changes in microbial composition at these different times were analyzed (Fig. 4a). At the phylum level, the dominant flora during the fermentation of *Cupei* was the Firmicutes, and its relative abundance remained above 80%. In the early stage of fermentation, Firmicutes were the main group, with Firmicutes gene abundance decreasing as fermentation time increased, while Proteobacteria became the second most plentiful flora during the fermentation process of the *Cupei*, with a relative abundance of nearly 20% on the 17th day of fermentation. Therefore, protozoa and Firmicutes are the two major microflora in the fermentation process of *Cupei*. Vinegar brewing is an open environment, and the fermentation process involves a large number of unknown and uncultured microorganisms. For a comprehensive description of microbial characteristics, a more scientific analysis of the microbial composition at the genus level is required. The relative abundance of the microbial community dynamics was determined at the genus level (Fig. 4b). Thirty genera of bacteria and four genera of fungi were identified. There were three genera with significant abundance. *Lactobacillus* is the uppermost genus in the whole *Cupei* fermentation process, followed by *Acetobacter* and *Weissella* (Fig. 4c), and the same findings held true for the other vinegars (Wang et al., 2016; Yun et al., 2019). The succession of dominant microbial species follows a clear pattern, and changes in microbial metabolites and substrate concentrations may facilitate a continuous succession of microbial species (Huang et al., 2022). *Lactobacillus genus* had the highest gene abundance in the early stage of fermentation, and its relative abundance reached 75.03%. In the early stage of fermentation, *Weissella* was the second dominant genus, with a relative abundance of 7.25%. In the middle of fermentation, the gene abundance of *Lactobacillus* and *Weissella* genus decreased to 65.87% and 2.55%, respectively. At this point, *Acetobacter* gradually replaced *Weissella* genus as the second most dominant genus, accounting for 8.61% of the gene abundance, which reached 16.42% on the day 17. Acetic acid and lactic acid were mainly produced at the upper and lower ends of the fermentation containers, respectively. Oxygen concentration is positively related to *Acetobacter* metabolism but negatively related to *Lactobacillus* metabolism (Zhang et al., 2020). The turning of fermented grains in the middle stage of fermentation resulted in an increase in oxygen content, which may explain the significant increase in gene abundance of *Acetobacter* on the day 17.

3.6. Microbial functional gene annotation analysis

Analysis of the KEGG pathways involved in each microbial gene during fermentation revealed that these genes belonged to 45 KEGG pathways in 6 categories (Fig. 5). Most of the genes related to the metabolic process fell into six categories, with a gene abundance of 64%. Genes related to organismal systems were the least abundant, and the gene abundance in the metabolism process classification gradually increased. In addition to global and overview maps, carbohydrate, and amino acid metabolisms were the two most annotated pathways. This result has also been observed in other fermented foods (Hu et al., 2021; Liu et al., 2019).

The results indicated significant differences in the abundance of *Cupei* metabolism genes at different fermentation times (Fig. 6). Gene abundance was the lowest on the third day of fermentation and gradually increased as fermentation time increased, with the highest gene abundance on the 11th day. At level 2, H11 showed the highest abundance of genes related to each metabolic pathway. In carbohydrate-, fatty acid-, and amino acid metabolism, as well as terpenoids, and polyketides. This study shows that H11 is the main stage of microbial enrichment, and a large number of microbes are involved in metabolism related to substrate decomposition. At level 3, the abundance of microbial genes related to histidine metabolism in H3 was higher. However, the abundance decreased gradually with fermentation, which may be the reason for the low histidine concentration during the fermentation stage. The genes associated with the synthesis of valine and leucine decreased, and those associated with their degradation increased, suggesting that amino acid catabolism, such as valine, gradually became active and converted to other compounds. In H11, genes related to arginine biosynthesis, glycine, serine, and threonine metabolism, phenylalanine metabolism, and lysine biosynthesis, bile acid synthesis, sphingolipid metabolism, taurine, and hypotaurine metabolism were prominently expressed. These changes in gene enrichment during

