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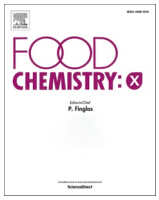

Correlation between physicochemical properties, flavor characteristics and microbial community structure in Dushan shrimp sour paste

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ABSTRACT

Dushan shrimp sour paste (DSSP), a traditional Guizhou condiment, and its unique flavor is determined by the fermentation microbiota. However, the relationship between the microbiota structure and its flavor remains unclear. This study identified 116 volatile flavor compounds using electronic nose and headspace solid-phase microextraction-gas chromatography mass spectrometry (HS-SPME-GC–MS) techniques, of which 19 were considered as key flavor compounds, mainly consisting of 13 esters and 1 alcohol. High-throughput sequencing technique, the bacterial community structure of nine groups of DSSPs was determined. Further analysis revealed *Vagococcus, Lactococcus*, and *Tepidimicrobium* as key bacteria involved in flavor formation. This study contributes to our understanding of the relationship between bacterial communities and the flavor formation, and provides guidance for screening starter culture that enhance the flavor of DSSP in industrial production.

1. Introduction

Guizhou's ethnic minority areas have long been renowned for their unique "acid" cuisine, such as Rice Acid Soup (Liu, Qin, Hu, & Miao, 2023), Red Sour Soup (Li et al., 2021), Suanyu (Liu et al., 2021) and Sour Meat (Wang et al., 2022; Wang, Liu, He, & Li, 2022). And the "three sours of Dushan", namely Dushan shrimp sour paste (DSSP), Dushan pickled vegetable, and stinky sour paste, whose history dates back to the Ming Dynasty, approximately over 400 years ago, and renowned for its distinctive taste (Xu et al., 2020). Generally, the production of DSSP occurs in household or commercial environments, employing traditional spontaneous fermentation techniques. The production of DSSP primarily use freshwater shrimp as the raw material, mixed with Chinese liquor, salt, glutinous rice sweet wine, garlic and red chili powder (Yang, Zhang, Mao, Zhou, & Li, 2018), ferment in jars with a "neck" structure, are filled with water to prevent air entry, ensuring anaerobic conditions inside the jar (Yang et al., 2018; Yang, Zhang, et al., 2018), ultimately forming a viscous semi-solid product with a unique aroma. The widespread use of DSSP in cooking various meat dishes, particularly in dishes like beef, fatty intestines, and pork ribs, which are popular among consumers (Zhang, 2023). Regional tourism's growth and poverty alleviation efforts in the western regions have presented novel development avenues for this local delicacy. Consequently, there's an increasing demand for large-scale industrial production of DSSP.

For the successful commercialization of fermented products, a thorough understanding of key microorganisms and their crucial role in impact flavor is essential (Gao et al., 2023). Consequently, understanding the relationship between microbiota and flavor in DSSP is paramount to ensuring product quality consistency. Despite the extensive research on the fermentation process of shrimp paste (Phewpan et al., 2020; Roh et al., 2009), which has unveiled the primary bacterial genera *Tetragenococcus*, *Lactobacillus*, *Salimicrobium* and *Halanaerobium* (Che, Yu, Sun, Lu, & Xie, 2021; Li, Lu, He, Sang, & Sun, 2021; Yang, Liu, Sang, & Sun, 2023). Research conducted by Deng et al. (Deng et al., 2022) revealed that the predominant volatile flavor compounds in shrimp paste encompassed long-chain alkanes, esters, and acids. Additional investigations demonstrated that high-salt shrimp paste exhibited an elevated content of acids, aldehydes, and heterocyclic compounds, resulting in a robust roasted flavor, whereas low-salt shrimp paste was abundant in esters, emitting a refreshing aroma profile (Yu, Lu, Zi, Yang, & Xie, 2022). Natural climatic conditions result in alterations of bacterial communities and metabolites during fermentation, subsequently leading to inconsistent product quality (Duan et al., 2016), which is influenced by multiple factors, including region, preparation method, and raw material (Sang et al., 2020; Xu et al., 2020; Xu, Kong, et al., 2020). The key difference between shrimp pastes and Dushan shrimp sour pastes were raw materials, production methods, and resulting taste profiles. Shrimp paste consists of small marine shrimp as the main

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ingredient and has a salty and umami taste with a strong aroma (Zhu et al., 2019), while Dushan shrimp sour paste has a unique sour and rancid odor. Although Yang et al. identified esters, acids, alcohols, aldehydes and heterocyclic as the primary flavor compounds in DSSP (Yang, Li, Xiong, He, & Zhou, 2020). To date, there is still a lack of in-depth understanding in the basic research on microbial diversity, flavor compounds, process optimization, and food safety related to DSSP, which will be the main obstacle hindering its further development.

The objective of this study was to elucidate the linkage between microbial composition and distinctive flavors in DSSP. Towards this objective, we performed comprehensive analyses of free amino acids, volatile flavor compounds, and microbial profiles across nine groups of DSSP samples selected based on their popularity in sensory evaluation (data not shown), employing HPLC, electronic nose, HS-SPME-GC–MS, and high-throughput sequencing methodologies. Additionally, we delved into the relationship between signature flavor components and microorganisms, determining the key functional bacteria within DSSP, thus laying a crucial foundation for enhancing its industrial-scale fermentation process.

2. Materials and methods

2.1. Sample collection

The study collected nine groups of spontaneous fermented Dushan shrimp sour paste (DSSP) originating from Dushan County, Duyun City, Guizhou Province, China. Specifically, these samples were sourced from nine workshops renowned for their highly rated DSSP available in local farmer's markets. Among the samples from the same batch in each workshop, three DSSP samples were randomly selected. The nine groups of DSSP samples were sequentially numbered XS1-XS9. These samples were properly preserved in foam boxes surrounded by dry ice and transported to the laboratory for uniform storage at −80 °C for subsequent analysis.

Despite the identical preparation process for these samples, there exists variation in the content levels of various ingredients. Firstly, local freshwater river shrimps were selected as the raw material. Following this, 8% to 10% salt and 4% to 6% Chinese liquor were incorporated, thoroughly mixed, and sealed in a jar for fermentation lasting between 9 and 12 months. Next, 8% to 13% white glutinous rice sweet wine, 8% to 13% red chili powder, 1% to 4% salt and 3% to 5% garlic are added, evenly mixed, and placed in a cool, shady area to ferment for a duration exceeding 3 months. The DSSP produced exhibited a viscous, semi-solid.

2.2. Determination of physicochemical analysis of shrimp sour paste

2.2.1. pH, total acidity (TA), amino acid nitrogen (AAN), nitrite, moisture and protein

The pH was measured using a digital PHS-3C pH meter (Leici, Shanghai, China). Total acidity (TA) was determined using acid-base titration according to the GB/T12456–2021 (China). The amino acid nitrogen (AAN) was assessed using the GB5009.235–2016 (China). Nitrite content was determined using the hydrochloride naphthodiamide method according to the GB 5009.33–2016 (China).

Moisture level was measured using the dry/wet weight method at 105 °C (Huang, Yu, Han, & Chen, 2018), and protein content was determined using the Kjeldahl method as specified in GB5009.5–20,116. All analyses were performed in triplicate.

2.2.2. Color difference

Color differences of DSSP were determined using a portable chromatic aberration meter (WSC–S, China), as previously described (Prihanto, Nurdiani, Jatmiko, Firdaus, & Kusuma, 2021). The parameters used to express the color were L* (lightness), a* (redness/greenness), b* (yellowness/blueness).

