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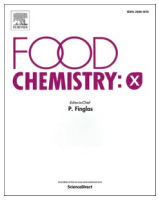

Effect of salt concentration on the quality and microbial community during pickled peppers fermentation

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ABSTRACT

This work aimed to investigate the effect of salt concentration on the quality and microbial community of pickled peppers during fermentation, and the cross-correlation between microorganisms and quality was also revealed. The results showed that 9 volatile flavor compounds were unique to the low salt concentration group (D group), which also contained higher content of FAA, lactic acid and acetic acid than high salt concentration group (G group). Meanwhile, the samples of D2 group have a better texture properties. *Firmicutes*, *Proteobacteria*, *Ascomycota*, *Lactobacillus*, *Pectobacterium*, and *Pseudomonas* were detected as the main microbial community during the fermentation with different salt concentrations. Furthermore, the correlations analysis results indicated that the salt concentration has a significant effect on the microbial community of pickled peppers ($p < 0.001$), and *Pediococcus*, *Lactobacillus*, *Cedecea*, *Issatchenkia*, *Pichia*, *Kazachstania*, and *Hanseniaspora* were significantly correlated with flavors, which played crucial roles in the unique flavor formation of pickled peppers.

1. Introduction

As a popular condiment, pickled peppers are a distinctive peppers product in the traditional fermented products in southwest China (Ye et al., 2020). As with most traditional fermented vegetables, pickled peppers is usually manufactured under spontaneous fermentation conditions based on empirical knowledge (Zhang et al., 2022). Fresh peppers are washed, and dried, and added with salt, water, baijiu, and a small amount of vinegar into pickle jars to undergo spontaneous fermentation at ambient temperature for 30 days. Finally, the pickled peppers with unique flavor are produced under the joint fermentation of various microbiota attached to the surface of the peppers (Xu et al., 2020). Pickled peppers are sour, spicy, and delicious, with a fresh and fragrant flavor, rich in nutrients, and can stimulate appetite. They also have the effects of improving the intestinal environment, promoting digestion, and preventing rheumatoid arthritis (Ye et al., 2022).

The flavor and taste of vegetables can be significantly improved after fermentation. As an important indicator, the composition and content of flavor compounds have a direct effect on the sensory quality of fermented vegetables (Yang et al., 2020a). However, the formation mechanism of fermented vegetable flavor is very complicated. The fermentation system consists of microbial communities, substrate, and environmental conditions. Throughout the entire fermentation process, there are a large number and diverse types of microbial communities involved. There is a wide variety of endophytic bacteria included in the raw material of pickled peppers, which is crucial to its fermentation (Xu et al., 2020). However, due to factors such as fermentation conditions and operating methods, miscellaneous microbiota may grow and produce odorous flavor compounds (Patel, Ruiz, Calderon, Marcelo, & Rojas, 2016). These odors can affect the quality of pickled peppers, even leading to their inedibility and loss of value. Therefore, the microbial community in pickled peppers with different fermented conditions may significantly affect their quality and characteristics during fermentation. High-throughput sequencing has been widely used to characterize the microbial ecosystem profiles of many fermented foods, including wine, vinegar, and soy sauce (Wu et al., 2015). Liu and Tong (2017) investigated the bacterial diversity of 10 Chinese fermented vegetables by using the method of 16S rRNA sequencing, and identified 12 phyla, 223 genera, and 348 species. Among them, *Firmicutes* and *Proteobacteria* were the main dominant bacteria, and *Lactobacillus*, *Bacillus*, *Weisella* and *Pediococcus* were the dominant genera. Mi et al. (2022) revealed the correlations between the microbial community and flavor compounds of

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Paocai, which indicated that several bacterial genera significantly correlated with flavors, including *Lactobacillus*, *Weissella*, *Leuconostoc*, and *Staphylococcus*, while fungi had weak correlations with flavors.

Salt plays an important role in the flavor and sensory quality of fermented vegetables. It can not only regulate the growth and metabolism of microbial communities (Yang et al., 2020b), but also affect the composition of flavor components such as acids, aldehydes, alcohols, and esters (Yang et al., 2020a). At the same time, it can also form a certain osmotic pressure, allowing soluble substances in vegetables, such as sugars, can seep out of vegetable cells for metabolism and utilization by microorganisms such as lactic acid bacteria (LAB) (Liang et al., 2020a). However, low salinity may cause fermented vegetables to become brittle, reduce their inhibitory effect on harmful microbiota, decrease their sensory quality, and even lead to food safety issues (Mi et al., 2022). High salinity can not only inhibit the activity of harmful microbiota, but also inhibit the activity of beneficial microbiota to a certain extent, which may affect the fermentation speed of vegetables, extend the production cycle, and not meet modern people's pursuit of low salt healthy food (Zandstra, Lion, & Newson, 2016). In the previous study, salt concentration significantly affected the fermentation of sauerkraut and radish pickles (Liang et al., 2020b; Ou et al., 2022; Xiong et al., 2016). Seo, Park, and E., Kim, E. J., Lee, K. I., Na, C. S., Son, H. S. (2017) studied the effects of different salinity on volatile flavor compounds in Kimchi, and found that bacterial diversities in Kimchi varied according to salinity. In recent years, many domestic and foreign scholars have shown that long-term excessive intake of salt in human daily diet will lead to the occurrence of hypertension, cardiovascular and cerebrovascular diseases (Xiong et al., 2016). The World Health Organization (2007) recommends that each person should consume no >5 g of salt per day. Since 2018, China has also launched the “Guide to Salt Reduction in China's Food Industry” for the first time, providing technical guidance suggestions, routes, and measures for salt reduction in food processing. In short, salt reduction has become one of the inevitable trends in the future development of the food industry around the world (Zandstra et al., 2016). In addition, the content of salt will also affect the subsequent fermentation of pickled food. Despite extensive research into the role of different influencing factors, including raw materials, fermentation environment, microbial communities, and processing methods, on the flavor and other quality attributes of pickled peppers products (Janiszewska-Turak et al., 2022; Xu et al., 2020; Ye et al., 2022; Zhang et al., 2022), the correlation among the salt concerntration, microbiota and quality in contributing to the fermentation process and final quality are not investigated.

Here, we hypothesize that salt concentration can influence the diversity of fermenting microorganisms, which in turn affects the quality of pickled peppers. This study aimed to examine the influence of salt concentration on the physicochemical properties, flavor compounds, and microbial community during the spontaneous fermentation of pickled pepper, which were run in parallel, and also to reveal the influence mechanism of microorganisms on the quality of pickled pepper by investigating the correlation between microbial community and physicochemical properties, flavor compounds in each case. We hope our research findings will provide the basis for the process optimization, quality assurance, and flavor control of pickled peppers.

2. Materials and methods

2.1. Preparation and collection of pickled peppers

Fresh *meiren* peppers with long finger type, thick round, bright red were originated in Yunnan Province, China. The 15 L pickle jars were rinsed with 100 °C water and dried under UV irradiation. The marketing standard pickled peppers of Guizhou Zunyi Xinjiayu Food Co. LTD were used as a control sample (M1 group). Peppers were washed, capped, cut into 3–4 cm small sections, and placed into pickle jars. Then, the brine with a salt content of 24% (high salt concentration group, G group) and 9% (low salt concentration group, D group) was prepared and mixed with peppers in a ratio of 6:4. Finally, the jars were water-sealed tightly and incubated at 18–25 °C. The pickled peppers or brine were sampled aseptically in the middle of each jar at four selected time points on days 7, 30, 60, and 90 of fermentation. In each group, three individual jars were analyzed, which formed three replicates.

2.2. Determination of physicochemical properties

The pH of the pickled peppers samples was obtained using a pH meter (PHS-3E, INESA China). The titratable acidity content (TTA) of pickled peppers samples was measured by acid-base titration, and N-(naphthyl)-ethylenediamine dihydrochloride was used to determine the nitrite content according to Du, Wu, Sun, and Yue (2013). The soluble protein (SP) content was determined by the Coomassie Brilliant Blue G250 method, and the chloride content was determined according to the GB5009.44–2016 method.

Textures of pickled pepper samples were measured by TMS-Pro texture analyzer (FTC, USA) according to the study (Ye et al., 2020). Each sample was carefully cut into small squares of 0.5 cm × 0.5 cm. The test mode of T.P.A. with the cylinder probe of P 0.5 was chosen for testing. The pre-test, test, and post-test speed were set at 2.0 mm/s, penetration distance was set at 40 mm, surface trigger force was set at 1.5 N, and the time between two compressions of 5.0 s was set for texture changes. Each sample was tested 20 times.

2.3. Determination of flavor compounds

2.3.1. Determination of organic acid content

The determination of organic acid content were carried out according to the method described by Zhang et al. (2022) with slight modifications. Briefly, 10 g sample was placed in a 50 mL centrifuge tube, and 20 mL deionization water were added. After extracted at 15000 r/min for 2 min and centrifuged at 4000 r/min for 5 min, the supernatant was filtered through a 0.45 μm microporous membrane and injected into an HPLC system (1260, Agilent, USA) equipped with a chromatographic column of Agilent C18 AQ (4.6 mm × 250 mm, 5 μm). The column temperature was set at 35 °C, the content of sample intaking was 10 μL, the flow rate was 0.5 mL/min, the mobile phase was 10 mM K_2HPO_4 (pH = 2.55) and the detection wavelength of ultraviolet detector was 210 nm. Organic acid standards were used as an external standard for the identification and quantification of organic acids including malic, lactic, acetic, citric, and fumaric acids. Each sample was extracted and analyzed in triplicate.

2.3.2. Determination of free amino acids (FAA) content

The determination of free amino acids content refers to the paper using High-Performance Liquid Chromatography (HPLC, 1260, Agilent, USA) (Qiu, Reynolds, Johanningsmeier, & Truong, 2020). The samples were dissolved with 0.02 mol/L HCl and made up to volume in a 10 mL volumetric bottle. Then, after 20 min of ultrasonic treatment, the solution was centrifuged at 6000 r/min for 5 min. Finally, the supernatant was removed and purified for HPLC analysis.

2.3.3. Determination of taste substances

Taste quality analysis of pickled peppers samples using an electronic tongue system (TS-5000Z, INSENT, Japan) equipped with six chemical sensors, including AAE (umami), AE1 (astringency), C00 (bitterness), CA0 (sourness), CT0 (saltiness), and GL1 (sweetness), was executed according to Sun et al. (2023). Potassium chloride in 30 mM and tartaric acid was chosen as a reference electrode. Pickled pepper samples in 4 mL mixing with 36 mL of deionization water were added to each sample cup and then infused twice for testing, and each infusion was measured three times. The sensor collects one data per second for 120 s, and the response value of the 120th second of each sensor was set for analysis when the sensor signal has been in a stabilized state.

2.3.4. Determination of volatile flavor compound by GC–MS

Determination of volatile flavor compounds in the fermentation process of pickled peppers samples with different salt concentrations was evaluated using GC–MS (6890 N-G5795B, Agilent, USA) according to the papers with some modifications (Yao et al., 2015). The volatile flavor compounds of samples was separated from the fiber coating, and was introduced in a splitless mode to the injector of GC–MS equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 μm) for thermal desorption at 250 °C for 5 min. The identification and quantification of volatile flavor compounds were analyzed using Helium as the carrier gas at the rate of 1.0 mL/min. The injector and detector temperature were set at 250 °C and 280 °C, respectively. The program of oven temperature was set as follows: at first, the parameter using 50 °C for 2 min, using the increasing rate of 5 °C /min until 180 °C, and then maintaining 5 min. Under ionization mode at 70 eV, MS was recorded in electron impact (EI). The quadrupole mass detector was maintained at 150 °C, the ion source was set at 230 °C and the transfer line temperature was chosen at 280 °C. The sweep range of MS was in 40–600 amu every second. Acetophenone in 1 mg/mL was chosen as an internal standard compound. The volatile flavor compounds were identified via comparing the MS with the MWGC data system library and linear retention index.