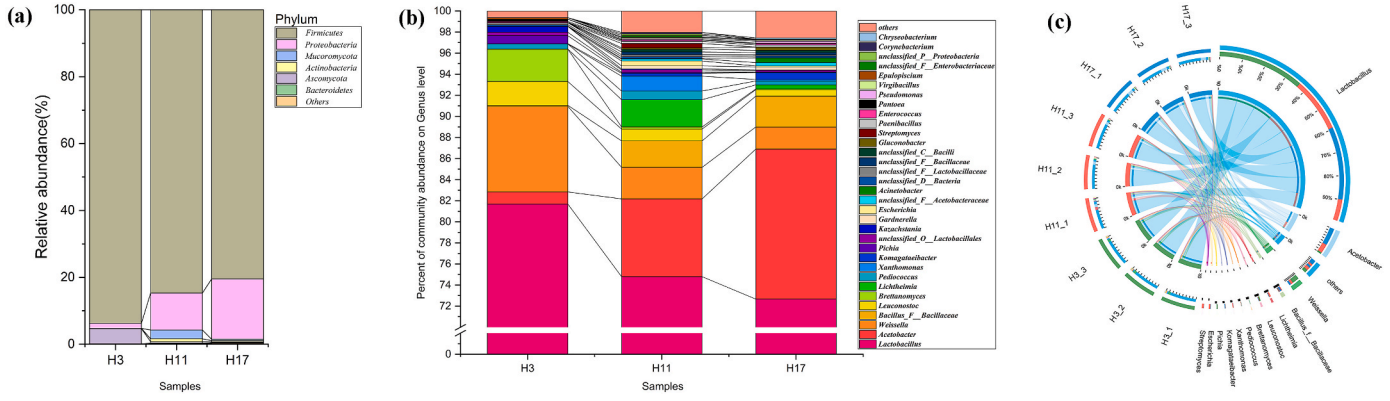

Fig. 4. Microbial community succession in different fermentation periods (a) Relative abundance of the bacterial flora of *Cupei* at different fermentation periods at the phylum level (b) Relative abundance of bacterial flora of Cupei at different fermentation periods at the genus level (c) Succession of species with significant abundance in SSV.

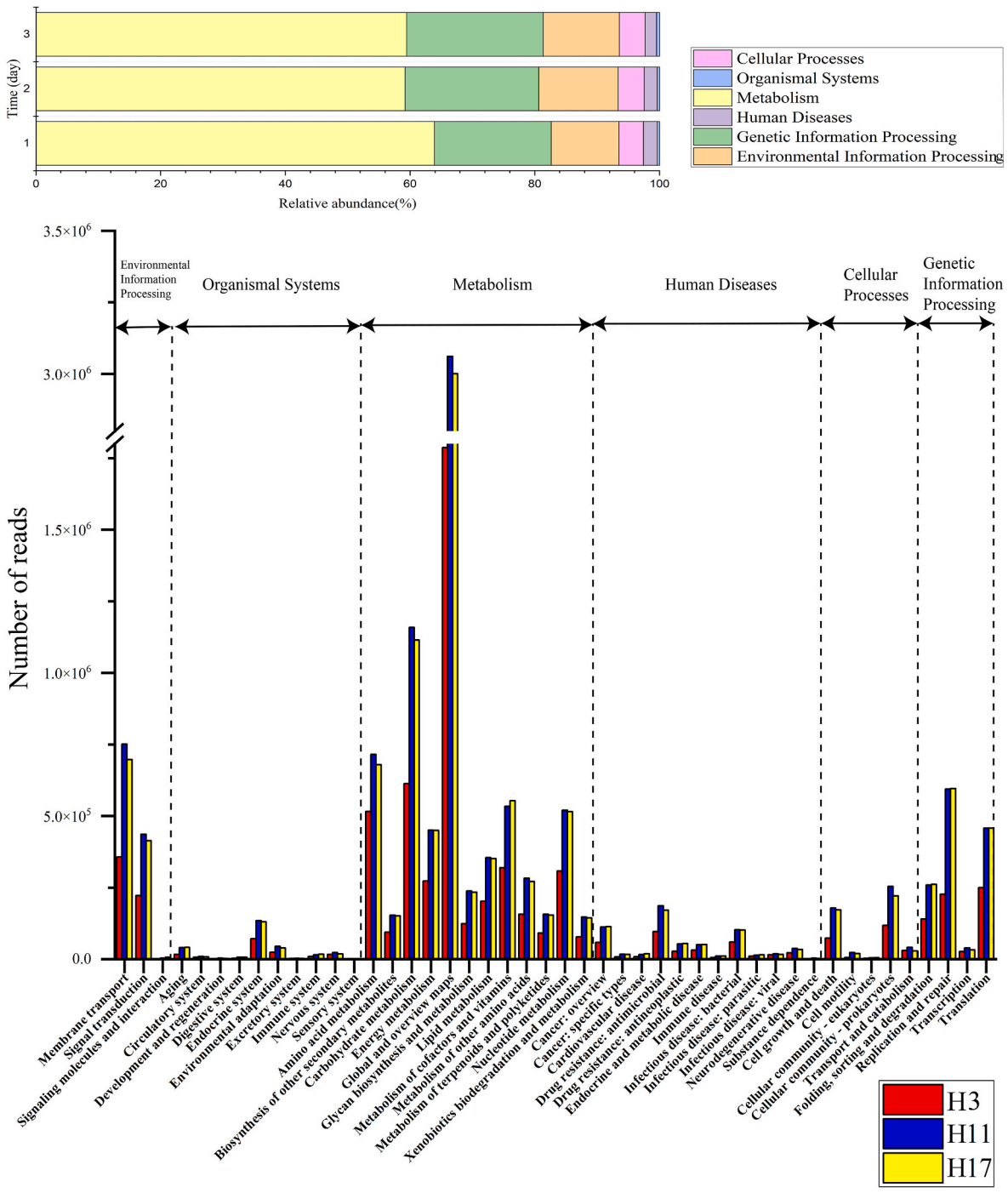

Fig. 5. Functional gene categories. Level 1, Level 2 based on KEGG.

fermentation indicate that substrate decomposition and flavor compounds production in SSV fermentation require a long process of microbial enrichment and growth. At the same time, it can also completely show the activity of microbes involved in the related metabolism at different stages. This provides a theoretical basis for identifying the key growth nodes of microbes during SSV fermentation.