2.3. Determination of free amino acids in shrimp sour paste by HPLC

The extraction and derivatization of free amino acids (FAAs) from 5.0 g of DSSP followed the protocol established by Yang et al. (Weiling, Na, Xiaojuan, & Qinghai, 2021), with slight modifications.

For the ultrasonic extraction of DSSP, 25 mL of 0.1 mol/L HCl were used at 25 °Cfor 30 min. Following the extraction, the filtrate passed through a 0.45 μm membrane before undergoing the derivatization process. The solution obtained was filtered again using a 0.22 μm membrane for HPLC analysis. FAAs were determined by a HPLC system with UV detection and a C18 column (4.6 mm x 250 mm, 5 μm, Dalian Elite). The HPLC parameters were set as follows: flow rate (1.0 mL/min), injection volume (10 μL), column temperature (35 °C); and detection wavelength (254 nm).

2.4. Electronic nose analysis

The PEN3 device (Airsense GmbH, Schwerin, Germany) was used for electronic nose analysis of DSSP samples by equipped with 10 kinds of semiconductor metal oxide chemical sensors (namely W1C W5S, W3C, W6S, W5C, W1S, W1W, W2S, W2W and W3S, respectively). These sensors showed different selectivity and sensitivity towards volatile compounds (Feng, Wang, Wang, Huang, & Kan, 2022). The performance description of *E*-nose gas sensor array were detailed by Yu et al. (Yu et al., 2022). DSSP samples (5.0 g) were added into a 25 mL glass vial, then balanced at room temperature for 30 min before measured. The measurement method was as follows: reset time was 1 s, pre-sampling time was 5 s, flow rate of carrier gas was 200 mL/min, sample measurement time was 240 s, and washing time was 100 s. The sensor response signal for the E-nose was expressed as the conductivity ratio (G/G0) (Liu et al., 2018).

2.5. Volatile flavor compounds by HS-SPME-GC–MS

The sample (3.0 g) was placed in a 20 mL headspace vial and 20 μL of 2,4,6-trimethylpyridine (TMP) at a concentration of 0.5 μg/μL was used as the internal standard. Following incubation at 60 °C for 10 min at 300 rpm, the sample was extracted by HS-SPME with a DVB-PDMS needle for 40 min under the same conditions. Subsequently, the extraction needle was inserted into the GC–MS injection port and desorbed at 250 °C for 10 min.

GC conditions: HP-5MS column (60 m × 250 μm × 0.25 μm); high purity helium (purity >99.99%) as carrier gas; flow rate of 1.0 mL/min; split-flow mode with a split-flow ratio of 2:1. The column was initially heated to 40 °C for 2 min, followed by a gradual increase to 120 °C at a rate of 3 °C/min for 5 min, and then further heated to 250 °C at a rate of 5 °C/min. The inlet temperature was maintained 260 °C.

Mass spectrometry conditions: the ionization source was an EI source, with a transmission line temperature of 230 °C, electron energy of 70 eV, and an ion source temperature of 280 °C.

2.6. DNA extraction and high-throughput sequencing analysis

The bacterial DNA from DSSP was extracted using CTAB. The V3-V4 regions of 16S rRNA genes were amplified with specific primer and the PCR am-plification was performed according to Wang (Wang, Liu, et al., 2022).Then, sequencing analysis was performed on the Illumina NovaSeq platform following the method described in Wu (Wu et al., 2021).

2.7. Statistical analysis

The statistical analyses were conducted using three biological replicates and the results were presented as mean values ± deviation errors, utilizing SPSS Statistics 26. To determine the significance of sample effects, Analysis of Variance (ANOVA) with Duncan's multiple comparison test was employed ($p < 0.05$). Origin 2021 was utilized for data

processing and the creation of graphs related to *E*-nose. Volatile compounds were subjected to partial least squares discriminant analysis (PLS-DA) using SIMCA 14.1. The data were analyzed on the free online platform of Wekemo Bioincloud (https://www.bioincloud.tech/).

3. Results and discussion

3.1. Physicochemical characterization and color difference of DSSP samples

The physicochemical properties of fermented foods significantly influenced microbial growth and metabolite biosynthesis. In this study, there existed variations in the physicochemical parameters of DSSP depending on the source (Table 1). The pH values of the DSSP samples ranged from 4.22 to 6.84, which were similar to those previously reported (Nakamura et al., 2022). Additionally, the TA content varied from 3.30 to 27.54 g/kg. The average AAN level of sample was 0.41 g/100 g. The nitrite content of DSSP ranged from 0.01 to 0.02 mg/kg, which was significantly below Huanghua shrimp paste (Li, Lu, et al., 2021). The abilities of LAB to deplete nitrite and restrict the growth of nitrate reducing bacteria could be the reason that limit the formation of nitrite (Yan, Xue, Tan, Zhang, & Chang, 2008).

The fermentation process induces proteolysis or autolysis, leading to the formation of taste-active amino acids and peptides (Zhao, Schieber, & Gänzle, 2016). According to the findings of Wang et al. study (Wang, Xia, Gao, Xu, & Jiang, 2017), the addition of LAB has been shown to enhance acid protease activities in fish meat, thereby promoting protein degradation. The average levels of moisture and protein of all DSSP samples were 63.44% and 5.54 g/100 g, respectively. The low protein contents of DSSP samples were attributed to the degradation of shrimp meat protein during the fermentation process. The highest a* value of XS8 (8.50) indicated more red color, potentially influenced by the diversity and quantity of red chili powder added. The highest b* value of XS5 (6.53) showed a relatively more yellow compared to other samples. The b* value correlates with a yellow pigment formed reactions between lipid oxidation products and amines found in phospholipid head groups or proteins (Liu, Wang, Zhang, Wang, & Kong, 2019).

3.2. Free amino acids of DSSP samples

FAAs serve as precursors for various volatile flavor compounds, including acids, alcohols, aldehydes, and esters, as well as other compounds incorporating N and S, all formed through reactions like transamination, dehydrogenation, decarboxylation, and reduction (Ardö, 2006). In this study, a total of 17 types of FAAs were detected in different DSSP samples (Table S1), and the total amino acid content ranged from 240.74 to 428.75 mg/100 g. Fig. 1A displayed that the total amino acid concentration and essential amino acids (EAA) were significantly different ($p < 0.05$) during the nine groups of DSSP samples. The concentration of FAA ranged from 104.48 to 217.2 mg/100 g, while XS1 had the highest level of total free amino acid (TFAA; 428.75 mg/100 g). According to previous researches (Zhang, 2023), the 17 kinds of FAAs could be grouped into three categories: umami amino acid, sweet amino acid and bitter amino acid. Noteworthy, the content of bitter amino acids exceeded the combined quantity of umami and sweet amino acids, accounting for 58.20% of the overall free amino acid levels (Fig. 1B). The combination of L-Arg and NaCl effectively mitigates the bitter taste sensation despite the presence of bitter-causing FAAs (Tazuko et al., 2004). The main amino acids were Gly, Met and Ile in different DSSP samples (Fig. 1C). For instance, sulfides compounds played a pivotal role in imparting the characteristic aroma of shrimp paste (Lv et al., 2020) and Met was widely recognized as a vital precursor for these volatile organic compounds (Sun et al., 2018).