2.3.5. Determination of volatile flavor compound by electronic nose

The determination of volatile flavor compounds via PEN3 electronic nose (Win Muster Airsense Analytics Inc., Schwerin, Germany) including a sensor array unit and pattern recognition software was also performed according to Yao et al. (2015). Different volatile flavor compounds correspond to different metal oxide semiconductors, including W1C, W1S, W1W, W2S, W2W, W3C, W3S, W5C, W5S, and W6S, and the correspondence is shown in Table S1. Samples (3.0 g) were put in a 20 mL airtight vial and incubated for 30 min at 60 °C. A hollow needle with tubing was used to pierce the seal of the vial and absorb the volatile gases from the headspace at a constant rate, and the measurement time was 120 s.

2.4. Microbial DNA extraction and sequencing

Genomic DNA of pickled peppers samples was extracted according to Yang, Fan, et al. (2022) using Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA), and the extraction method was carried out by following the experimental operating guidelines provided by the manufacturer. Agarose gel electrophoresis in 1% and densitometry were used for appraising the quality and integrity of genomic DNA, respectively. After having been qualified, database construction and sequencing were carried out.

The V3-V4 region of 16S rRNA genes in bacteria was amplified with primer 338F/806R. Fungi genes of the internal transcribed spacer (ITS) region were amplified by the primers ITS1F and ITS2R. The PCR programs were performed as previously described (Kong et al., 2022). After being eluted by Tris-HCl and detected via electrophoresis on a 1% agarose gel, amplicons were quantified using QuantiFluor™-ST (Promega, USA) and applied to construct library PE 2 × 300 according to the standard of Illumina MiSeq (Illumina, San Diego, USA). The gene amplicons were sequenced by Illumina Miseq PE300 (Majorbio Biopharm Technology Co., Ltd., Shanghai, China). Diversity of bacterial and fungus accompanying correlation analysis were obtained on the free online platform of Majorbio Cloud Platform (www.majorbio.com). After having been merged by FLASH and quality filtered, the sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold by UPARSE, and the UCHIME was employed to identify chimeras.

2.5. Statistical analysis

Each test was performed in triplicate, and all the results values were displayed as "mean±standard deviation (SD)" of "n" replicates. The data were analyzed using the method of "Analysis of variance (ANOVA)" via the Statistical Package for Social Sciences (SPSS 18.0, SPSS Inc., Chicago, IL, USA). $p < 0.05$ were considered as statistically significant.

3. Results and discussion

3.1. Physicochemical properties of pickled peppers with different salt concentrations

3.1.1. pH and TTA

The pH and TTA are the most important indicators for measuring the fermentation process of pickled peppers (Liu et al., 2020). The changes in pH and TTA content of spontaneously fermented pickled peppers with different salt concentrations are presented in Table 1. The change in pH is mainly related to the metabolism of LAB and other microorganisms, thus affecting the fermentation process of pickled peppers (Liu et al., 2020). As is shown, during the fermentation process, the pH of the D group ranged from 4.27 to 4.39, whereas that of the G group was from 4.11 to 4.20. When fermented for 30 d, the pH of the two groups was the closest to that of the M1 group, and the pH of the G group was significantly better than that of the D group ($p < 0.05$). Furthermore, under the same fermentation time, the pH of the G group was lower than that of the D group, which may be because the low salt concentration has an inhibitory effect on the acid-producing metabolism, and microorganisms in the fermentation system metabolize and decompose sugars to produce acids, leading to a decrease in pH. A lower pH is beneficial for controlling the growth of harmful microorganisms and the generation of odors in the fermentation product, thereby extending the shelf life of the product (Yang, Fan, et al., 2022).

During the fermentation of pickled peppers, the TTA content of D and G groups increased gradually, which due to the undissociated organic acids accumulated, and there was no significant difference in the fermentation stage above 30 d ($p > 0.05$, Table 1). The TTA content of the D group reached a maximum of 2.58 g/kg at 90 d, while that of the G group reached a maximum of 3.54 g/kg at 60 d. The TTA content of the two groups was quite different from that in the M1 group, which may be due to the growth of LAB being affected, resulting in the different acid production abilities of fermented pickled peppers with different salt concentrations (Hong, Zeng, Li, Yang, & Jiang, 2016). The TTA content of the pickled peppers was significantly different between the two groups throughout the fermentation process ($p < 0.05$). Furthermore, the TTA content in the G group increased much more slowly than those of the D group, implying that the fermentation rate was closely related to salinity, which was in accordance with the research reported by Mi et al. (2022).

3.1.2. Nitrite

Nitrite, as a precursor of a known carcinogen nitrosamine, is considered an important parameter to evaluate the edible safety of fermented foods. Previous studies have shown that both *Escherichia coli* and Marine fungi can produce nitrate reductase (Bartholomew, 1984), which could catalyze NAD(*P*)H reduction of nitrate (NO_3—) to nitrite (NO_2—). As can be seen from Table 1, during the fermentation period, the nitrite content in pickled peppers with different salt concentrations showed a similar pattern of first accumulating to peak values and then decreasing, and they all peaked at 30 d of fermentation (1.40 mg/kg for D group and 1.14 mg/kg for G group, respectively). These results may be due to the growth and propagation of a large number of miscellaneous microbiota in a relatively short fermentation time, so that the content of nitrite rose to the peak, but it was also far lower than the maximum limit (20 mg/kg) stipulated by National Standard for Food Contaminant in Food Safety (GB 2762-2022). The reason for the "nitroso peak" in the early stage of the fermentation may be that the number of LAB is low in fresh vegetables, which is still in a stage of proliferation and has not formed an acidic environment, so various harmful bacteria will multiply rapidly, resulting in an increase in nitrite content (Du et al., 2013). In the late

Table 1
Physicochemical properties of pickled pepper with different salt concentrations during fermentation.

Group	pH	Titratable acidity (g/kg)	Nitrite(mg/kg)	Soluble protein (mg/g)	Chloride(g/100 g)	Texture properties			
						Firmness (N)	Elasticity (mm)	Viscosity (N)	Chewiness (mJ)
M1	4.21 ± 0.055^{D}	4.50 ± 0.18^{A}	0.29 ± 0.15^{C}	0.77 ± 0.081^{ABC}	4.51 ± 0.04^{C}	47.00 ± 5.94^{AB}	0.41 ± 0.01^{ABC}	10.88 ± 3.04^{BC}	4.44 ± 1.24^{AB}
D1	4.28 ± 0.015^{Cc}	2.04 ± 0.10^{Fb}	0.51 ± 0.11^{Bb}	0.59 ± 0.06^{Eb}	2.82 ± 0.04^{D}	38.30 ± 2.88^{CDa}	0.38 ± 0.03^{BCb}	10.66 ± 1.34^{BCDa}	4.06 ± 0.63^{ABa}
D2	4.27 ± 0.026^{Cc}	2.52 ± 0.00^{Ea}	1.40 ± 0.20^{Aa}	0.76 ± 0.11^{ABCab}	2.89 ± 0.11^{D}	25.69 ± 2.98^{EFc}	0.49 ± 0.06^{Aa}	9.65 ± 1.11^{CDEa}	4.76 ± 1.11^{ABa}
D3	4.32 ± 0.011^{Bb}	2.52 ± 0.00^{Ea}	0.47 ± 0.16^{Cb}	0.82 ± 0.063^{ABa}	2.89 ± 0.04^{D}	33.07 ± 4.67^{DEab}	0.24 ± 0.04^{Dc}	5.60 ± 1.47^{Fb}	1.36 ± 0.51^{Db}
D4	4.39 ± 0.011^{Aa}	2.58 ± 0.10^{Ea}	0.51 ± 0.04^{Cb}	0.60 ± 0.12^{DEb}	2.94 ± 0.04^{D}	28.04 ± 4.07^{EFbc}	0.25 ± 0.06^{Dc}	6.74 ± 1.10^{EFb}	1.73 ± 0.68^{CDb}
G1	4.11 ± 0.01^{Gd}	3.12 ± 0.21^{Cb}	0.38 ± 0.07^{Cb}	0.58 ± 0.027^{Ec}	7.52 ± 0.16^{Bb}	45.56 ± 1.57^{ABCab}	0.43 ± 0.03^{AB}	12.63 ± 1.72^{ABCa}	5.45 ± 0.91^{Aa}
G2	4.20 ± 0.00^{Da}	3.42 ± 0.18^{Bab}	1.14 ± 0.07^{Ca}	0.74 ± 0.033^{BCDb}	7.40 ± 0.14^{Bb}	23.4 ± 4.27^{Fc}	0.43 ± 0.08^{AB}	7.47 ± 1.12^{DEFb}	3.24 ± 0.89^{BCb}
G3	4.17 ± 0.00^{Eb}	3.54 ± 0.21^{Ba}	0.45 ± 0.12^{Cb}	0.89 ± 0.11^{Aa}	8.14 ± 0.04^{Aa}	48.56 ± 5.43^{Aa}	0.36 ± 0.03^{BC}	14.44 ± 2.15^{Aa}	5.18 ± 1.05^{Aa}
G4	4.14 ± 0.02^{Fc}	3.48 ± 0.10^{Ba}	0.48 ± 0.04^{Cb}	0.63 ± 0.031^{CDEbc}	8.04 ± 0.07^{Aa}	40.55 ± 3.64^{BCb}	0.34 ± 0.02^{C}	13.31 ± 1.96^{ABa}	4.57 ± 0.85^{ABab}

Note: the above values are expressed as means ± SD obtained across triplicate measurements. Lowercase letters indicate significant difference ($p < 0.05$) of samples within the group; Capital letters indicate significant difference ($p < 0.05$) of samples among groups. M1 represents the marketing standard pickled peppers of company; D1 represents pickled peppers fermented for 7 d in low salt concentration; D2 represents pickled peppers fermented for 30 d in low salt concentration; D3 represents pickled peppers fermented for 60 d in low salt concentration; D4 represents pickled peppers fermented for 90 d in low salt concentration; G1 represents pickled peppers fermented for 7 d in high salt concentration; G2 represents pickled peppers fermented for 30 d in high salt concentration; G3 represents pickled peppers fermented for 60 d in high salt concentration; G4 represents pickled peppers fermented for 90 d in high salt concentration.

stage of fermentation, the acidity formed in the metabolic process of LAB will inhibit the growth of miscellaneous microbiota to some extent, and the nitrite will be degraded by acids or enzymes, therefore, the content of nitrite will be reduced (Mi et al., 2022). The overall variation of nitrite content in the D group was 0.47–1.40 mg/kg, while that of the G group was 0.38–1.14 mg/kg and there was no significant difference between the two groups ($p > 0.05$). The nitrite content of both groups was higher than the M1 group, and only that in G group was closest to the M1 group at 7 d of fermentation.