3.7. Exploration of the microbial distribution and metabolic mechanisms associated with different flavor compounds

Combined with the KEGG database (https://www.kegg.jp/) and related literature (Wu et al., 2021a), 99 enzymes related to the enzymatic reaction of SSV flavor formation were obtained (Fig. 7). The results showed that 14 genera were most closely related to substrate decomposition and flavor formation. Although multiple enzymes have been annotated by these microbes, only *Lactobacillus*, *Weissella*, and *Acetobacter* are present at high levels. In addition, the number of fungi annotated was far lower than that of bacteria. However, a few fungi, such as *S. cerevisiae*, *Kazachstania*, *Pichia,* and *Lichtheimia,* are annotated with a variety of enzymes. With the extension of the fermentation duration, the abundance of enzyme encoding genes regulating substrate decomposition and flavor formation increased. This shows that the decomposition of the substrate and the formation of flavor mainly occur after three days and these processes are facilitated by microbes that harbor genes encoding enzymes related to substrate decomposition and flavor formation.

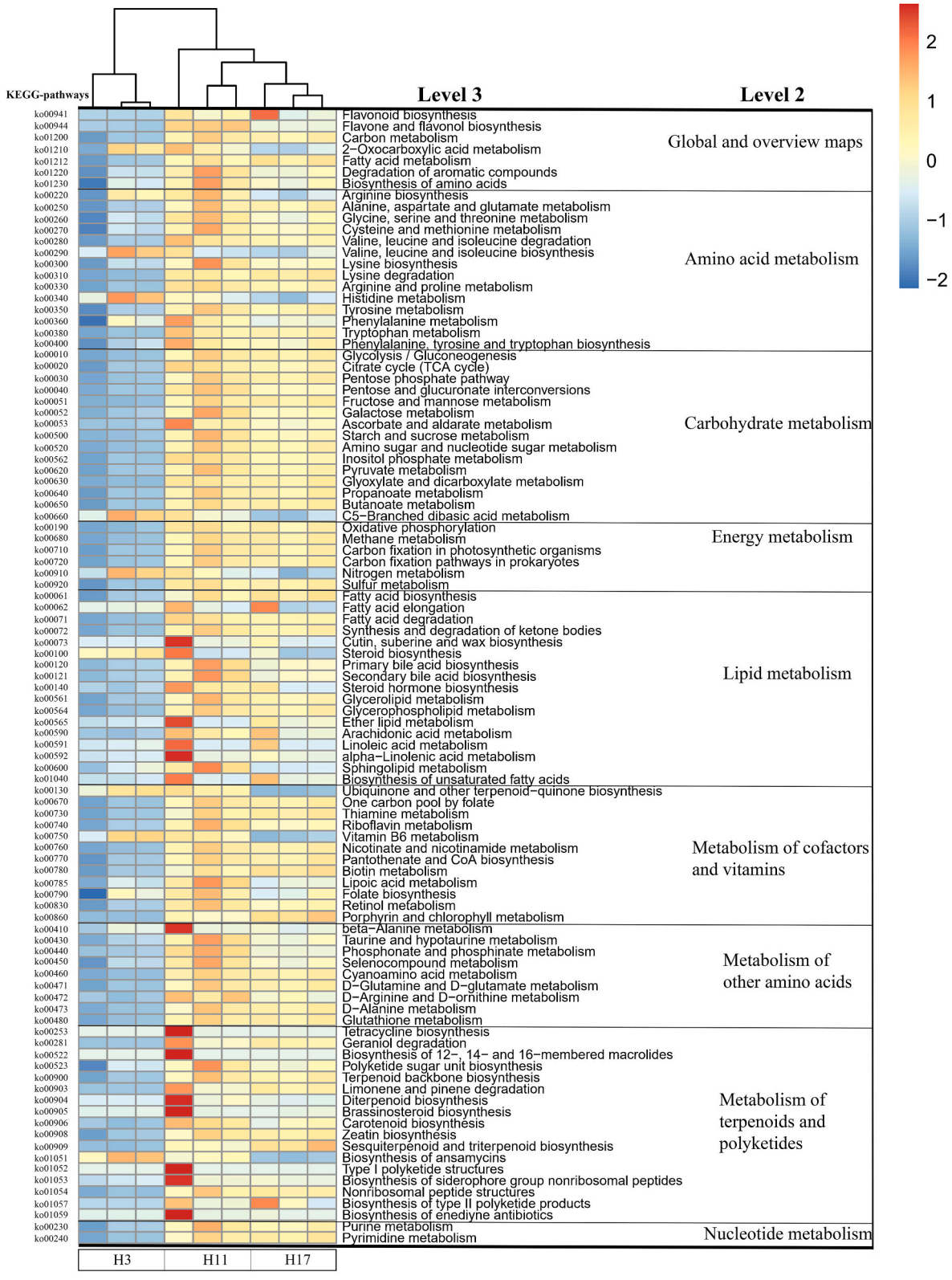

Fig. 6. Functional gene categories. Level 2, Level 3 based on KEGG.