To investigate the contribution of FAAs to the taste of DSSP, the Taste Activity Value (TAV) of FAAs were determined (Table S2). The TAV was widely used to assess the intensity of flavor. If TAV > 1, the substance contributes more to overall flavor (Gong et al., 2024). All FAAs (TAVs <1) were not taste-active compounds among the 9 group DSSP samples. Fortunately, the bitter FAA does not seem to impact the flavor of DSSP. We speculate that is might be due to their high threshold. The EAA/TFAA ratios ranged from 0.34 to 0.55 (Table S1), similar with the reference value of 0.40 reported by the FAO/WHO in 1973. Leu and Lys were identified as the limiting amino acids in shrimp paste by Li et al. (Li et al., 2023). However, the consumption of shrimp sour paste, a distinct seasoning in Chinese cuisine often paired with meat dishes, was mainly influenced by its taste rather than nutritional value.

3.3. Volatile flavor compounds of DSSP samples

3.3.1. Analysis of aroma patterns by E-nose

Volatile compounds serve as crucial indicators for assessing the flavor profile of condiments and indirectly influence the overall organoleptic quality of condiments. The E-nose was used to evaluate the overall flavor profile of all traditional fermented DSSP samples (Fig. 1D). The sensors W1C, W5S, W1S, and W2S showed the highest response values for DSSP, suggesting that the aroma characteristics may be attributed to the presence of aromatic ingredients, nitrogen oxides, short-chain alkanes, alcohols, aldehydes, and ketones. Undergoing metabolic transformations during prolonged fermentation, prominent bacterial strains such as *Pseudomonas* and *Marinilactibacillus* produce inorganic sulfur compounds and nitrogen oxides, collaborating with long-chain alkanes to form the major flavor compounds of shrimp paste (Yao, Zhou, Hadiatullah, Zhang, & Zhao, 2021). Previous studies have shown that shrimp paste has the highest response value for W1S, a lower response value for W2W, and almost no response for W6S and W5C (Li et al., 2023; Li et al., 2023).

The PCA analysis revealed distinct clustering patterns among DSSP samples, with PC1 and PC2 contributing significantly to the variance, and notable variations observed in specific samples. The PC1 and PC2 contribute 65.83% and 19.61% of the variance, respectively (Fig. 1E).

Table 1
Physicochemical indexes of Dushan shrimp sour paste (x ± s).

Sample	pH	TA(g/kg)	AAN (g/100 g)	Nitrite(mg/kg)	Moisture (%)	Protein (g/100 g)	color deviation		
							L*	a*	b*
XS1	6.21 ± 0.04^{e}	7.20 ± 0.01^{e}	0.55 ± 0.01^{a}	0.02 ± 0.00^{ab}	58.27 ± 1.50^{de}	6.27 ± 0.57^{a}	31.61 ± 0.96^{c}	5.37 ± 0.72^{d}	3.50 ± 0.12^{d}
XS2	6.75 ± 0.05^{b}	4.49 ± 0.01^{g}	0.49 ± 0.01^{b}	0.01 ± 0.00^{b}	59.45 ± 0.84^{d}	6.16 ± 0.37^{ab}	34.57 ± 1.03^{a}	8.34 ± 0.51^{a}	5.94 ± 0.31^{a}
XS3	6.42 ± 0.03^{d}	5.71 ± 0.42^{f}	0.36 ± 0.01^{d}	0.01 ± 0.00^{b}	56.80 ± 0.91^{e}	5.86 ± 0.09^{ab}	31.98 ± 0.60^{c}	6.78 ± 0.26^{c}	4.54 ± 0.30^{c}
XS4	6.53 ± 0.06^{c}	4.52 ± 0.01^{g}	0.48 ± 0.00^{b}	0.01 ± 0.00^{b}	58.59 ± 1.69^{de}	6.39 ± 0.39^{a}	32.52 ± 1.09^{bc}	7.93 ± 0.38^{ab}	5.04 ± 0.64^{bc}
XS5	6.84 ± 0.02^{a}	3.30 ± 0.43^{h}	0.43 ± 0.01^{c}	0.01 ± 0.00^{b}	68.09 ± 0.16^{ab}	5.35 ± 0.09^{bc}	33.97 ± 0.50^{ab}	7.09 ± 0.46^{c}	6.53 ± 0.16^{a}
XS6	5.27 ± 0.04^{f}	10.20 ± 0.41^{d}	0.45 ± 0.02^{c}	0.01 ± 0.00^{b}	65.99 ± 0.09^{bc}	5.51 ± 1.08^{abc}	31.54 ± 1.10^{c}	4.37 ± 0.27^{e}	3.10 ± 0.08^{d}
XS7	4.22 ± 0.01^{h}	21.36 ± 0.44^{c}	0.33 ± 0.01^{e}	0.01 ± 0.00^{b}	64.14 ± 1.97^{c}	4.25 ± 0.47^{d}	32.78 ± 1.01^{bc}	5.23 ± 0.51^{d}	4.84 ± 0.17^{bc}
XS8	3.95 ± 0.01^{i}	24.55 ± 0.46^{b}	0.37 ± 0.01^{d}	0.02 ± 0.00^{a}	69.11 ± 0.93^{a}	4.80 ± 0.03^{cd}	32.33 ± 0.48^{c}	8.50 ± 0.31^{a}	5.29 ± 0.37^{b}
XS9	4.29 ± 0.01^{g}	27.54 ± 0.39^{a}	0.24 ± 0.01^{f}	0.01 ± 0.00^{b}	70.50 ± 0.41^{a}	5.26 ± 0.22^{bc}	34.09 ± 0.54^{ab}	7.32 ± 0.30^{bc}	6.10 ± 0.46^{a}

Note: values were expressed as means ± standard deviation ($n = 3$). Different letters within the same column indicate significantly different, $p < 0.05$.
TA: total acidity; AAN: amino acid nitrogen; L*: Color lightness, a*: a measure of red-green characteristics, b*: a measure of yellow-blue properties.