3.1.3. Soluble protein (SP)

The SP content of pickled peppers in the D and G groups both showed a trend of first increasing and then decreasing (Table 1). The SP content of the D group ranged from 0.59 to 0.82 mg/g, while that of the G group ranged from 0.58 to 0.89 mg/g. There was no significant difference between the two groups during fermentation ($p > 0.05$). At 30 d of fermentation, the SP content in both groups was closest to the M1 group, and at 60 d of fermentation, their SP content reached the maximum value. This result was probably because the proteins trapped in the pickled peppers were hydrolyzed into low-MW proteins and peptides, or the starch was degraded, which reduced the interaction with the proteins (Li & Wang, 2020). Therefore, the solubility of proteins in pickled peppers increased after fermentation. Similarly, Ma et al. (2017) demonstrated that the content of SP in traditional northeastern suancai also increased in the middle period of fermentation. From this, it can also be seen that the optimal fermentation time for pickled peppers is between 30 and 60 d. In the optimal fermentation period, the final fermentation quality and stability could be ensured, and the problems during the fermentation process could be greatly reduced.

3.1.4. Chloride

The chloride content of the pickled peppers samples in the D and G groups did not change significantly in the early stage of fermentation ($p < 0.05$, Table 1). The chloride content of the D group remained stable at 30–60 d of fermentation, which only increased slightly with the extension of fermentation time, but there was no significant difference ($p > 0.05$). While the chloride content of the G group ranged from 7.40 to 8.14 g/100 g, which had a significant difference from that of the D group during fermentation ($p < 0.05$). Higher salt concentration and osmotic pressure contribute to the extraction and diffusion of juice, which may affect the growth of the microorganisms and the release of organic compounds in pickled peppers. This also leads to a significantly higher content of TTA in section 3.1.1. A similar result was also obtained in kimchi (Ahmadsah, Min, Han, Hong, & Kim, 2015). Neither of the chloride content in the D and G groups exceeded the specified limit (15.0 g/100 g).

3.1.5. Texture properties

The texture properties of pickled peppers after fermentation are also important indicators that must be considered if the product is expected to be recognized by consumers (Ye et al., 2020). The texture properties (firmness, elasticity, viscosity, and chewiness) of pickled peppers with different salt concentrations are presented in Table 1. As shown, the firmness of both D and G groups mainly showed a decreasing trend during the fermentation and reached the highest point of 33.07 and 48.56 N at the fermentation of 60 d, respectively, which was significantly higher than other results ($p < 0.05$). The firmness in Sichuan pickles (Liu, Peng, et al., 2019) and bamboo shoots (Zheng et al., 2013) also decreased during fermentation. Moreover, the firmness of the D group was significantly lower than that of the G group during the fermentation ($p < 0.05$), except for D2 group vs G2 group. These may be related to the production of lactic acid produced by LAB during the fermentation process. This result was consistent with the results reported by Zhang and Zheng (2015) on changes in the firmness of bamboo shoots during fermentation.

As the fermentation progressed, the elasticity and chewiness of the D group reached their maximum values at the fermentation of 30 d, with the chewiness remaining stable within the same range as the M1 group. In the later stage of fermentation, the elasticity and chewiness of the D group began to sharply decrease and stabilized again ($p < 0.05$), which should be related to the softening of the pickled peppers tissue during the fermentation process. The elasticity of the G group did not change much during fermentation, while the chewiness showed a trend of first decreasing and then increasing. Both of them remained stable within the same range as the M1 group during the later stages of fermentation. The elasticity and chewiness of group D were significantly lower than those

of group G ($p < 0.05$), except for D2 group vs G2 group. Our results were consistent with previous studies reported by Ou et al. (2022).

The viscosity of the D group gradually decreased from 7 to 60 d of fermentation and then stabilized at a level significantly lower than that in the M1 group ($p < 0.05$). While that of the G group gradually decreased from 7 to 30 d of fermentation, and then began to rise. After 90 d of fermentation, its viscosity was not significantly different from that in the M1 group ($p < 0.05$). Simaliarly, the viscosity of group D were significantly lower than those of group G ($p < 0.05$), except for D2 group vs G2 group.

3.2. Flavor compounds profiles of pickled peppers with different salt concentrations

3.2.1. Organic acids

The organic acids produced by microbial metabolism during the fermentation process of pickled peppers can not only alter the microenvironment of fermentation materials, but also provide a unique flavor to fermented food and improve its nutritional value and safety. As presented in Table S2, five organic acids, including malic acid, lactic acid, acetic acid, citric acid, and fumaric acid, were detected in all samples. Among them, the contents of lactic acid, acetic acid, and citric acid were higher, accounting for >90% of the total organic acid content in all samples. The results indicated that these three acids were the most abundant organic acids in pickled peppers fermentation, which is similar to the result obtained by Chen et al. (2022). As the fermentation progressed, the lactic acid content in both D and G groups showed an upward trend, while the citric acid content showed an opposite downward trend. This might be due to the use of citric acid by LAB in fermentation to generate substances such as acetic acid and lactic acid (Yang, Sun, et al., 2022). Furthermore, lactic acid and acetic acid were the two most abundant organic acids accumulated throughout fermentation in D group, the content of which peaked at day 60 of fermentation (3433.84 ± 3.10 and 1432.84 ± 2.77 mg/kg, respectively), which was closest to M1 group, and decreased slightly afterward. While the content of citric acid was the highest (3809.41 ± 2.49 mg/kg) in the G group, with a level significantly higher than that in the M1 group ($p < 0.05$), which also peaked at 60 d of fermentation. The growth rate of lactic acid content in the G group was slower than that D group, which may be due to the inhibition of LAB proliferation by high salt concentration. Although the content of fumaric acid was the lowest, it also played an important role in enriching the acidity and fullness of pickled peppers.

3.2.2. Free amino acids (FAA) profiles

Next, the dynamic change of FAA during the fermentation of pickled peppers was performed. FAA are important flavor compounds in pickled peppers, which can impart various flavors such as bitterness, sweetness, and umami to the product (Ye et al., 2020). The flavor characteristics of FAA are closely related to their content and threshold value, which is one of the important indexes to evaluate the quality of product. As shown in Table S3, 15 FAA were detected in all samples, and the content of sweet amino acids (SAA) and umami amino acids (UAA) was higher than others, which were the main contributors to the taste of pickled peppers. The total content of FAA in the D group was significantly higher than that in the G group ($p < 0.05$), and was close to the M1 group, which represented that salt concentration has a considerable effect on the taste properties of pickled peppers. Besides, the SAA content in the D group was >5 times that in the G group, and showed an upward trend with the extension of fermentation time. The possible reason for the result might lie in the fact that in the fermentation process, suitable temperature and lower salt concentration made protease activity higher, which degraded proteins in pickled peppers into amino acids (Chen et al., 2024). As the fermentation progressed, the content of UAA in the D group gradually decreased, while that in the G group generally showed a gradual upward trend and slightly decreased at 90 d of fermentation. The increase in the FAA contents might be due to lactic fermentation, where the SP was hydrolyzed to FAA by peptidases secreted by LAB (e.g., *L. plantarum*) during fermentation (Wu et al., 2015).

3.2.3. Taste substances

The electronic tongue system can detect sour, bitter, umami, bitter aftertaste, astringent aftertaste, astringency, richness, and saltiness. As illustrated in Fig. S1A, compared with the M1 group, pickled peppers fermented with different salt concentrations during different fermentation time had significant differences in sour taste, bitter taste, fullness, and saltiness. The sour taste of pickled peppers in the G group gradually decreased with the extension of fermentation time, however, there was no significant difference in the acidity of the D group throughout the entire fermentation stage and it was lower than that in the G group. This may be due to the production of lactic acid, butyric acid, and other substances by microorganisms (such as LAB) in the G group during the early fermentation process. As the fermentation time is prolonged, these acidic substances could combine with other substances to form esters and other flavor compounds (Shi et al., 2023). Similarly, the bitterness of pickled peppers in the G group was lower than that in the D group during the whole fermentation stage. With the extension of fermentation time, the response values of the bitterness in the G group gradually decreased and reached their minimum after 90 d of fermentation, but there was no significant change in the D group. In terms of richness, the samples in the D group were slightly better than the G group in the short term. However, there was no significant difference between the two groups when fermented for 90 d. In terms of saltiness, there was no significant difference in the fermentation process of pickled peppers, but the response values in the G group were higher than those in the D group.

3.2.4. Volatile flavor compounds profiles

3.2.4.1. Analysis of GC–MS. The volatile flavor compounds play an important role in consumer acceptance, and they can contribute to the organoleptic properties of fermented vegetables (Liang et al., 2020a). The volatile flavor components in pickled peppers fermented with different salt concentrations are presented in Table S4. A total of 218 volatile flavor compounds were detected on 7 d, 30 d, 60 d, and 90 d of pickled peppers, including 51 esters, 6 aldehydes, 21 alcohols, 28 ketones, 49 hydrocarbon compounds, and other types. Among them, many flavor compounds were present in the early fermentation stage but disappeared by the end of fermentation. The main reason is likely that many flavor compounds are synthesized from a variety of precursor substances (such as acids and alcohols), impacting the content of flavor compounds (Yao et al., 2021). 3 esters (the methyl salicylate and phenyl ethyl acetate had the highest content in D group), 4 alcohols (e.g., linalool and phenylethanol), 3 hydrocarbons (e.g., heptadecane), and 1 aldehyde (the pentadecanal had the highest content in G group) were all detected in M1, D and G group. Methyl salicylate and linalool provide an herbal flavor to pickled peppers (Patel et al., 2016). As reported, linalool, geraniol, benzaldehyde, and phenylethyl alcohol were detected in Paocai, Suancai, pickled peppers, and Sichuan Dongcai (Wu et al., 2015; Yao et al., 2015), and some of them were determined as the crucial or potential markers in Paocai (Luo, Li, Chen, Liu, & Pu, 2017). However, geraniol, benzaldehyde, and phenylethyl alcohol were not detected in pickled peppers in our study, which may be due to many factors, such as vegetable species, regions, fermentation conditions, microbiota, etc.

5 esters, 3 hydrocarbons, and 1 ketone were uniquely presented in the D group, and 3 esters and 1 alcohol were uniquely presented in the G group during the whole fermentation period, which revealed that salt concentration can remarkably affect the type and content of flavor compounds in pickled peppers. In addition, the phytane in hydrocarbons was not detected in the D and G groups after 7 d of fermentation, while

in the remaining fermentation periods, it was detected in both salinity groups and its content in the G group was significantly higher than that in the D group ($p < 0.05$).

The largest proportion of esters among the detected volatile flavor compounds was about 22%, and the total relative content of which was the highest (30%) in the D3 group (Table S4). The results showed that samples in low salt concentration showed a higher aromatic potential after fermentation for 60 d. This result may be because alcohols and organic acids, as synthetic precursors of ester compounds, combine with acids to form esters in late fermentation. Therefore, esters were the main volatile flavor compounds of pickled peppers. Ye et al. (2022) also reported that esters were the most important volatile flavor compounds in fermented peppers.