Lactobacillus and *Weissella* are extensively involved in starch and cellulose degradation. *Acetobacter, Lichtheimia*, and *Pantoea* are also involved in starch metabolism. In addition to starch and cellulose, arabinoxylan is the primary sugar in bran and can be used by microbes. There are few microbial processes related to arabinoxylan degradation. Among them, *Lactobacillus* and *Bacillus* contained enzyme genes involved in arabinoxylan metabolism, which were labeled on days 3 and 11, respectively, of fermentation. *Lactobacillus* and *Paenibacillus* were annotated by enzymes related to arabinoxylan degradation, with levels increasing with prolonged fermentation times. Galactose and xylose, which are the main sugars of the arabinoxylan branched chain, may also participate in the decomposition reaction. Among them, aldose 1-

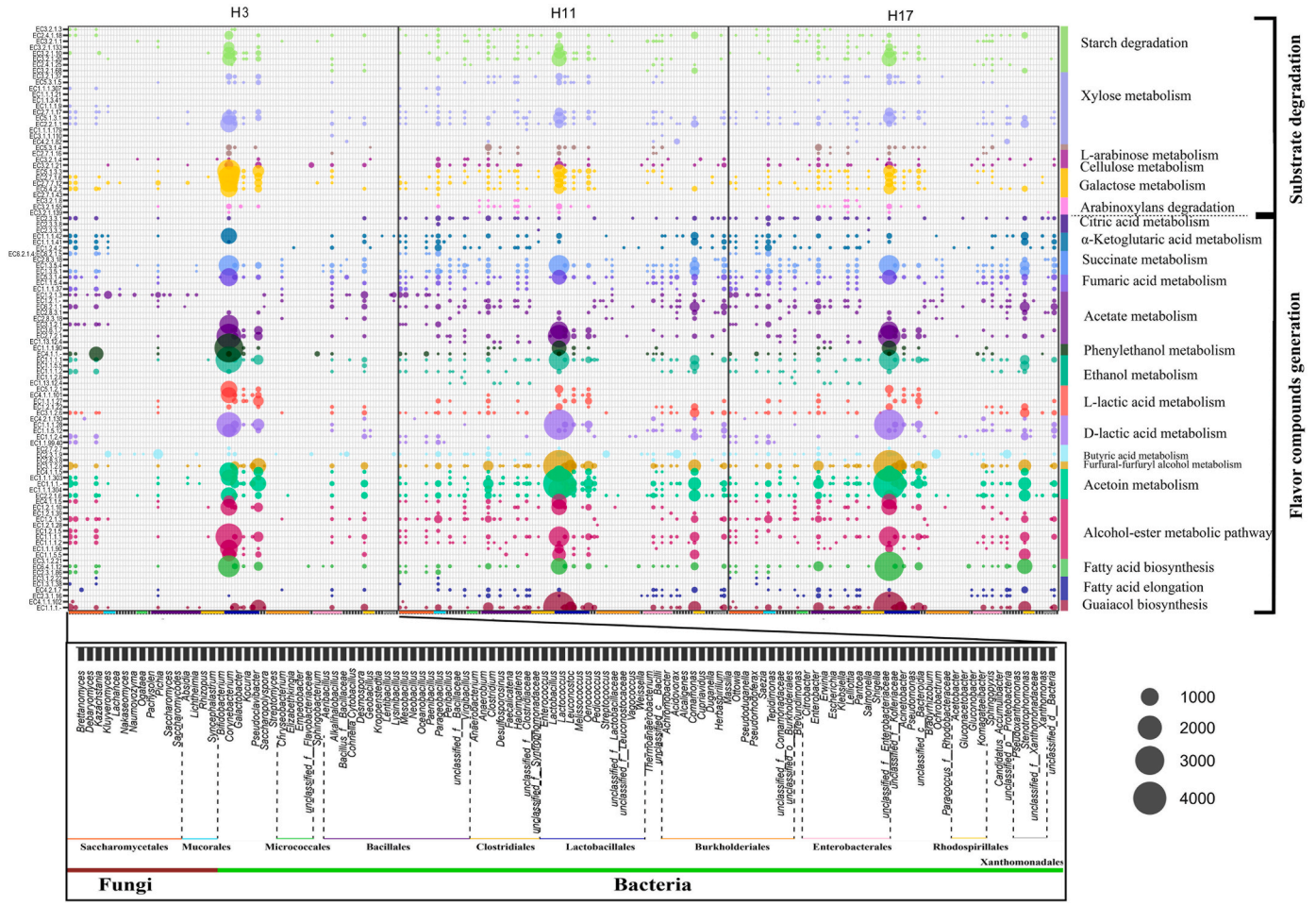

Fig. 7. Distribution and enzyme gene abundance of microbial associated with substrate degradation and flavor metabolism pathways during SSV fermentation The diameter of bubble correlates with the abundance of enzymes.