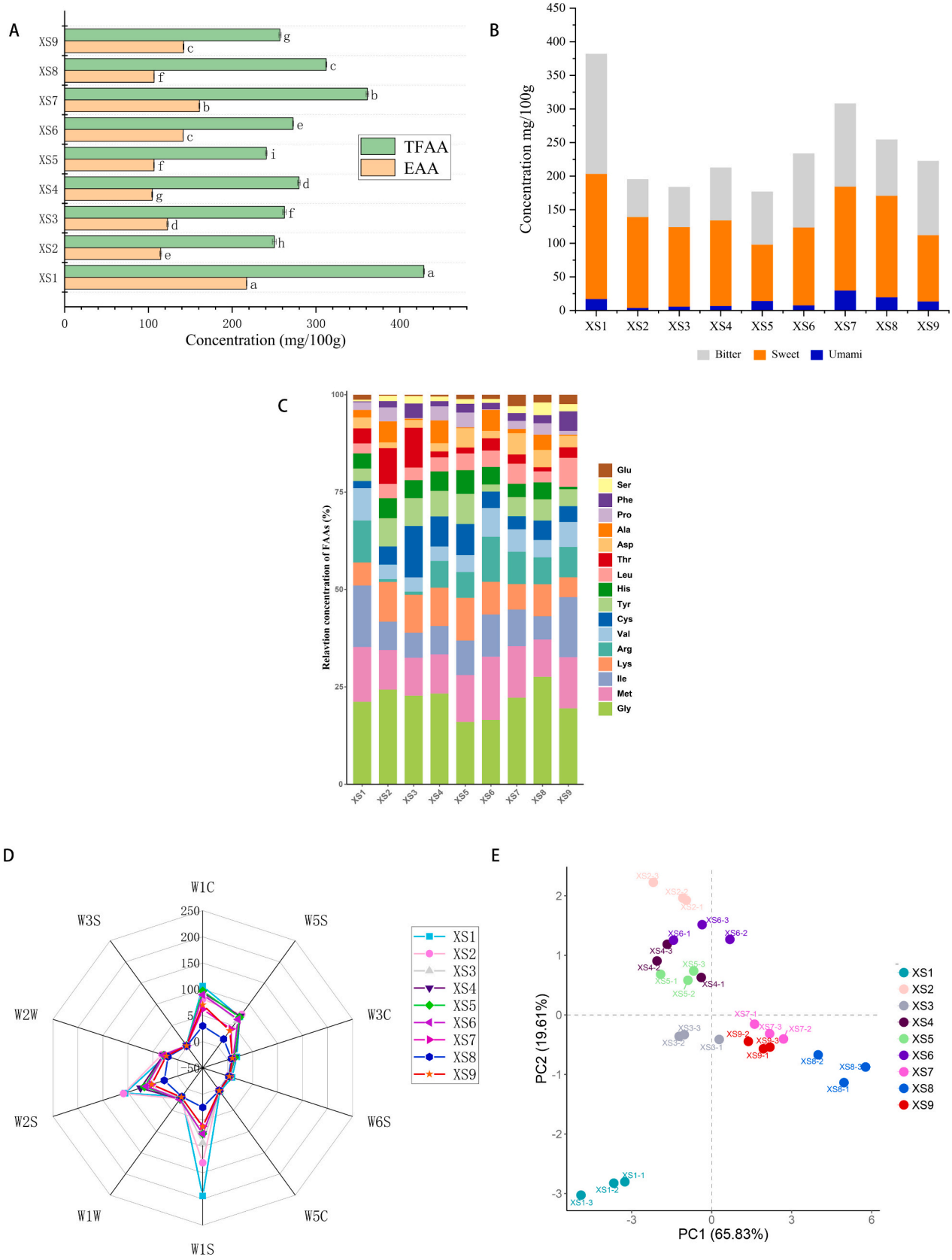

Fig. 1. Contents of TFFA and EAA obtained of DSSP (A); compositions of free amino acids in 9 groups of umami, sweet and bitter amino acids (B); the relative abundances of FAA (C); the spider diagram of the E-nose analysis (D) and PCA scores plot of E-nose data (E).

The cumulative contribution of PC1 and PC2 reached 85.44%, effectively distinguishing between DSSP samples and providing potential clustering results. The findings indicated that the samples can be broadly classified into two distinct regions. Notably, the XS1 sample appeared to be relatively isolated, whereas the remaining samples showed a higher level of clustering, which may be related to its relatively high conductivity for ratios short-chain alkanes (W1S = 194.16) and aromatic components (W1C =105.68). Moreover, the XS7 and XS9 samples showed a significant similarity, whereas the XS4 and XS5 samples were more concentrated. That suggested they may have comparable concentrations of key chemical components or share similar origins or processing conditions. Environmental factors play a significant role in shaping the volatile aroma compounds produced during spontaneous fermentation of shrimp paste (Lu, Liu, Xu, & Xie, 2022).

3.3.2. Analysis the volatile components by HS-SPME-GC–MS

To investigate the aroma characteristics of DSSP, volatile components were analyzed by HS-SPME-GC–MS techniques. As shown in Table S3, a total of 116 volatile substances were identified from nine groups of shrimp sour paste, including esters (62), alcohols (16), hydrocarbons (9), acids (7), amines (3), ketones (3), sulfides (2), aldehydes (2) and other aroma compounds (12). The types and concentrations of volatile compounds varied greatly in the nine groups of DSSP (Fig. 2 and Table S3). In this study, XS7 had the lowest count of volatile compounds (32 kinds) (Fig. 2A). The type of volatile compounds affects the complexity of the DSSP aroma, and Upset plots (Fig. 2B) were used to visualize the variations in volatile compound species among the nine groups. Among the samples, XS5 had the richest variety of volatile compounds, which had 35 identical volatile substances with XS2, XS3, and XS4, and 30 identical substances with XS1. The hierarchical cluster analysis (HCA) divided the nine groups of DSSP samples into two categories based on their volatile components, with XS7 belonging to the first category and the remaining samples grouped into the other category (Fig. 2C). In addition, XS1 contained 98.22% of the total volatile compounds and had the highest ester concentration, reaching 880.12 μg/g. Specifically, esters constituted 74.56%, 91.51%, and 87.5% of the total volatile compounds in XS5 (218.94 μg/g), XS6 (197.01 μg/g), and XS8 (104.61 μg/g), respectively (Fig. 2D and E).

The main flavor compounds in DSSP include esters (Table S3). These esters, typically generated through esterification or lipid oxidation, have fruity and sweet aromas (Cano-García, Rivera-Jiménez, Belloch, & Flores, 2014), contributing to the pleasant flavor of DSSP. Notably, the detection thresholds for esters are relatively low. In DSSP, the majority of esters detected were ethyl esters, which may be linked to acid esterification and alcoholic fermentation (Wei et al., 2020). And NaCl promotes bacterial esterification, ultimately leading to the formation of ester compounds (Hu et al., 2020). LAB acyltransferase reactions can be used to break down glycerides to release fatty acids (through hydrolysis) as well as to synthesize ester compounds (through alcoholysis) (Holland et al., 2005). Among the nine groups of DSSP samples, there are 10 common esters, including ethyl 3-phenylpropionate, ethyl linoleate, palmitic acid ethyl ester, ethyl oleate, ethyl 4-methylvalerate, ethyl 9-hexadecenoate, ethyl myristate, ethyl 2-methylbutyrate, ethyl acetate and pentanoic acid ethyl ester. However, their concentrations exhibited significant variations. The highest total content was ethyl 3-phenylpropionate (731.2 μg/g), followed by ethyl linoleate (423.5 μg/g), palmitic acid ethyl ester (285.96 μg/g), ethyl leate (233.56 μg/g), and ethyl 4-methylvalerate (182.06 μg/g). Ethyl 3-phenylpropionate was the key aroma substance and played the key role in the overall aroma of *Huangjiu* (Wang et al., 2020). Ethyl linoleate may be produced by garlic added to DSSP (Park et al., 2014), which has many physiological functions, such as enhancing immunity, reducing cholesterol and blood lipid levels (Koo et al., 2014).

Analysis of Table S3 revealed that the concentration of alcohols and acids trailed only behind esters. The formation of alcohols is likely due to the reduction of aldehydes, whereas esters are produced through microbial esterification of acids and alcohols (Wang et al., 2024; Wang, Zeng, Qiu, Han, & Wang, 2024). The presence of acids can provide the basis for the subsequent formation of flavor substances such as esters and aldehydes, which is an important contribution to the formation of DSSP. The production of organic acids established a low-pH acidic milieu, effectively enhancing the inhibition of various spoilage microorganisms (Wang, Chen, et al., 2024). Alcohols played a crucial role in enhancing the flavor and aroma, phenylethyl alcohol, DL-2-octanol and 1-pentanol have distinctive medicinal, wine-like, flowery, rose, honey and sweet (Zhang et al., 2020), which provide a unique flavor profile of DSSP.

Alkanes, amines, aldehydes, ketones, and sulfides were identified in the 9 kinds of DSSP. Alkanes have a high threshold level and are commonly found in the volatile components of crustaceans, fish and other aquatic products, which contributed less to the flavor of DSSP. Trimethylamine was present only in XS6, with a concentration of 0.09 μg/g. The fishy and ammoniacal odors associated with aquatic products such as fish and shrimp are related to trimethylamine (Jaffrès et al., 2011; Liu, Liu, He, Song, & Chen, 2015). Benzaldehyde is a product of the Strecker reaction of the amino acid and presents a pleasant nutty flavor (Fan et al., 2017), and benzaldehyde was detected only in XS4 and XS5. Ketones exhibit a significantly higher threshold, often exhibiting creamy, delicate, and fruity aromas (Rossana, Mitsuya, & Yutaka, 1996), which may have a certain enhancement of the flavor of DSSP. However, among all samples, XS1 had the highest ketone content of 3.43 μg/g.