3.2.4.2. Analysis of electronic nose. Fig. S1B is a radar plot drawn by combining the data from all samples in M1, D, and G groups after 78 s of stabilization. As is shown, the response values of W1C, W5C, W3S, and W3C sensors were almost the same in the D and G groups, indicating that the content of aromatic components (including ammonia) in pickled peppers fermented with different salt concentrations during different fermentation time was similar to that of short- or long-chain alkanes. However, the response values of W2W, W1S, and W1W sensors in D and G groups during the fermentation process had significant differences, indicating that the content of sulfide and methyl compounds in different salinity pickled peppers during the whole fermentation period was significantly different. Combined with all the results, the response value of nitrogen oxide (W5S) was the highest, suggesting that it contributed the most to the aroma of pickled peppers. PCA can more intuitively reflect the overall relationship between odor substances and salt concentration of pickled peppers. As illustrated in Fig. S1C, under the correlation matrix mode, the cumulative sum of the differentiating contribution rate of the two principal components was >90%, thus, they have represented the main information characteristics of all pickled peppers samples. Except for the intersection of samples in D2 and G3

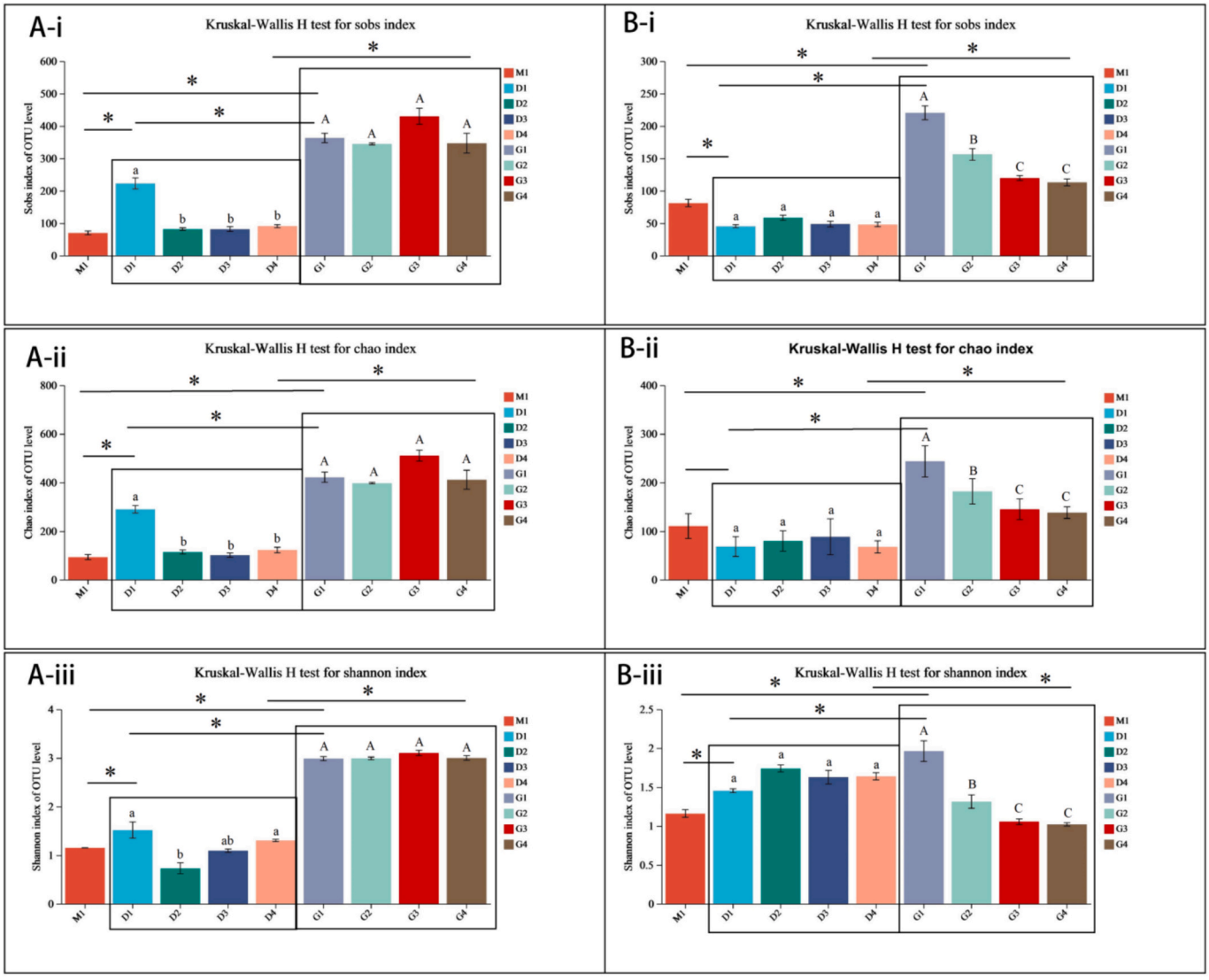


Fig. 1. Alpha diversity of pickled pepper with different salt concentrations during fementation. A: Bacteria. B: Fungi. i: Sobs index. ii: Chao index. Iii: Shanno index. Different capital letters (A-C) indicate significant differences among fungi ($p < 0.05$); different lowercase letters (a-c) indicate significant differences among bacteria ($p < 0.05$); * indicates significant difference among groups ($p < 0.05$). M1 represents the marketing standard pickled peppers of company; D1 represents pickled peppers fermented for 7 d in low salt concentration; D2 represents pickled peppers fermented for 30 d in low salt concentration; D3 represents pickled peppers fermented for 60 d in low salt concentration; D4 represents pickled peppers fermented for 90 d in low salt concentration; G1 represents pickled peppers fermented for 7 d in high salt concentration; G2 represents pickled peppers fermented for 30 d in high salt concentration; G3 represents pickled peppers fermented for 60 d in high salt concentration; G4 represents pickled peppers fermented for 90 d in high salt concentration.

groups, the remaining samples could be significantly distinguished by the electronic nose, indicating that pickled peppers with different salt concentrations during the whole fermented period had their unique flavor characteristics.

3.3. Microbial profiles of pickled peppers with different salt concentrations

3.3.1. Alpha diversity analysis of microbial community

A total of 3,401,717 and 3,177,152 raw reads were acquired, and an average of 41,407 and 42,621 high-quality sequences per sample were recovered from nine spontaneously fermented pickled peppers for bacteria and fungi, respectively. The rarefaction curves of the microbial community (Fig. S2A&B) tended to be flat as the number of sequences increased, and good' s coverage surpassed 99.7% in all samples (data not shown), which indicated that sequencing depth in this study was adequate and the majority of microbial phylotypes present in pickled peppers had been covered. Fig. 1 displays the alpha diversity including Sobs, Chao, and Shannon indexes of bacterial (A-i, ii and iii) and fungal (B-i, ii and iii) microbiota. Sobs index means the number of OTUs in the sample, and Chao and Shannon indexes indicate the abundance and diversity of samples at the OTUs level, respectively (Liu, She, et al., 2019). As a whole, the bacterial numbers, abundance, and diversity of pickled peppers with different salt concentrations were higher than those of fungi.

As presented in Fig. 1A, the OTUs numbers, abundance, and diversity of bacterial microbiota in the G group were higher than that of the D group. In the beginning, both D1 and G1 groups possessed higher values of numbers, abundance, and diversity of OTUs than the M1 group. That means M1 samples with materials addition, the bacteria in the pickled peppers production liquid carried out the structure and diversity reaction quickly, at the early stage of fermentation. The G1 group showed higher values of the numbers, abundance, and diversity of OTUs than the D1 group, observably ($p < 0.05$). There were no significant differences ($p \geq 0.05$) among the samples in the G group as the fermentation time extended, thus, high salt concentration has a stable fermented bacterial system. While a decrease of OTUs numbers, abundance and diversity in low salt concentration from the D1 group to the D2 group were observed, and this might mean the strain content and species reduced during pickled peppers fermentation at low salt environment. Therefore, it could be speculated that strains with high osmotic pressure should be present in the pickled peppers fermentation broth. At the end of fermentation, the diversity of OTUs in the D4 group showed a lower level than that in the G4 group, indicating that high salinity would not decrease the diversity of pickled peppers at the OTUs level. Similarly, the bacterial composition in paocai also positively correlated with salinity (Liu, She, et al., 2019).

At the same time, the OTUs numbers and abundance of fungal microbiota in the G group were higher than that in the D group, as showed in sobs and chao index (Fig. 1B-i and ii). As fermentation time prolonged, there were no significant changes ($p \geq 0.05$) existing in the numbers, abundance, and diversity of fungal microbiota OTUs in the D group, whereas that in the G group significantly decreased ($p < 0.05$). The same phenomenon would be obtained in the bacterial results, the numbers, abundance, and diversity of fungal microbiota OTUs in the G group were significantly higher than those in the M1 group ($p < 0.05$). That means in M1 samples with high salt addition, the fungal microbiota in the pickled peppers production liquid occurring changes in the structure and diversity quickly. It is worth noting that the fungal diversity in the D group was significantly increased ($p < 0.05$) compared to M1, indicating that the fungal species and diversity of pickled peppers were increased under low-salt conditions. In contrast to the bacterial results, the diversity of fungal microbiota OTUs in the D4 group showed a higher level than that in G4, significantly ($p < 0.05$), and this is in agreement with the study on Yan-cai vegetable pickles with different salinity (Liu, Kuda, Takahashi, & Kimura, 2018).