epimerase (EC5.1.3.3), galactokinase (EC2.7.1.6), and glucose phosphomutase (EC5.4.2.2), as enzymes related to galactose degradation, had the highest levels of gene annotation at day 3, mainly *Lactobacillus* and *Weissella* were annotated. *Lichtheimia* and *Bacillus* had more galactose-degrading enzyme-related genes on day 11. The decomposition of cellulose and arabinoxylan produces large amounts of xylose. *Lactobacillus*, *Acetobacter*, and *Wessilla* have annotated ketopentose ribulose-phosphate 3-epimerase (EC5.1.3.1), glycolaldehydetransferase (EC2.2.1.1), and xylulokinase (XY2.7.1.17) in xylose metabolism. Under the action of microbes, the different substrates in the raw material of the SSV provide carbon sources and nutrients for microbial growth and metabolism. Simultaneously, microbial communities metabolize substrates for growth while producing metabolic products with different flavors.

Organic acids are the main flavor compounds of SSV, *Brettanomyces*, *Lactobacillus*, *Weissella*, *Acetobacter*, and *Pichia*, which contain genes encoding enzymes related to alcohol metabolism, such as alcohol dehydrogenase (E1.1.1.1) (E1.1.5.5) and alcohol dehydrogenase (NADP+) (E1.1.1.2), which provide alcohol to *Acetobacter*. Furthermore, *Acetobacter* is the main contributor to acetic acid metabolism, mainly involving three enzymes, of which the acetyl-activating enzyme (Y6.2.1.1) was annotated at high levels on the 11th day of fermentation. However, acetic acid production starts during the early stage of fermentation, and the less abundant acetic acid bacteria are obviously not the early producers of acetic acid. *Lactobacillus*, *Weissella*, *Pediococcus*, and *Leuconostoc* also have several genes encoding for enzymes related to acetic acid metabolism. *Lactobacillus* and *Acetobacter* are acetic acid-producing strains used in the fermentation of Amazon cocoa beans (Serra et al., 2019). Acetic acid is mostly produced by bacterial fermentation in two main ways: (1) *Acetobacter* oxidize ethanol to acetic acid under aerobic conditions in neutral or acidic media. (2) Production of acetic acid from two molecules of carbon dioxide (CO_2) by heterofermentative lactic acid bacteria through the Wood-Ljungdahl Pathway (Vidra and Németh, 2017). It can be seen that heterofermentative lactic acid bacteria are important strains for forming acetic acid in the early and middle stages of SSV. *Lactobacillus* is thought to be the main microbial species that contains genes for enzymes related to lactic acid metabolism. In addition, *Weissella*, *Acetobacter*, *Bacillus*, *Pichia, and Pediococcus* have many genes encoding enzymes involved in lactic acid metabolism. Lactic acid is mainly produced by lactic acid bacteria, such as *Lactobacillus* and *Weissella*. The high abundance of lactic acid bacteria during fermentation is why the concentration of lactic acid in bran vinegar is greater than that of acetic acid.

The annotated levels of enzymes related to butyric acid were not high during the fermentation process. *Lichtheimia* and *Bacillus* were the main microbial species containing the acetyl coenzyme A C-acetyltransferase (EC 2.3.1.9) gene. This reached its highest point on the 3rd day of fermentation, decreasing in abundance as the fermentation time increased. *Comamonas* was the main microbial species containing the glyoxalase II (EC3.1.2.6) gene, with the highest gene abundance on the 11th day of fermentation, followed by no significant change. *Clostridium* may produce butyric acid, and anaerobic *Bacillus* is a dominant potential producer of butyric acid (Chai et al., 2019; Yuan et al., 2022).