3.3.3. PCA and PLS-DA analysis of volatile components

PCA and PLS-DA were used to analysis the differences in volatile components in DSSP from different sources. The PCA plot (Fig. 3A) showed that XS1 was significantly different from the other groups of samples in terms of distance. Additionally, an in-depth assessment of volatile flavor components was conducted using partial least squares discriminant analysis (PLS-DA), with the findings presented in Figs. 3B-3D. PLS-DA scatterplot showed distinct differences among the DSSP samples (Fig. 3B). Additionally, the PLS-DA model's reliability was affirmed through a 200-permutation test validation, resulting in intercept values of 0.384 and − 0.688 for R2 and Q2, respectively (Fig. 3C). To further investigate the flavor differences among the DSSP, significant variables were identified through the variable projected importance (VIP), and volatile compounds with VIP > 1 and $P < 0.05$ were defined as key characteristic volatile compounds of the DSSP. Consequently, 19 significant volatile compounds were identified (Fig. 3D). The 19 key flavor compounds included 2-nitroethanol (A3), acetic acid (A9), ethyl butyrate (A31), ethyl 2-methylbutyrate (A35), pentanoic acid ethyl ester (A41), ethyl 4-methylvalerate (A11), phenol (A47), ethyl phenylacetate (A70), 3-phenylpropionic acid methyl esterer (A73), cis-anethol (A74), indole (A76), ethyl 3-phenylpropionate (A78), ethyl caprate (A80), ethyl myristate (A98), methyl palmitate (A102), ethyl 9-hexadecenoate (A103), palmitic acid ethyl ester (A104), ethyl linoleate (A114), ethyl oleate (A115). Indole, a volatile compound, is associated with a pleasant aroma at lower concentrations but transforms into a strong and persistent fecal odor at higher concentrations, significantly contributing to the unique smelling and tasting characteristics of *Chouguiyu* (Yang et al., 2020; Yang, Li, et al., 2020).

In order to normalize the values, the data on volatile compounds ($\log_{10(x)}$) were subjected to logarithmic and unit-variance scaling and a hierarchical clustering heat map analysis was carried out to visualize the content of volatile compounds in the samples (Fig. 3E). Hierarchical clustering analysis showed that the 19 volatile compounds with VIP > 1 could be clustered into three groups of 9 groups of DSSP, while XS2, XS4 and XS5 clustered together, indicating that they contain similar key flavor substances.

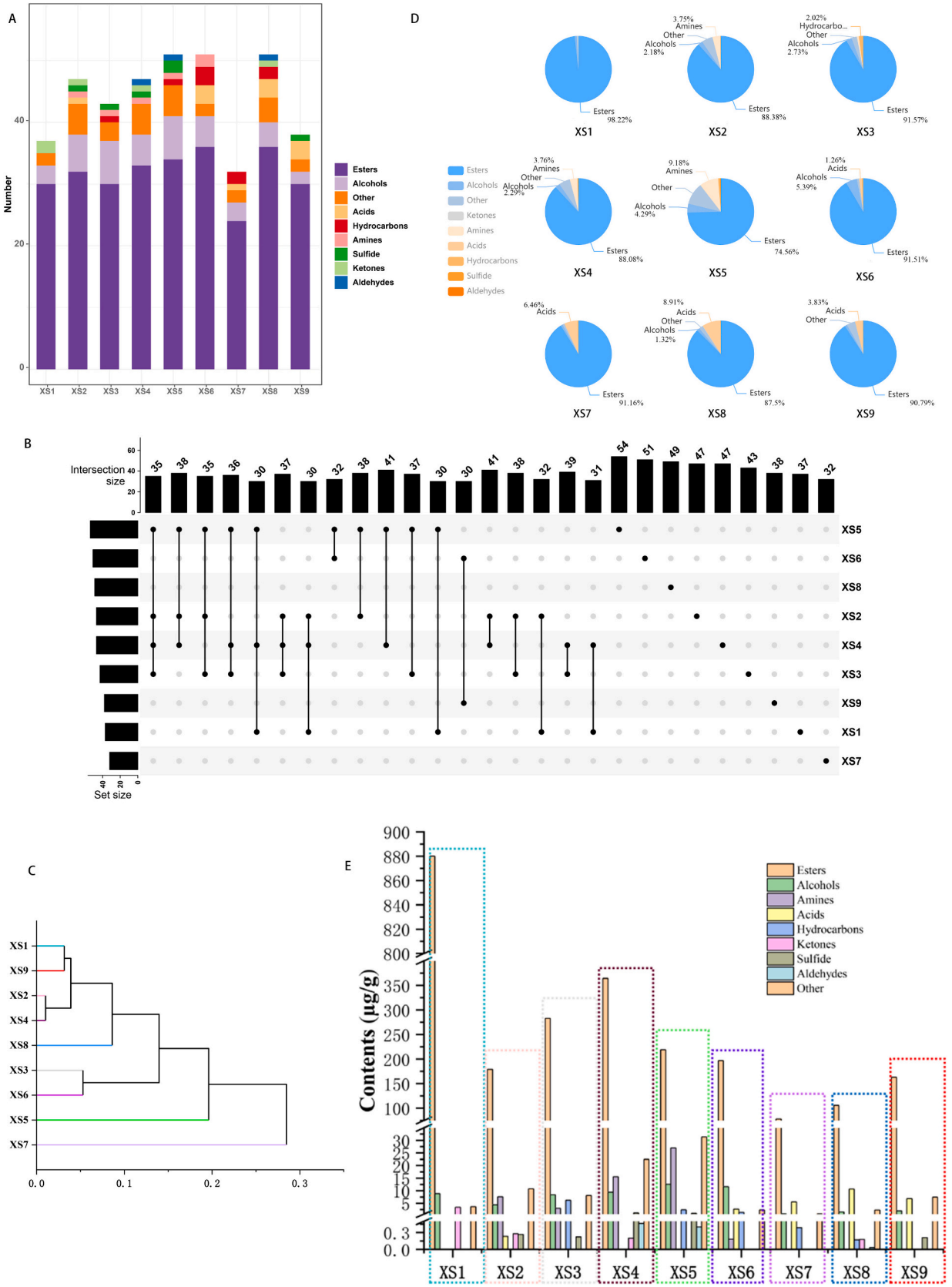

Fig. 2. Identification analysis of volatile substances. Number of volatile compounds (A); Upset Venn diagram (B); HCA analysis (C); proportion of each group of volatile compounds (D); concentrations of each group of volatile compounds (E).