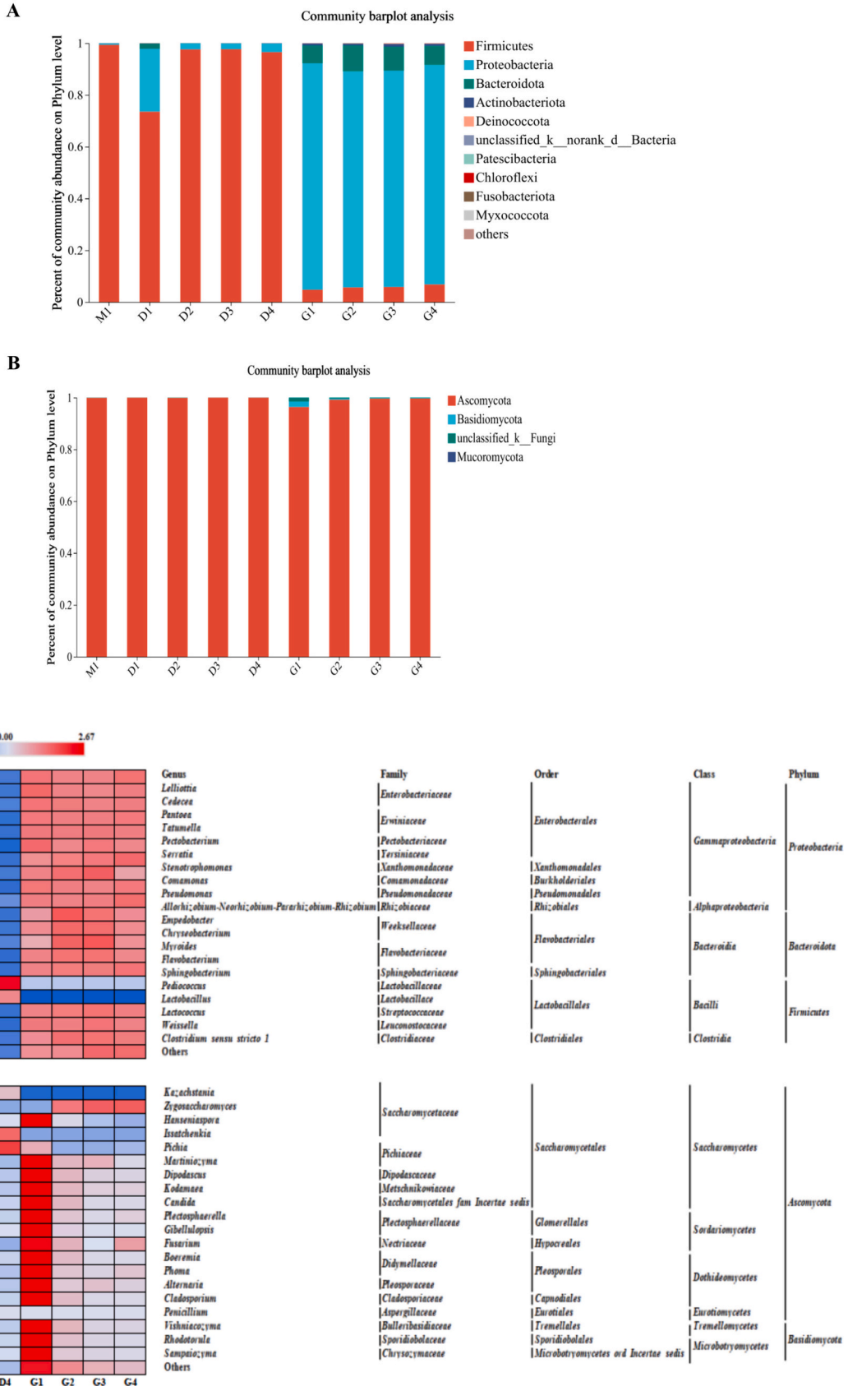
3.3.2. Comparison of microbial profiles

Pickled peppers are a spontaneous fermentation process and rely on a naturally occurring microbial community present in materials. The salt addition could affect the diversity of the microbial community during the fermentation. The Illumina HiSeq platform was employed to sequence the V3-V4 regions of the 16S rRNA gene and the ITS region of fungi genes of 54 samples, thereby obtaining >0.34 million (bacteria) and 0.31 million (fungi) clean tags after concatenation and quality control. The clean reads with an average length of 376 bp (bacteria) and 356 bp (fungi) were clustered into 1391 (bacteria) and 448 (fungi) OTUs at 97% similarity level, respectively. These OTUs were clustered into 25 phyla, 65 classes, 150 orders, 265 families, 544 genera, and 833 species for bacteria, and 4 phyla, 21 classes, 43 orders, 93 families, 161 genera, and 258 species for fungi, respectively. In this study, microbial taxonomic composition was investigated at the level of phylum and genus in different salinity pickled peppers.

At the phylum level of the bacterial community, 25 phyla were observed in total, and the top 10 relative abundances were illustrated in Fig. 2A. As is shown, >90% of the annotated reads were assigned to *Firmicutes* and *Proteobacteria*, and the predominant phylum in G group were completely different from those in D group during the fermentation. In the early stage of fermentation, we found the relative abundance of *Firmicutes* in the D and G groups was lower than that in the M1 group, while the relative abundance of *Proteobacteria* and *Bacteroidota* was higher than that in the M1 group. With the extension of fermentation time, the relative abundance of *Firmicutes* in the D group significantly increased and was still considered the most predominant phylum (with an average relative abundance of over 97.26%), followed by *Proteobacteria* that covered nearly 2.63% of annotated reads. These results were close to that in the M1 group. While for the G group, *Proteobacteria* (84.83%) was the absolute predominant phylum, followed by *Bacteroidetes* (8.40%) and *Firmicutes* (5.74%). Both of the predominant phylum of D and G groups in the later stage of fermentation remain unchanged, suggesting that the corresponding fermentation environment may inhibit the growth and reproduction of other bacteria. The sum of the relative abundance of the predominant phyla in both groups was >98%, which fully indicated that the bacterial community structure of pickled peppers in different fermentation processes was stable at the phylum level. These results were consistent with the research of Mi et al. (2022), which focused on the bacterial community structure during the fermentation of radish paocai.

During the whole fermentation period, there were only 4 fungal phyla were detected in all samples (Fig. 2B), viz., *Ascomycota*, *Basidiomycota*, *Mucoromycota*, and others, and *Ascomycota* was the most predominant phylum since it approximately accounted for 99% of fungal phyla. These results are similar to previous research on fermented vegetables (Liang et al., 2020b; Liu, She, et al., 2019). Furthermore, with the prolongation of fermentation time, the relative abundance of predominant phylum in both the D and G groups did not change significantly, and was similar to the M1 group. The above results were different from the changes in the bacterial community, indicating that the effect of salt concentration on the fungal community was less than that on the bacterial community at the phylum level.

A detailed analysis of the microbial community at the genus level was performed, and the top 20 genera of microbiota in all samples were selected, and the rest were labeled as "others" (Fig. 2C). Many genera of LAB were widely reported as the dominant microorganisms of fermented vegetables, including *Lactobacillus*, *Lactococcus*, *Weissella*, *Pediococcus* and *Leuconostoc* (Rao et al., 2019), and this study showed similar result that *Lactobacillus*, *Pediococcus*, *and Serratia* were primary genera and shared by all groups. Throughout the entire fermentation period, the most dominant genus in group D was *Lactobacillus*, which was similar to group M1, whereas in group G it was *Pectobacterium* and *Pseudomonas*, which probably suggested that the main dominant genera varied in pickled peppers with different salt concentration during fermentation and the genus *Lactobacillus* were less salt-tolerant than others. Mi et al.



(caption on next page)

Fig. 2. Relative abundance of microbial community. A, and B represent the phyla of bacteria and fungi, respectively. C present the heatmap of relative abundance of bacteria and fungi at genus level. The red color of the spots in the panel represent greater abundance of genera, while the blue color represent lesser abundance of genera. The taxa information (family, order, class and phylum) is shown on the right. M1 represents the marketing standard pickled peppers of company; D1 represents pickled peppers fermented for 7 d in low salt concentration; D2 represents pickled peppers fermented for 30 d in low salt concentration; D3 represents pickled peppers fermented for 60 d in low salt concentration; D4 represents pickled peppers fermented for 90 d in low salt concentration; G1 represents pickled peppers fermented for 7 d in high salt concentration; G2 represents pickled peppers fermented for 30 d in high salt concentration; G3 represents pickled peppers fermented for 60 d in high salt concentration; G4 represents pickled peppers fermented for 90 d in high salt concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(2022) and Liang et al. (2020b) also suggested that the relative abundance of *Lactobacillus* in the bacterial community decreased as salt concentration increased. As a major part of the LAB, *Lactobacillus* could metabolize sugar into the primary byproduct lactic acid, which lowers the pH and increases the content of TTA. Besides, *Lactobacillus* can also produce ethanol, and acetic acid, and inhibit the growth of pathogenic bacteria. Therefore, some species affiliated with *Lactobacillus* were used as starter cultures for controlled fermentation in the production of fermented vegetables (Yang et al., 2019). In the D group, *Lactococcus* was dominant in the early stage of fermentation and it was decreased during the fermentation, which was in accordance with the previous study of suancai fermentation (Yang et al., 2016). However, it was reported that the growth of *Lactococcus* was not affected by salt addition in suancai fermentation, which was different from the pickled peppers fermentation (Liang et al., 2020b). *Pediococcus* were observed with a high abundance in the later stage of fermentation, which was commonly detected in fermented vegetables, such as paocai and spicy cabbage (Liu, She, et al., 2019). In the previous study, *Weissella* was the dominant genus in the high salt concentration of suancai and radish paocai fermentation, which is similar to our results of pickled peppers (Liang et al., 2020b; Mi et al., 2022). *Pseudomonas*, which can break down proteins and is significantly related to nitrogen-containing metabolites, is the first strain to be shown to produce compounds of phenazines, a biologics with a broad spectrum of antimicrobial activity (Qiu, Liu, Kang, Ye, & Ge, 2022).

A total of 161 fungal genera were detected during pickled peppers fermentation at the genus level. The genera detected in this study, such as *Zygosaccharomyces*, *Pichia*, *Candida*, *Cladosporium*, *Issatchenkia*, *Alternaria*, etc. (Fig. 2C), were also observed in previous studies on suancai or other fermented vegetables (Liu, Peng, et al., 2019; Xiao et al., 2018). Yeast was found to be the main fungal microorganism, which has been reported to enhance the flavor and quality of fermented vegetables (Xiong & Li, 2021). As shown in Fig. 2C, *Kazachstania*, *Issatchenkia*, and *Pichia* were the dominant strains in the D group, which were similar to the M1 group. While for the G group, the dominant strains were *Zygosaccharomyces*, *Cladosporium*, and *Dipodascus*. You et al. (2021), Che (2020) and Yang, Li, Liu and Tian (2022) found that *Kazachstania turicens*, *Kazachstania Humilis*, *Issatchenkia*, and *Dipodascus* were the main flavor substances during the fermentation process of grains, but whether they were related to the formation of pickled peppers flavor compounds remains to be further studied. Interestingly, *Pichia*, recognized as being involved in firmness decline and tissue impairment of fermented vegetables (Fan et al., 2022), was detected in the G group with a relative abundance of 9.72%–25.18% whereas in the D group was 8.64–50.36%. This result was consistent with that in section 3.1.5. With the progress of fermentation, due to the strong acidophilic and ethanol tolerance characteristics of *Issachenkia* and *Pichia* (Liu, 2011), their abundance in the D group increased continuously, while there were no similar changes in the G group, indicating that excessive salt concentration significantly inhibits the reproduction of these flavor yeast. The relative abundance of *Zygosaccharomyces* increased from 0.03% to 71.10% during fermentation, which has been reported to significantly reduce the content of nitrite and biogenic amines in kimchi and increase its flavor compounds (Wu, Zheng, Huang, & Zhou, 2014).

3.3.3. Multivariate analysis of microbial community

Based on the abundance of the microbial profiles, PCoA was conducted to discern the pickled peppers fermented with different salt concentrations (Fig. 3A and B). The repeatability of the samples within the group was good, the first two canonical axes explained the variation of 69.46% and 23.29% in the bacterial community data, and 55.89% and 18.23% in the fungal community data, respectively. PCoA analysis could distinguish D and G groups ($R^2 = 0.897$, $p < 0.01$ for bacterial community and $R^2 = 0.891$, $p < 0.01$ for fungal community), indicating that the microbial community in pickled peppers was significantly affected by the salt concentration. In addition, we further used ANOSIM to evaluate the impact of fermentation time on the microbial community during the fermentation process of pickled peppers under low and high salt concentrations, revealing the reasons for the differences in the microbial community of pickled peppers with different salt concentrations during fermentation. From Table S5, it can be seen that there were significant differences in the microbial community of pickled peppers under different salt concentrations and fermentation times ($p < 0.01$), except for the changes in the bacterial community in the G group, suggesting that fermentation time can also regulate the diversity of microbial community during the fermentation process of pickled peppers.