The middle and late stages of fermentation are the most active stages

of microbial metabolism. The high frequency of turning *Cupei* promotes the growth of aerobic microbes, which makes the tricarboxylic acid cycle more active. *Acetobacter*, *Bacillus*, and *Lichtheimia* were the main microbial species involved in the expression of genes related to citric acid metabolism. A-ketoglutaric acid is an important intermediate product in the tricarboxylic acid cycle, and *Acetobacter*, *Lactobacillus*, *Xanthomonas*, and *Lichtheimia* are the major organisms harboring genes encoding isocitrate dehydrogenase (NADP+) (EC1.1.1.42) and isocitrate dehydrogenase (NAD+) (EC1.1.1.41). *Lactobacillus* and *Acetobacter* are the main microbial species harboring genes encoding enzymes related to succinic acid metabolism, such as succinyl coenzyme A (EC2.8.3.18) and succinate dehydrogenase (EC1.3.5.1:1.5.4). Meanwhile, *Xanthomonas* and *Lichtheimia* also contain genes for enzymes related to succinic acid metabolism, and the gene abundance gradually increased after 11 days of fermentation. *Lactobacillus, Acetobacter,* and *Xanthomona*s were involved in fumaric acid metabolism, and among them L-arabinose isomerase (EC5.3.1.4) was highly annotated. The growth of these aerobic microbial species enriched various organic acids in SSV. Phenylethanol can be produced by two pathways: the glucose anabolic pathway or the corresponding amino acid biosynthesis pathway involving valine, leucine, isoleucine, and phenylalanine (Li et al., 2008). *Lactobacillus*, *Acetobacter*, *Weissella*, and *Pichia* are the main microbial species containing the acylamidase (EC3.5.1.4) and aryl alcohol dehydrogenase (EC1.1.1.90) genes in the metabolism of phenylethanol. The genus *Pichia* contains phenylpyruvate decarboxylase (EC4.1.1.-) during the early stages of fermentation. In addition, *Xanthomonas*, *Lichtheimia*, and *Bacillus* are microbial species with genes for enzymes related to phenylethanol metabolism.

Furfural can be produced by the Maillard reaction or dehydration of sugar, and while 2-furan methanol is a furfural degradation product (Cocchi et al., 2011), its catabolism is dependent on the expression of the acetoacetylglutathione hydrolase (EC3.1.2.6) gene. *Lactococcus, Leuconostoc, Xanthomonas, Oenococcus, Pediococcus, Unclassified-f- Leuconostocaceae, Bacillus, Weissella, Acetobacter, and Lactobacillus* contain acetyl acetoacetylglutathione hydrolase (EC3.1.2.6)-related genes. *Acetobacter*, *Lactobacillus*, *Oenococcus*, and *Wessila* are most closely related to furfural metabolism and are the main microbial species containing the acetoacetylglutathione hydrolase (EC3.1.2.6) gene. Acetoin is a prerequisite for the synthesis of tetramethylpyrazine (Zhong et al., 2020), and is also a characteristic flavor of SSV. Acetoin can also be metabolized to 2,3-butanediol. Furthermore, 2,3-butanediol is positively correlated with *Lactobacillus* and *Streptococcus* during wine fermentation (Chen et al., 2020). *Acetobacter pasteurianus* has high acetoin production capacity (Zhao and Yun, 2016). Therefore, 2,3-butanediol and tetramethylpyrazine formation may be related to *Acetobacter pasteurianus*as well as. *Lactobacillus, Bacillus, Lichtheimia, Weissella, Acetobacter, and Oenococcus* annotated the genes related to acetoin metabolism.

The characteristic aroma of SSV is imparted by a large number of esters, such as ethyl phenylacetate and phenethyl acetate, which are often produced by the reaction of alcohols and acids catalyzed by esterase (J. Wang et al., 2022). Butanoic acid reacts with alcohol under the catalysis of esterase or lipase to form ethyl butyrate with banana and strawberry flavors (Xu et al., 2020). Paraldehyde is an acid-catalyzed trimer of acetaldehyde (Gunasekaran and Abraham, n.d.). In this study, *Lactobacillus*, *Weissella,* and *Acetobacter* were the main microbes harboring genes encoding for enzymes involved in ester metabolism, which also play an important role in the fatty acid synthesis. *Acetobacter, Pantoea,* and *Bacillus* spp. Contribute significantly to the elongation of fatty acid chains. Ferulic acid is a natural precursor of aromatic compounds such as guaiacol (Mishra et al., 2014). *Lactobacillus*, *Acetobacter*, *Oenococcus*, *Weissella*, *Bacillus*, and *Debaryomyces* produced 2-methoxy-4-methyl-phenol and 4-ethyl-2-methoxy-phenol through phenacrylate decarboxylase (EC4.1.1.102) and butanol dehydrogenase (EC1.1.1.-) pathways. *Lactobacillus* and *yeast* are reported to be the dominant microbial genera for alcohol formation during the fermentation of Xiaoqu liquor, whereas *Lactobacillus* and *Weissella* are probiotics in pineapple peel (Hanya et al., 2020; Tallei et al., 2022). *Lichtheimia, Acetobacter, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Leuconostocaceae,* and *Weissella* are the main microbial genus involved in guaiacol synthesis related to enzyme genes.

In summary, 15 microbial genera (*Lactobacillus*, *Weissella*, *Acetobacter*, *Bacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Leuconostocaceae*, *Lichtheimia*, *Pichia*, *Brettanomyces*, *Pantoea*, *Kazachstania*, and *Xanthomonas*) were most closely related to the flavor of SSV. *Lactobacillus*, *Acetobacter*, *Bacillus*, *Weissella*, *Lichtheimia*, *Pichia*, and *Oenococcus* may play crucial roles in flavor formation and substrate degradation.