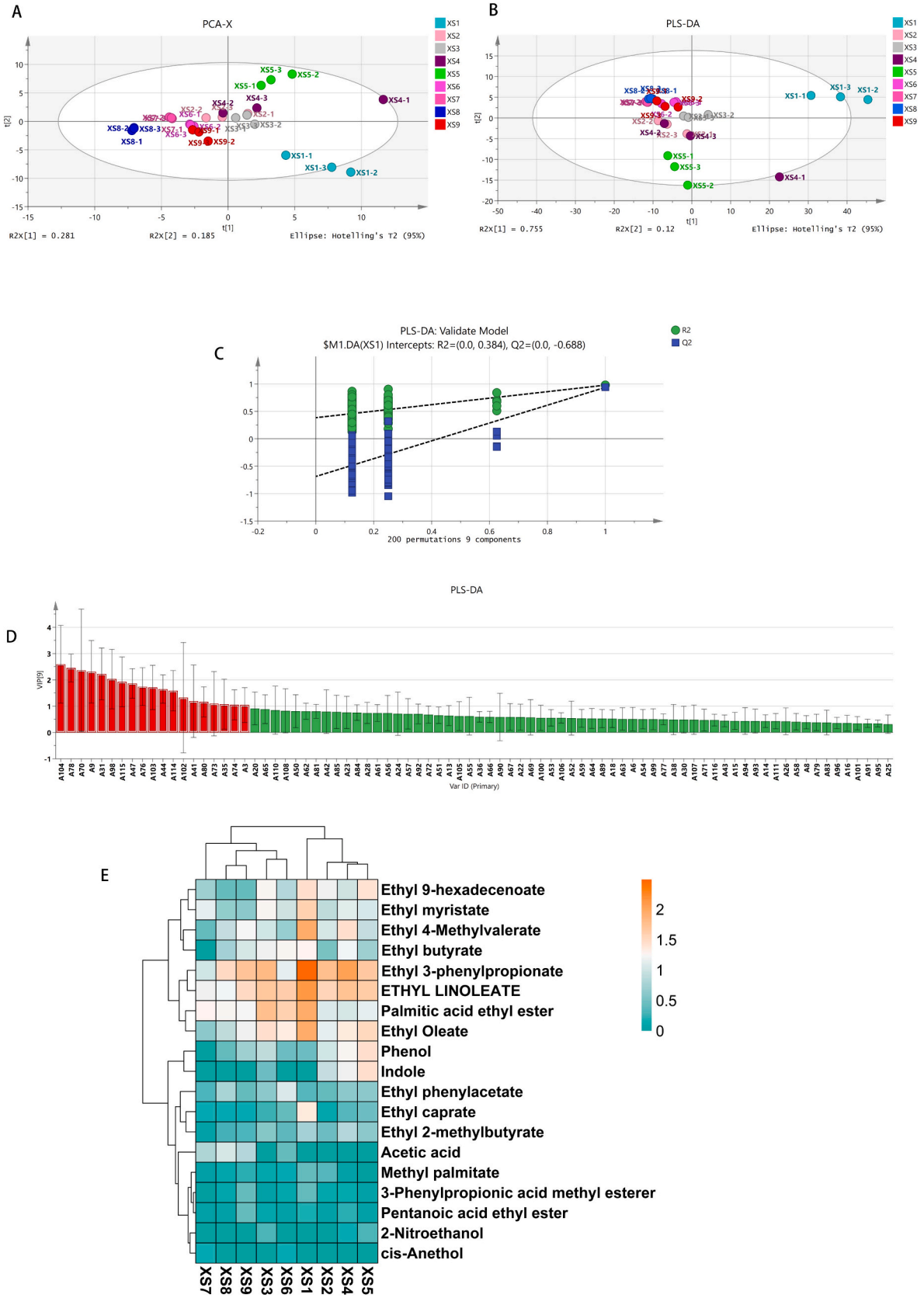

Fig. 3. PCA analysis based on the data obtained by HS-SPME-GC–MS (A); PLS-DA clustering analysis (B); performance of permutation test (C); VIP scores of volatile flavor substances (D); heatmap of the content of volatile compounds (VIP > 1) (E).

3.4. The Illumina HiSeq sequencing discovered the bacterial diversity of DSSP

The metabolic activity of fermenting microorganisms governs the flavor characteristics and safety of fermented foods. Analyzing bacterial diversity serves as an effective approach to evaluate the composition of microbial communities in fermented aquatic products. The alpha-diversity indices mainly include ACE, Chao1, Shannon, Simpson and Goods-coverage indices of bacterial communities in DSSP samples, which were employed to assess species richness, are displayed in Fig. 4A-4E. A greater Shannon index corresponds to a reduced Simpson index, signifying heightened richness and even distribution within the bacterial community of the samples. The Shannon diversity index of XS4 was the highest, indicating that the bacterial community of XS4 exhibited the greatest level of diversity. Flower plot analysis is a method used to display the common and particular OTUs in different groups. As shown in Fig. 1F, 50 OTUs were identical across all samples. The maximum number of special OTUs (1541) was found in XS4.

Cluster analysis showed that XS2, XS3, and XS4 clustered together, indicating a comparable bacterial community structure across these three sample groups (Fig. 4G), with while the rest of the samples clustered into another group (except XS1). The composition and structure of the bacterial community (including uncultivable and unstudied) in spontaneous fermentation of DSSP samples, classified at the phylum and genus levels. Based on the species annotation, *Firmicutes* (average abundance: 60.19%), *Proteobacteria* (average abundance: 33.62%) were the dominant bacteria in all samples at the phylum level (Fig. 4H). That result was consistent with shrimp paste by Yao et al. (Yao et al., 2021) and fermented fish reported by Sun et al. (Sun et al., 2022). The correlation between *Firmicutes* and carbohydrate metabolism, as well as the association of *Proteobacteria* with amino acid and lipid metabolisms, has been established in previous studies (Gao et al., 2023).

Lactic acid bacteria (LAB) consisted of many genus including *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Pongsak & Parichat, 2010). At the genus level (Fig. 4I), a total of 28 bacterial genera with the relative abundance over 1% were observed. Genera with a relative abundance greater include *Lactobacillus*, *Pseudomonas*, *Vagococcus*, *Erwinia*, *Tepidimicrobium*, *Peptoniphilus*, *Enterobacter*, *Tetragenococcus*, and unclassified. It was observed that relative abundance of *Erwinia* (23.35%), *Tepidimicrobium* (19.98%) and *Tetragenococcus* (9.01%) were the main bacteria in XS1. *Lactobacillus* showed the highest relative abundance in XS8 (64.57%) and XS9 (58.83%). That may be attributed to the addition of more fermented glutinous rice. Qan et al. study found that *Lactobacillus* and *Enterobacter* were the dominant bacterial of rice wine (Qian et al., 2023). *Lactobacillus* could generate a variety of antimicrobial metabolites, which was associated with amino acid metabolism during fermentation (Han et al., 2023). Furthermore, *Pseudomonas* emerged as the first dominant bacteria in XS5 (50.31%), XS6 (36.91%), and XS7 (45.28%). The relative abundance of *Vagococcus* in samples XS2, XS3, and XS4 are 22.26%, 32.58%, and 19.65%, respectively. The *Vagococcus* can produce energy necessary for fermentation, thereby promoting the process (Wang, Xiang, Zhang, Hou, & Guo, 2021).

Calculate β diversity using Bray-curtis distance to study the similarity between different DSSP samples. This study conducted a microbial diversity analysis of XS based on the Bray-Curtis distance, and the results are depicted in Fig. 4J. Based on PCoA1 (30.2%) and PCoA2 (17.7%), the DSSP samples were categorized into two distinct groups. XS2, XS3, and XS4 were clustered into a single group, indicating their similarity in characteristics. The remaining samples formed a separate group, suggesting differences from XS2, XS3, and XS4. The NMDS analysis of the bacterial community structure of the two groups based on the Bray-Curtis distance was shown in Fig. 4K and that were also consistent with the heatmap clustering analysis (Fig. 4G). DSSP samples from different groups showed a little overlapping regions in space, indicating differences in the bacterial community structure.