LEfSe is an algorithm for high-dimensional biomarker discovery, which identifies abundant features, characterizing the meaningful differences between two or more biological conditions (Liang et al., 2020a). The LEfSe analysis of microbial community during pickled peppers fermentation with different salt concentrations is presented in Fig. 3C-F. The results showed that the genera *Kazachstania* and *Lactobacillus* were the microbial biomarkers in M1. *Pediococcus* and *Pichia* were the microbial biomarkers that distinguished D4 from the other eight groups. *Pectobacterium*, *Pseudomonas*, *Pantoea*, *Cedecea*, *Kluyvera*, *Hanseniaspora*, *Cladosporium*, *Gibberella*, *Dipodascus*, *Sporidiobolus*, *Boeremia*, and *Phoma* were the microbial biomarkers significantly enriched in group G1, while *Empedobacter* was the microbial biomarker in group G2. *Phoma* and *Cladosporium* are both dominant molds in the environment and common pathogens, which are often parasitic on plants and cause various diseases (Zhu & Huang, 2021). And they may migrate from raw materials or the environment to the brewing process, which needs to be prevented and controlled. *Devosia*, *Achromobacter*, *Shewanella*, and *Glutamicibacter* were the microbial biomarkers in group G3, while *Lelliottia*, *Patulibacter*, *Novosphingobium*, *Sanguibacter*, *Salana*, *Curtobacterium* and *Zygosaccharomyces* were the microbial biomarker in group G4. While no biomarkers at the genus level were found in the D1, D2, and D3 groups (LDA scores >4, $p < 0.05$), which may be due to the community composition of these three groups being extremely similar to others. These results indicate that there were significant changes in the distribution of bacterial and fungal community structures in pickled peppers with different salinity from the beginning to the end of fermentation. These microbial biomarkers of pickled peppers with different salinity at different fermentation stages collectively determine the different qualities of pickled peppers.

3.3.4. Network inference of microbial species in pickled peppers with different salt concentrations

With the progress of fermentation, various microorganisms perceive and regulate each other through group effects, contributing to the microbial diversity of pickled pepper products. The relationship between microorganisms of picked peppers with different concentrations based

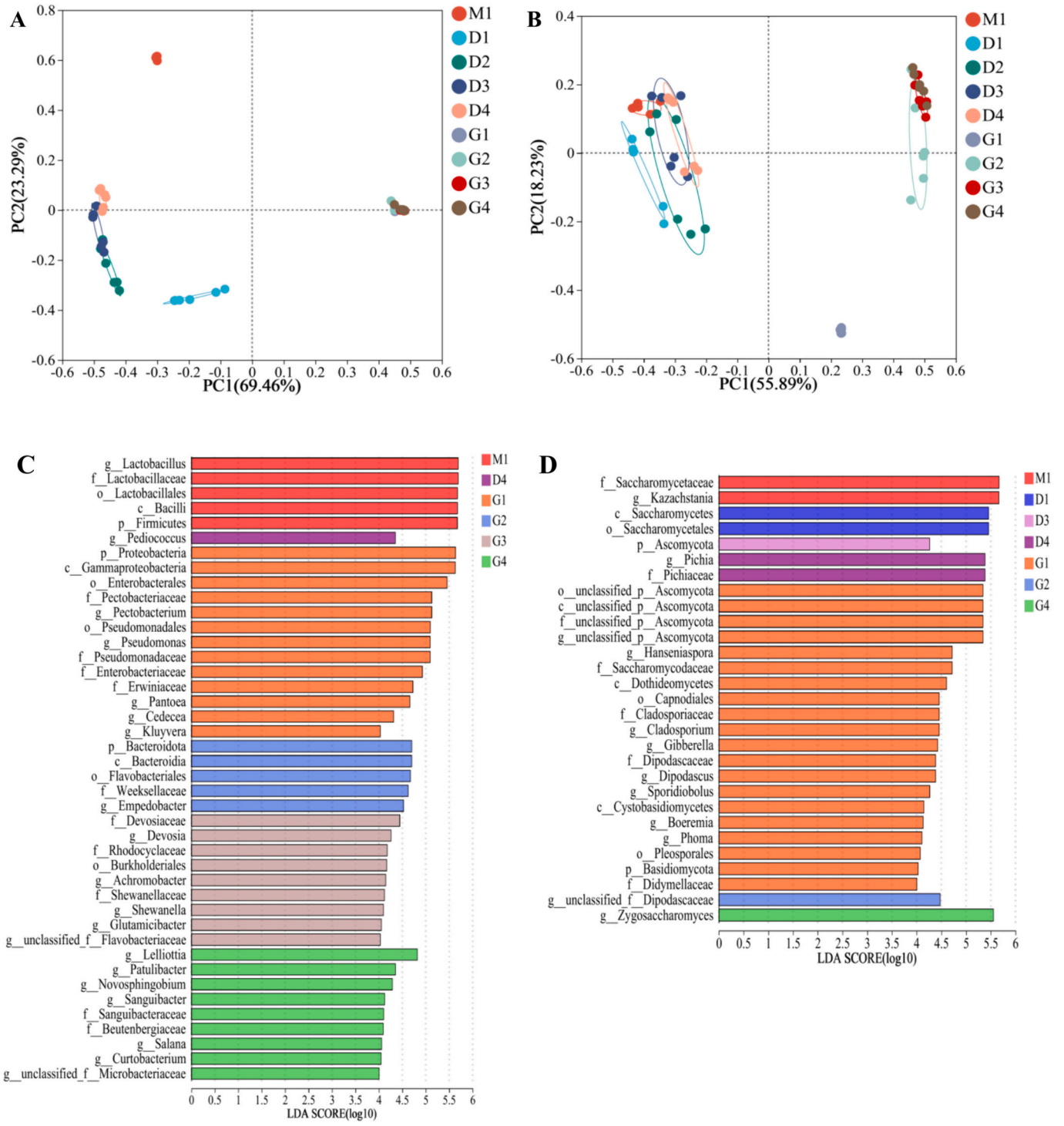


Fig. 3. PCoA score plots of bacterial (A) and (B) fungal community. The microbial communities of pickled pepper samples were analyzed using the LDA effect size algorithm to determine the optimal characteristic taxa and rank them according to the effect size (C and D). LDA scores identified the magnitude of differentiation; and the score threshold was 4.0. Cladogram shows that yellow dots are unimportant bacteria in any groups; other colored dots are important bacteria in the group labeled with the same color (E and F). (C-E: Bacteria; D-F: Fungi). Labels beginning with p_ indicate phylum; c_, class; o_, order; f_, family; g_, genus. M1 represents the marketing standard pickled peppers of company; D1 represents pickled peppers fermented for 7 d in low salt concentration; D2 represents pickled peppers fermented for 30 d in low salt concentration; D3 represents pickled peppers fermented for 60 d in low salt concentration; D4 represents pickled peppers fermented for 90 d in low salt concentration; G1 represents pickled peppers fermented for 7 d in high salt concentration; G2 represents pickled peppers fermented for 30 d in high salt concentration; G3 represents pickled peppers fermented for 60 d in high salt concentration; G4 represents pickled peppers fermented for 90 d in high salt concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

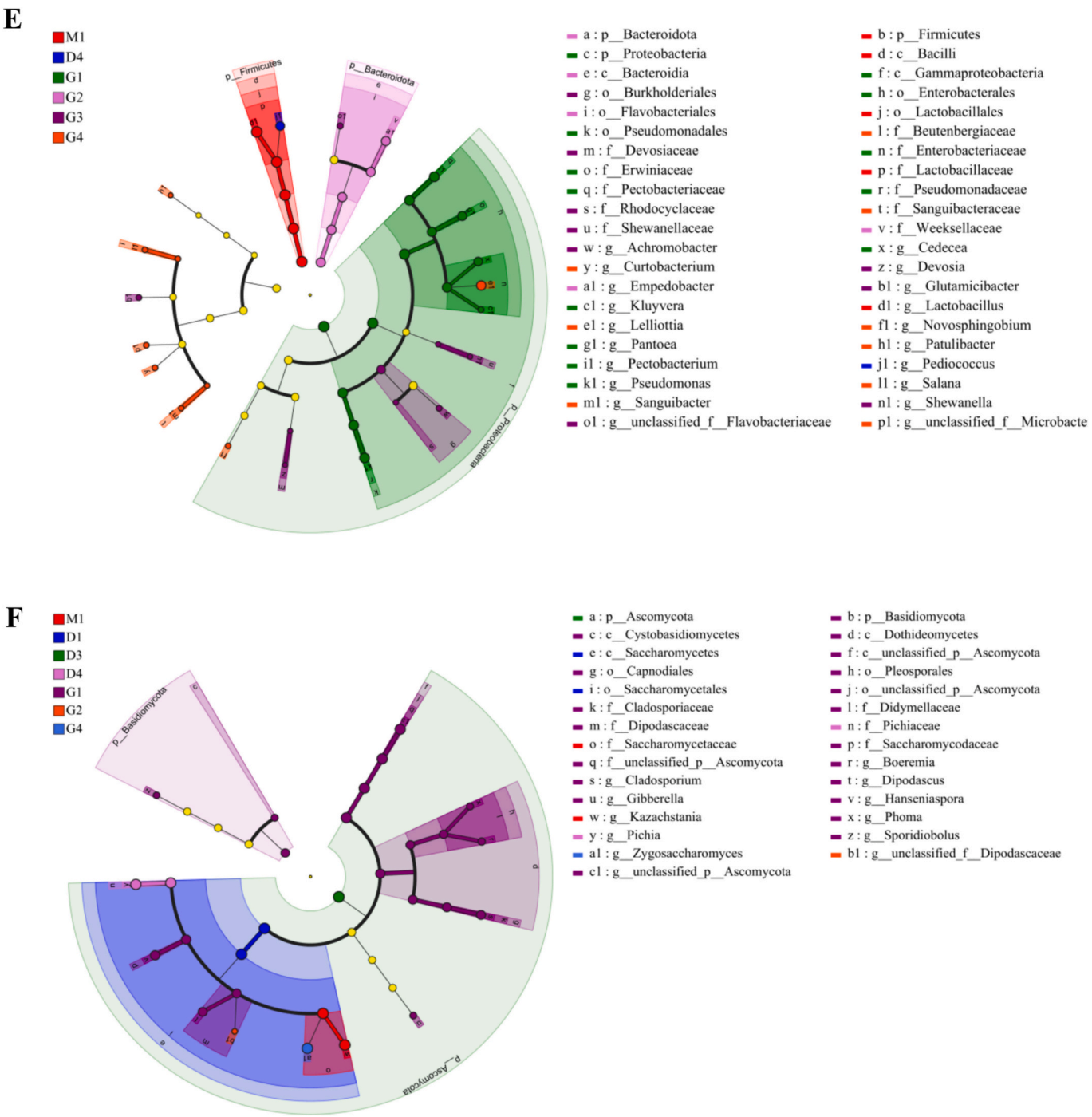


Fig. 3. (*continued*).

on the relevant network diagram was studied, and the clustering coefficients for different species were calculated (Fig. 4A-D). The genus-level correlation network diagram revealed that there were significant interactions between different microbial genera in picked peppers with different salt concentrations (red indicates a positive correlation, green indicates a negative correlation; and the thickness of the line indicates the size of the correlation coefficient). As shown in Fig. 4A, there was a significant positive correlation between *Lelliottia* and *Pectobacterium* in the D group, with a clustering coefficient of 0.95 (clustering refers to the clustering coefficient of the node; the larger the node, the more important it is). *Pseudomonas* also showed a significant positive correlation with *Stenotrophomonas* and *Chryseobacterium*, with clustering coefficients of 0.91 and 0.90, respectively. In addition, the number of genera associated with *Lactococcus*, *Pseudomonas*, and *Empedobacter* were the largest (degree = 18) (degree represents the number of nodes connected to another node), and there were 14, 13, and 11 positively related genera, respectively, which proved that they played an important role in the entire bacterial diversity system of low salt concentration pickled peppers. Previous reports have shown that *Lactococcus* and *Pseudomonas* were the dominant bacteria and crucial contributors during the production of fermented foods, such as cheese, sufu, soy sauce, liquor, tea, and vinegar (Gu et al., 2018; Li et al., 2019). On the other hand, for G group, *Lelliottia* presented a significant positive correlation with *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, with a clustering coefficient of 0.81 (Fig. 4B). *Chryseobacterium* presented a significant positive correlation with *Empedobacter*, *Myroides*, and