3.8. Construction of a flavor metabolic pathway for SSV

Although numerous enzymes are involved in substrate metabolism and flavor formation, only a few are involved in the substrate decomposition and flavor formation pathways. To explore the metabolic pathways of the different flavor substances produced by microbes during the fermentation of SSV, we constructed a metabolic network of the main flavor substances of SSV based on the annotation results of the KEGG metabolic pathway and existing vinegar studies (Fig. 8; Wu et al., 2017; 2021b). Sixty enzymes were found to be involved in substrate decomposition and flavor formation in SSV during fermentation, and 19 microbial genera were found to be involved in substrate decomposition and characteristic flavor formation. Furthermore, *Lactobacillus* and *Bacillus* are commonly involved in the decomposition of substrates such as starch and arabinoxylan, while *Lichtheimia* and *Xanthomonas* are essential microbes for cellulose degradation. In addition, *Weissella* has a crucial role in starch degradation. In the flavor-formation pathway, *Lactobacillus* and *Bacillus* are commonly involved in the formation of flavors such as butyric acid, lactic acid, ethyl coupling, and acetic acid. *Pichia. Kazachstania, Brettanomyces,* and *Acetobacter* were involved in the formation of 3-(methylthio)-1-propanol. In addition, *Oenococcus* and *Lenconostoc* were the main genus responsible for the production of acetoin, while *Lichtheimia, Pediococcus,* and *Lenconostoc* are the main genera involved in lactic acid metabolism and *Acetobacter, Xanthomonas,* and *Lichtheimia* play crucial roles in the tricarboxylic acid cycle and acetic acid metabolism.

The Intermediate products, pyruvate, and acetyl-CoA, play a crucial role in the flavor metabolic network and participate in the synthesis of many flavor compounds. *Lichtheimia, Acetobacter, Bacillus,* and *Xanthomonas* are mainly involved in converting acetyl-CoA to acetic acid. *Lichtheimia*, *Acetobacter*, *Brettanomyces*, *Xanthomonas*, *Bacillus*, and *Kazachstania* can oxidize acetaldehyde to acetic acid using aldehyde dehydrogenase (EC1.2.1.3). *Lactobacillus, Brettanomyces, Lichtheimia, Acetobacter, Xanthomonas, Bacillus,* and *Pichia* are the main aerobic microbes involved in the tricarboxylic acid cycle. The significant increase in organic acid content may be related to these microbes. Butyric acid is mainly produced by the butyryl-CoA and butyrate kinase pathways, *Lactobacillus* and *Bacillus* are the main microbes forming these two pathways.

Lactobacillus, Weissella, Acetobacter, Lichtheimia, Oenococcus, Pediococcus, and *Xanthomonas* achieved conversion of 2,3-butanediol and acetoin by biocatalysis. The metabolism of amino acids may be derived from the proteins in the raw material, and methionine is a precursor substance for 3-(methylthio)-1-propanol (Du et al., 2021). Throughout the formation, *Lactobacillus*, *Weissella*, *Acetobacter*, *Lenconostoc*, *Xanthomonas*, *Pichia*, *Kazachstania*, and *Brettanomyces* are the main contributing genera.

Lactobacillus, *Weissella*, and *Pediococcus* spp. are potential producers of phenylethanol. Therefore, *Lactobacillus, Weissella*, *Acetobacter*, *Bacillus*, *Lichtheimia*, *Pediococcus*, *Oenococcus*, *Lenconostoc*, *Pichia*, *Kazachstania*, Xanthomonas, and *Brettanomyces* may be the main contributors to flavor metabolism. In addition, the compounds in the aging stage may be mainly generated by chemicals such as furfural and pyrazine produced by the Maillard reaction (Durán-Guerrero et al., 2015; Liang et al.,