To achieve a comprehensive understanding of the DSSP ecosystem, the LEfSe analysis was employed to investigate the differences among bacterial groups and determine the characteristic microorganisms associated with each group (Fig. 4L and M) (Wang, Zeng, et al., 2024). After LEfSe analysis was indicated that there were 31 biomarkers with LDA scores >4. These 31 biomarkers consist of three classes, seven orders, seven families, and 14 genera. Additionally, microbial biomarkers at the genus level were identified: *Peptoniphilus* in XS6, *Peptostreptococcus* and *Enterobacter* in XS2; *Vagococcus* in XS3; *Tetragenococcus*, *Tepidimicrobium*, and *Erwinia* in XS1.*Tetragenococcus* is a common member of the microbial consortia food fermented under high salt conditions (Link & Ehrmann, 2023). *Tetragenococcus* may improve flavoring, taste characteristics, and safety of shrimp sauces (Gao et al., 2023). Furthermore, the addition of *Tetragenococcus muriaticus* can improve the microbial community of low-salt fish sauce and enhance the variety and concentration of its volatile flavor compounds (Chunsheng et al., 2022). In Fig. 4Land 4M, the samples XS5, XS7, and XS8 were not included, which suggested that these three groups have no significant difference in relative abundance when compared to the other groups.

3.5. Correlation analysis between microbiota and physicochemical indicators and volatile compounds

The microbial communities' composition in fermented foods is influenced by various factors, including chemical indicators, nutrients, and microbial metabolites. The effect of physicochemical properties on the top 20 bacterial (Fig. 5A) genera was revealed by redundancy analysis (RDA). The results showed that RDA1 and RDA2 accounted for 26.99% and 22.08% of the total variation, respectively. The variables pH ($R^2 = 0.56$, $P = 0.0015$), AAN ($R^2 = 0.058$, $P = 0.0005$), TA ($R^2 = 0.70$, $P = 0.0005$), and Moisture ($R^2 = 0.72$, $P = 0.0005$) exhibited significant correlations with the DSSP within the bacterial communities. The dominant strain *Lactobacillus* was negatively correlated with pH and AAN but positively correlated with water content and TA. While *Vagococcus*, *Erwinia*, *Tepidimicrobium*, *Peptoniphilus*, *Enterobacter* and *Tetragenococcus* were positively correlated with pH and AAN, and negatively correlated with TA and moisture. The distribution of the bacterial community. Li et al. found that in low-salt fermented shrimp paste, *Tetragenococcus* was positively associated with AAN and TVB-N (Wenya, Si, Xiaochang, Yaxin, & Jilu, 2022).

Microbial interactions play a crucial role in maintaining the stable coexistence of microbial communities and have an important impact on the quality characteristics of fermented foods (Yang et al., 2022). Based on Spearman's rank correlation, the interactions between the first 20 genera of bacteria (Fig. 5B) were explored separately to determine the co-occurrence patterns of bacterial communities in DSSP. In the positive correlation network of bacterial communities (red), there were 20 valid connectivity sites, and strong connectivity sites (≥6 edges) were mainly distributed *Firmicutes* and *Proteobacteria*, including *Vagococcus*, *Lactococcus*, *Peptoniphilus*, *Peptostreptococcus*, *Tepidimicrobium* and *Enterobacter*. In addition, a significant positive correlation was observed between *Vagococcus* and *Tepidimicrobium*, *Peptoniphilus*, *Enterobacter* and *Peptostreptococcus*. The LAB including *Vagococcus*, *Peptostreptococcus*, as well as *Lactococcus* played an important role in the hydrolysis preparation of these umami peptides and in the formation mechanism of *Chouguiyu* (Yang et al., 2022).

The Spearman correlation analysis also revealed a correlation between the top 20 genus and 19 key flavor compounds (VIP > 1 and $p < 0.05$) (Fig. 5C). *Peptoniphilus, Vagococcus* and *Tepidimicrobium* displayed a significant positive correlation with indole, ethyl 3-phenylpropionate, ethyl linoleate, ethyl myristate and ethyl 9-hexadecenoate. The main flavoring substances in stinky tofu brine are indole, p-cresol, phenol and 3-methylindole (Tang et al., 2022). Ethyl oleate displayed a significant positive correlation with *Vagococcus, Lactococcus, Tepidimicrobium, Erwinia, Enterobacter* and *Tetragenococcus.* The LAB strains undergo esterification reactions through an alcoholysis mechanism, primarily