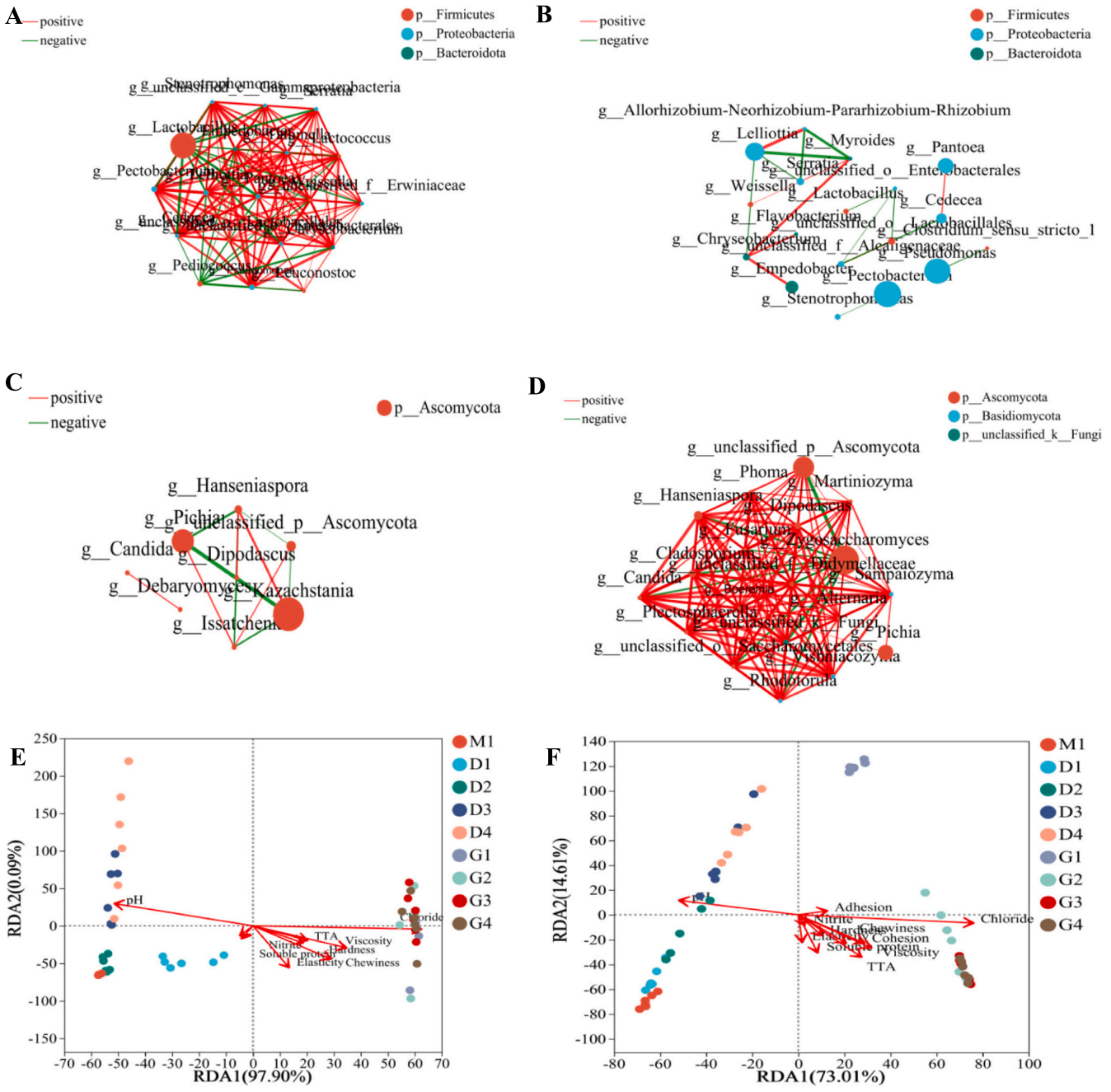


Fig. 4. Network analysis of the top 20 species of pickled pepper with diffferent salt concentrations. Different colored dots represent different genera of different phyla. The size of the dot indicates the abundance of the genus (A-B: Bacteria; C-D: Fungi; A and C: Low salt concentration; B and D: High salt concentration). CCA analysis of the correlation between physicochemical properties and microorganisms of pickled pepper (E: Bacteria, F: Fungi). The blue arrow and triangle represent the correlation between species and physicochemical properties. The longer the arrow, the stronger the correlation. The red arrow represents physicochemical properties. M1 represents the marketing standard pickled peppers of company; D1 represents pickled peppers fermented for 7 d in low salt concentration; D2 represents pickled peppers fermented for 30 d in low salt concentration; D3 represents pickled peppers fermented for 60 d in low salt concentration; D4 represents pickled peppers fermented for 90 d in low salt concentration; G1 represents pickled peppers fermented for 7 d in high salt concentration; G2 represents pickled peppers fermented for 30 d in high salt concentration; G3 represents pickled peppers fermented for 60 d in high salt concentration; G4 represents pickled peppers fermented for 90 d in high salt concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Flavobacterium, with clustering coefficients of 0.74, 0.73 and 0.66, respectively. The important genera in the entire bacterial diversity system of high salt concentration picked peppers were *Serratia*, *Myroides*, *Lelliottia*, and *Chryseobacterium*, all with degree values of 4.

The correlations between the fungal genera of picked peppers with low and high salt concentrations are shown in Fig. 4C&D. In the D group, *Hanseniaspora* were positively correlated with *Kazachstania* and *Dipodascus*, with clustering coefficients of 0.77 and 0.67, respectively. And *Issatchenkia* and *Hanseniaspora* were the important genera with the degree of 5. In the case of the G group, almost all the genera, except for *Pichia* and *Zygosaccharomyces*, were positively correlated with each other. For example, *Phoma*, *Cladosporium*, *Sampaiozyma*, and *Boeremia*

all showed positive correlations with each other. While there was a negative correlation between *Zygosaccharomyces* and other genera.

3.4. Correlations between microbial community and physicochemical properties

RDA analysis was performed to reflect the relationship between the microbial community and physicochemical properties, including pH, TTA, nitrite, SP, chloride, and texture properties (Guan et al., 2022). As illustrated in Fig. 4E and F, the RDA1 and RDA2 ranking axes were 97.90% and 0.09% for bacteria, 72.87% and 13.20% for fungi, respectively, and the sum of the two ranking axes was 97.99% for bacteria and 86.07% for fungi, respectively. These results indicated that the first two axes of redundancy analysis can better reflect the internal correlations between physicochemical properties and the microbial community of pickled peppers. As shown in the Figures, the longer the arrow, the stronger the correlation (Yao et al., 2021). The results indicate that the most important physicochemical properties shaping the microbial community was chloride ($r^2 = 0.894$, $p < 0.001$ for bacterial community, $r^2 = 0.832$, $p < 0.001$ for fungal community), followed by pH ($r^2 = 0.745$, $p < 0.001$ for bacterial community, $r^2 = 0.397$, $p < 0.001$ for fungal community), viscosity ($r^2 = 0.404$, $p < 0.001$ for bacterial community, $r^2 = 0.222$, $p = 0.004$ for fungal community), chewiness ($r^2 = 0.501$, $p < 0.001$ for bacterial community, $r^2 = 0.117$, $p = 0.04$ for fungal community), and TTA ($r^2 = 0.144$, $p = 0.021$ for bacterial community, $r^2 = 0.236$, $p = 0.002$ for fungal community). They were the most important properties influencing the variation of microbial flora.

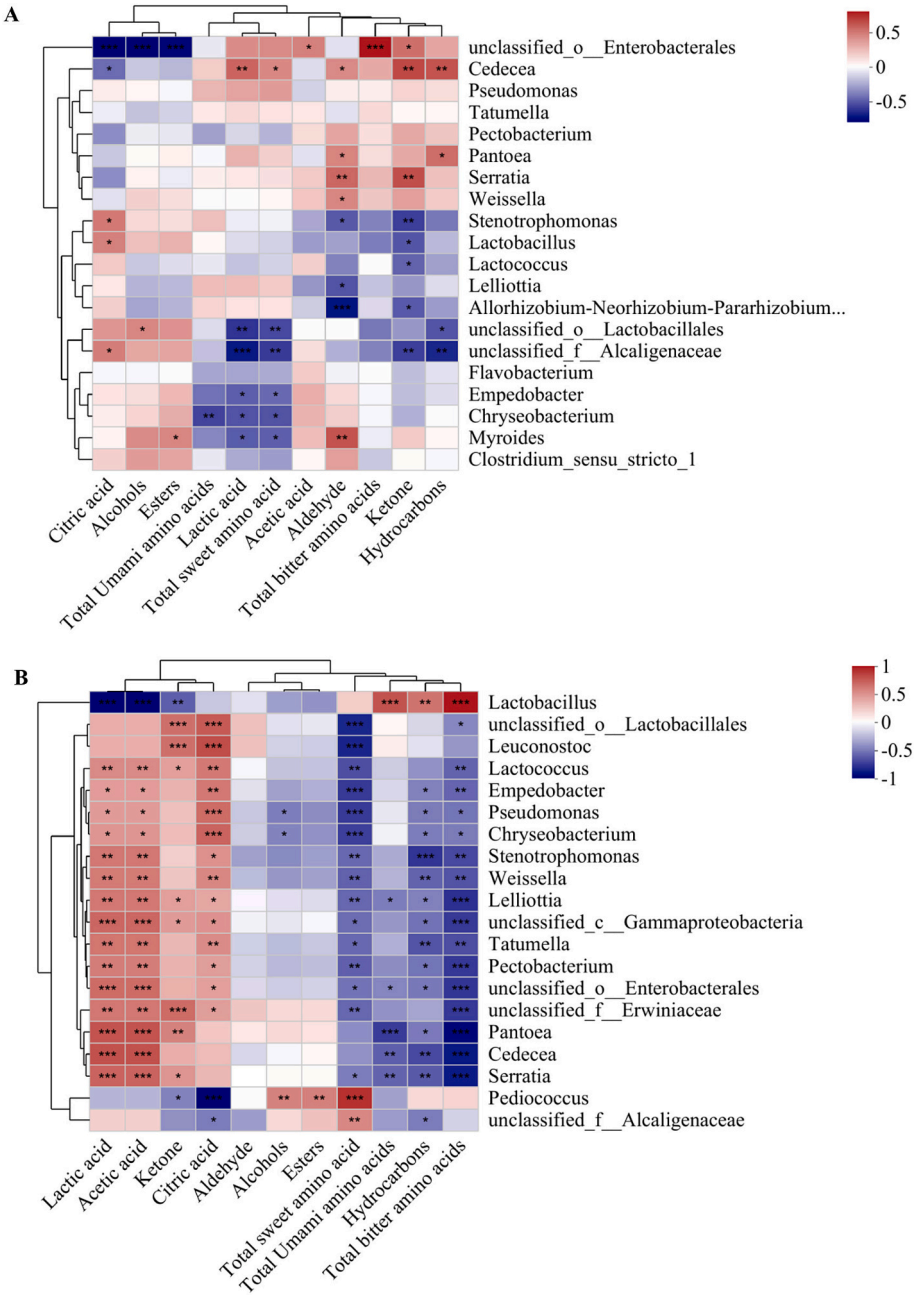


Fig. 5. Heat map of the correlation between flavor compounds and microbial communities of pickled pepper with diffferent salt concentrations (A-B: Bacteria, C-D: Fungi; A and C: Low salt concentration; B and D: High salt concentration). * represents the correlation between flavor compounds and species, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