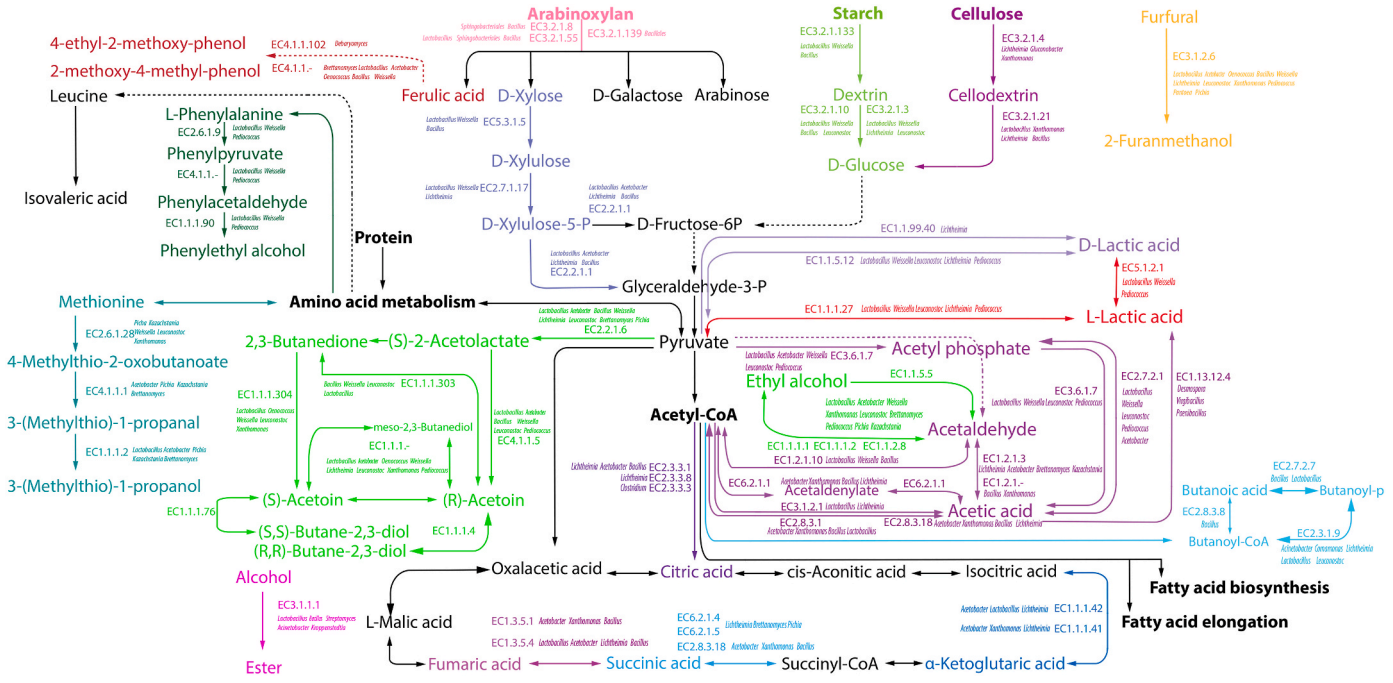

Fig. 8. Metabolic network of key flavor formation of SSV.

2016). However, the metabolic reactions leading to volatile compounds such as benzaldehyde during the aging stage, require further research.

The mechanism of some of the main aroma production during the fermentation stage is still unclear. Related studies have shown that some of the main aromas are produced by microbial metabolism. For example, furanone production may be derived from yeast metabolism, and the production of 3-methyl-1-butanol may be related to lactococci and yeasts (Yan et al., 2022). Branched-chain aldehydes (butanal, 3-methyl-, and 2-methyl-propanal) are produced by microbes via the Ehrlich pathway or the amino acid biosynthesis pathway, in which 3-methyl-butanal can be converted from leucine, *Lactobacillus* and *Bacillus* have leucine dehydrogenase, and 3-methylbutanol can be formed from 3-methyl- butanal by reduction (Smit et al., 2009).

4. Conclusion

In this study, the characteristic flavors of SSV, their stages of production in the brewing process, and the correlation between microbial and key enzymes were explored through a flavor-orientaion and metagenomic approach, and the dominant flora that influence flavor formation was explored. Alanine, proline, valine, histidine, and leucine were the main amino acids in SSV, whereas lactic-, acetic-, and succinic acid were the main organic acids. Furthermore, 30 species of volatile compounds, including dihydro-5-pentyl-2(3H) -furanone, 2-methyl-propanal, 2,3-butanediol, 3-methyl-butanoic acid, butanoic acid, 4-methyl-pentanoic acid, and 2,4-bis (1,1-dimethyl ethyl)-phenol, were the critical flavors of SSV. Pyrazines and furfural are produced during the aging stage, and non-enzymatic reactions produce these compounds. Most of the other compounds are produced during fermentation. Metagenomic technology was used to analyze the microbial structure during the fermentation stage. *Lactobacillus*, *Weissella*, *Acetobacter*, *Bacillus*, *Lichtheimia*, *Pediococcus*, *Oenococcus*, *Lenconostoc*, *Pichia*, *Kazachstania* and *Brettanomyces* were the key genus involved in substrate degradation and flavor formation. Heterotypic fermentative lactic acid bacteria play an important role in acetic acid metabolism in SSV. In this study, the main flavor and production stages were determined during vinegar fermentation, and the dominant flavor-related flora were identified. Based on this, the functional microbial properties of SSV can be determined, and the characteristic flavor of SSV can be reproduced through community construction. The metagenomic technology based on DNA level can study microbial diversity, population structure, and metabolic function potential, but it is limited to distinguishing the expression and activity of genes, and it is also difficult to distinguish the activity of microorganisms detected in samples. To solve this problem, transcriptome technology should be used to elucidate the microbial activity during SSV fermentation at the RNA level and to determine the expression levels of microbial enzymes associated with flavor production at different fermentation times.

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CRediT authorship contribution statement

Na Li: Writing – original draft, conceived the research, analyzed the data. **Junjie Fu:** conceived the research, analyzed the data. **Guirong Zhang:** analyzed the data. **Jun Liu:** Supervision, designed and supervised the experimental work. **Zhongxuan Li:** Funding acquisition, provided research funding. **Rui Luo:** assisted the experiment, wrote the manuscript. All authors critically read and approved the manuscript. **Li Li:** Supervision, designed and supervised the experimental work, Funding acquisition, provided research funding.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

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