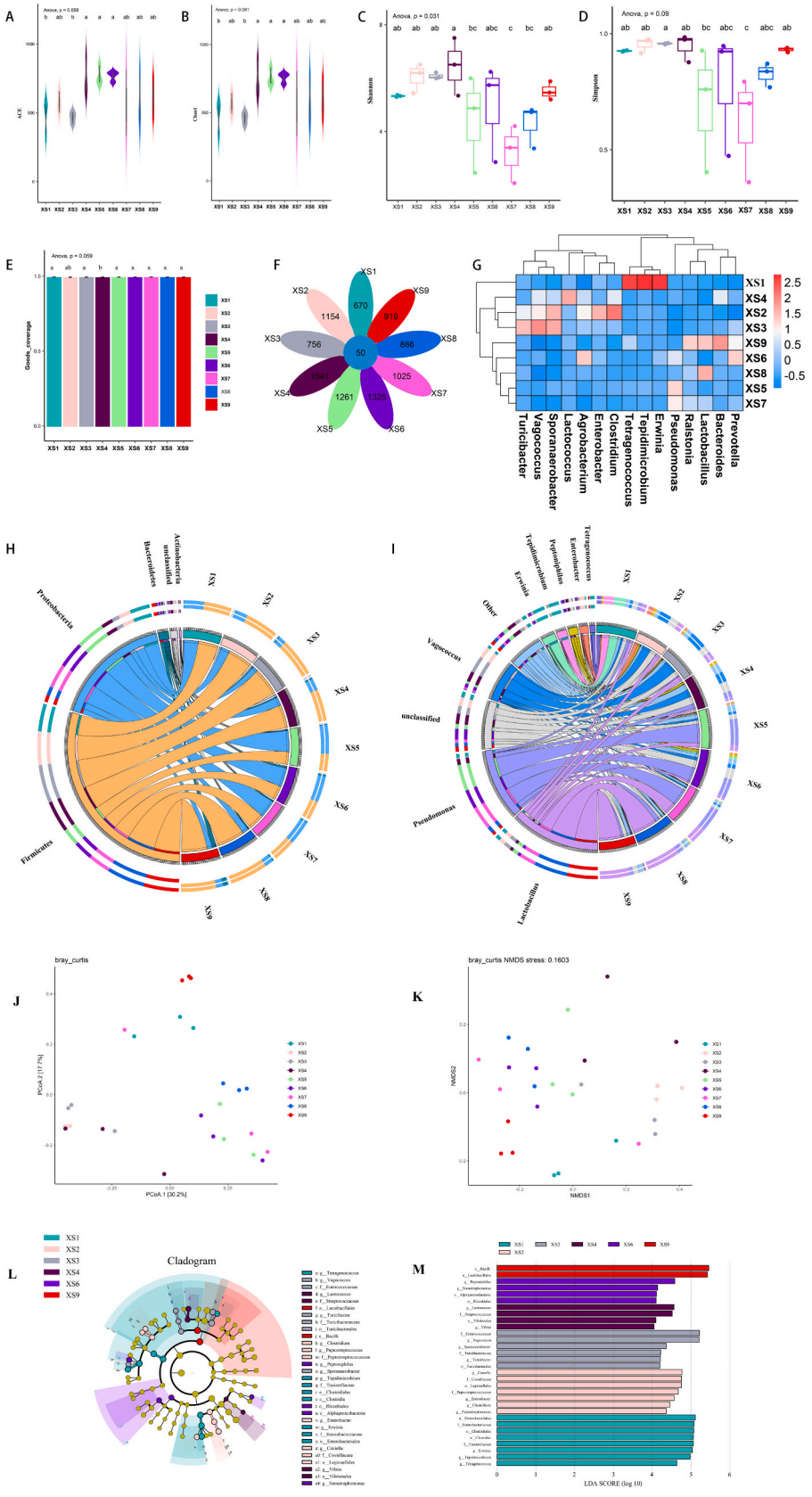

Fig. 4. The α-diversity index (A-E), petal plot (F), the heatmap of the relative abundance of top 15 genera at the genus level (G); the relative abundances of microbiota at phylum (H) and genus (I) level; Principal coordinate (J) and Non-metric multidimensional scaling (NMDS) plots (K) based on Bray-Curtis distance; LefSe multilevel species hierarchical tree diagram (L) and LDA value distribution histogram (M) (LDA > 4 was considered to be the differential characteristic taxon).

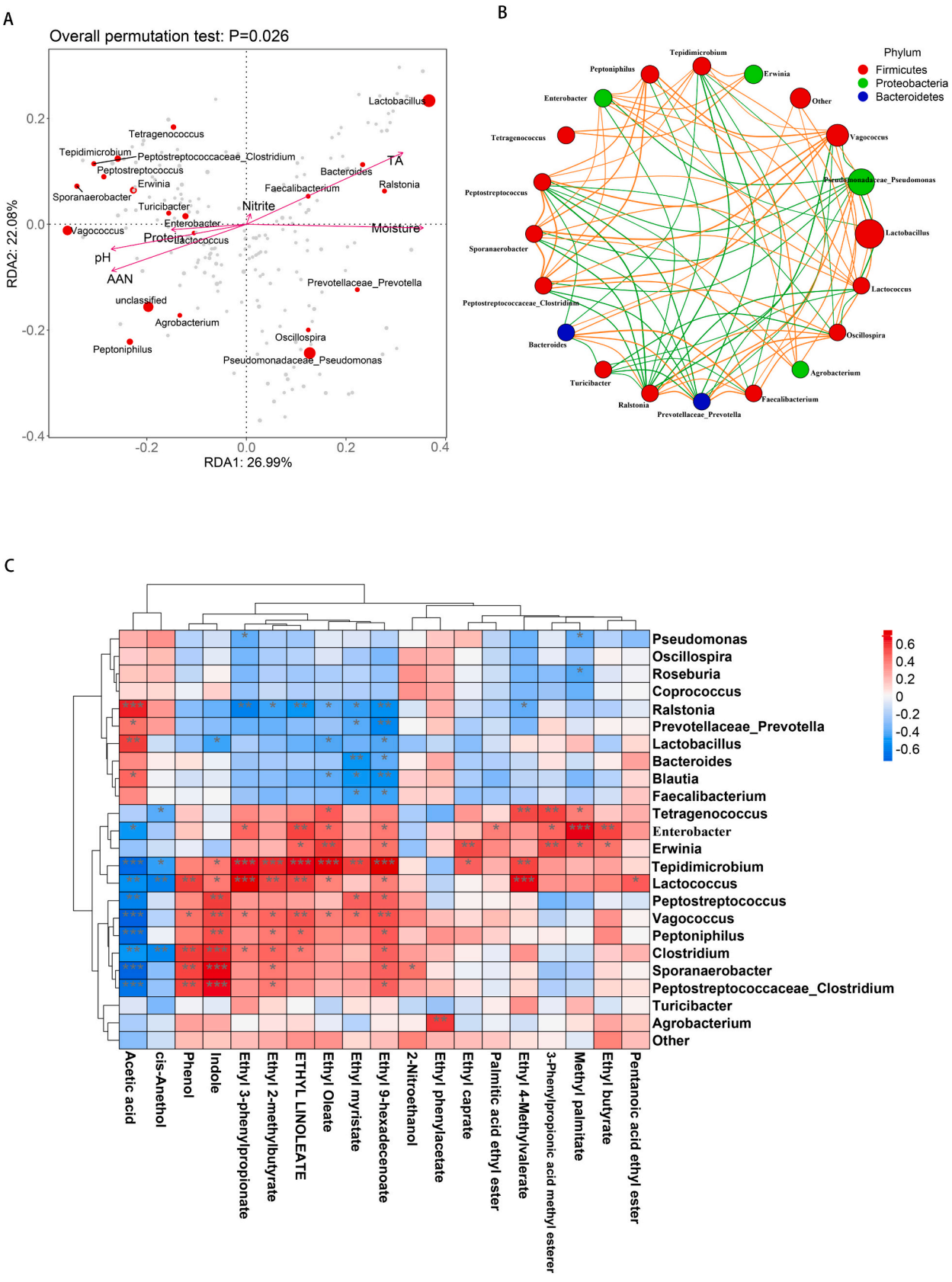

Fig. 5. Redundancy analysis was performed on important species and environmental factors (A); genus correlation network based on Spearman's rank correlation analysis (B); Spearman's correlation heatmaps between dominant bacterial communities and volatile flavor compounds (C). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

generating ethyl butyrate and ethyl hexanoate in milk products (Abeijón Mukdsi, Medina, Alvarez, & González, 2009), while mainly producing ethyl 2-hydroxy-3-methylbutanoate and ethyl 2-hydroxy-4-methylpentanoate in wine (Gammacurta et al., 2018). Phenol had positive correction with *Vagococcus* and *Lactococcus*, but indole had a negative correlation with *Lactobacillus*. LAB (*Vagococcus* and *Lactococcus*) have been identified as contributors to inhibiting TVB-N, leading to a moderate fermentation and good quality of *Chouguiyu* (Yang et al., 2021). The screening criteria of key functional bacteria in the DSSP was based on the fact that they maintain a high relative abundance in fermented foods and were highly correlated with important influence on the characteristic flavor. Therefore, *Vagococcus, Lactococcus*, and *Tepidimicrobium* were identified as key bacteria responsible for flavor compound production, thereby influencing the overall sensory experience of DSSP. Furthermore, these bacteria can be used as starter culture in DSSP production to enhance product safety and improve flavor.

4. Conclusion

In this study, we identified 19 key flavor compounds in DSSP, including 13 esters, 1 alcohol, 1 acid, 2 amines and 2 others. At the phylum level, *Firmicutes* and *Proteobacteria* were the dominant bacteria. Additionally, *Lactococcus, Vagococcus* and *Tepidimicrobium* at the genus level were identified as core functional bacteria, which were involved in the formation of key flavor compounds of DSSP. This study aims to our understanding of the relationship between bacterial communities and the flavor formation, and provide guidance for starter culture screening in the industrial production of DSSP. Future research will utilize peptidomics and metagenomics to delve deeper into the key proteases in DSSP, while analyzing the flavor intensity of DSSP using electronic tongue and revealing its flavor mechanism through molecular docking techniques.

CRediT authorship contribution statement

Xiaojuan Song: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Dan Liao:** Validation, Investigation, Data curation. **Yan Zhou:** Writing – review & editing, Formal analysis. **Qun Huang:** Writing – review & editing, Resources, Methodology. **Shicheng Lei:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Xiefei Li:** Software, Methodology, Formal analysis.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101543.

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Further-reading

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