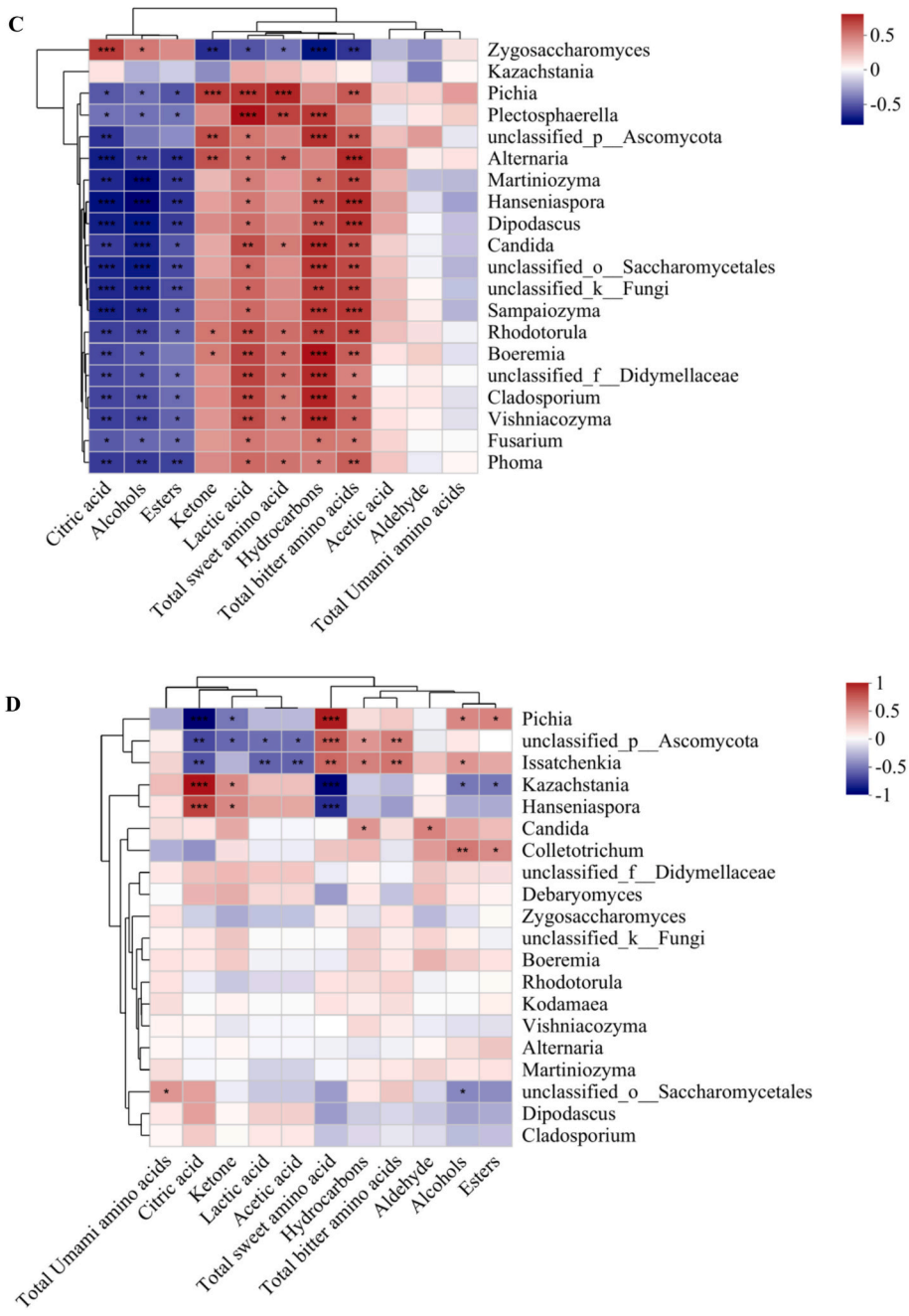


Fig. 5. (*continued*).

3.5. Correlations between microbial community and flavor compounds

Generally, the flavor compounds were associated with the microbiota in fermented food (Xiao et al., 2018). The Spearman's rank correlations were calculated to analyze the correlations of the top 20 abundant bacteria and fungi genera with flavor compounds, FAA, and organic acids in pickled peppers with different salt concentrations (Fig. 5A-D). For the D group, *Pediococcus* showed a significant positive correlation with alcohols, esters, and total sweet-amino acids ($p \leq 0.01$, Fig. 5A, colors closer to red represent a stronger positive correlation). Our results were similar to the report that *Pediococcus* could increase short chain fatty acids and esters during food fermentation (Liu, Wang, Sun, & Ni, 2020), and provided a new reference for the contribution of *Pediococcus* to the aroma of pickled peppers. In addition, various LAB genera (*Lactobacillus*, *Leuconostoc*, and *Lactococcus*) also have strong correlations with most flavor compounds during fermentation processes. Other genera had a positive correlation with ketone, lactic acid, acetic acid, and citric acid, while had a negative correlation with other flavor compounds. Among them, *Pantoea*, a common dominant bacterium in plants, was reported to produce volatile flavor compounds, such as phydroxyphenylethyl alcohol and methyl 4-hydroxybenzeneacetate, and possesses antibacterial properties (Shen et al., 2012). The correlations between flavor compounds and bacterial genera in the G group seem not as significant as that in the D group (Fig. 5C). *Cedecea*, *Pantoea*, and *Serratia* were positively correlated with ketone, hydrocarbons, aldehyde, lactic acid, and total sweet amino acids (TSAA), while were negatively correlated with citric acid. *Lactobacillus*, *Stenotrophomonas*, *Empedobacter*, *Chryseobacterium*, *Myroides*, and *Clostridium_sensu_stricto_1* showed a positive correlation with alcohols, esters, and citric acid. *Clostridium_sensu_stricto_1*, as an obligatory anaerobic bacterium, was

identified to be the functional microbes responsible for the production of characteristic flavor compounds (Guan et al., 2022).

As presented in Fig. 5C, based on the correlation analysis, *Pichia* and *Issatchenkia* in the fungal genera of the D group were positively correlated with TSAA, hydrocarbons, esters, and alcohols, while they were negatively correlated with citric acid, lactic acid, acetic acid, and ketone. *Candida* and *Colletotrichum* also showed a positive correlation with esters, alcohols, aldehyde, hydrocarbons, and ketone. He et al. (2019) found that *Candida* was involved in improving the flavor components of *Aspergillus*-type douchi during koji-making and significantly increased the content of amino acids, organic acids, and unsaturated fatty acids. Besides, *Kazachstania* and *Hanseniaspora* were positively correlated with citric acid, ketone, TUAA, lactic acid, and acetic acid, while they were negatively correlated with TSAA, alcohols, esters, and hydrocarbons. While for the G group, the top 20 abundant fungi genera, except for *Kazachstania* and *Zygosaccharomyces*, all presented a negative correlation with citric acid, alcohols, and esters, while presenting a positive correlation with ketone, lactic acid, TSAA, hydrocarbons and total bitter amino acids (Fig. 5D). The correlations between *Zygosaccharomyces* and above flavor compounds was exactly opposite to the above results. *Hanseniaspora* and *Pichia* are all non-*Saccharomyces* yeasts (Jolly, Varela, & Pretorius, 2014). Non-*saccharomyces* yeasts are usually found on the surface of fruits, which are the largest yeast group in the early stages of fermentation, and many publications and patents have revealed the importance of them (Casas-Godoy, Arellano-Plaza, Kirchmayr, Barrera-Martínez, & Gschaedler-Mathis, 2021). Most Non-*saccharomyces* yeasts cannot be fermented on their own or are not used to ferment wine, but they can have a positive effect on the quality and flavor after fermentation. Recently, there has been increasing interest in the use of Non-*saccharomyces* yeasts for acid reduction and taste enhancement through multi-strain fermentation (Liu, Laaksonen, Li, Gu, & Yang, 2022).

Due to the strong positive correlations between the genera and the flavor compounds in pickled peppers with different salt concentrations, they were the main microbial community that determined the flavor compounds in pickled peppers. Furthermore, the correlations between flavor compounds and main genera in fermented pickled peppers with different salt concentrations were greatly different.

3.6. Effect of salt concentration on metabolic pathways

The prediction of functional genes illustrates that the functions of bacterial genes were mostly related to metabolism (75.19%), environmental information processing (8.60%), genetic information processing (6.62%), cellular processes (4.25%), human diseases (3.74%), organismal systems (1.59%) (Fig. S3). Among the genes related to metabolism, 14.38% were related to carbohydrate metabolism, 8.92% to amino acid metabolism, 5.06% to energy metabolism, 4.73% to metabolism of cofactors and vitamins, 3.64% to nucleotide metabolism, and 2.98% to lipid metabolism. Thus, the microbial activity in fermented pickled peppers was mainly related to carbohydrate metabolism, amino acid metabolism, and energy metabolism. Previous studies have shown that the synergy between different nucleotides and amino acids has an important effect on taste (Sato, Ohgami, & Kaneniwa, 2015). Carbohydrate metabolism was more abundant in the D group, while amino acid metabolism was enriched in the G group. In general, genes involved in carbohydrate metabolism were more enriched than those related to amino acid metabolism. In previous studies, it has been proven that carbohydrate metabolism was closely related to the presence of *Firmicutes*, while amino acid and lipid metabolism was closely related to the presence of *Proteobacteria*, and these results corresponded to section 3.3.2 (Wang et al., 2021). Microorganisms were able to produce flavor compounds through amino acid metabolism, and the genes involved in amino acid metabolism may be related to the nutritional value of the product (Bhutia, Thapa, Shangpliang, & Tamang, 2021). These Functional predictions showed that the majority of the functional information on the bacterial community is beneficial in pickled peppers.

4. Conclusion

In this study, the physicochemical properties, characteristic flavor compounds, and microbial community of pickled peppers with different salt concentrations during natural fermentation were investigated, and the correlations between these factors were further explored. Different trends of physiochemical properties changes were observed in the pickled peppers with different concentrations during fermentation. Compared with the high salt concentration pickled peppers, the low salt concentration pickled peppers fermented for 30 d have a better texture properties. Meanwhile, 9 volatile flavor compounds were unique to the low salt concentration samples throughout the fermentation period, which also contained more and higher content of FAA, while 4 volatile flavor compounds were unique to the high salt concentration samples. Lactic acid and acetic acid were the two most abundant organic acids accumulated throughout fermentation in the low salt concentration samples while it was citric acid for the high salt concentration samples. *Pediococcus*, *Lactobacillus*, *Issatchenkia*, *Cedecca*, *Pichia*, *Kazachstania*, and *Hanseniaspora* were vital for forming the unique flavor of pickled peppers with different salt concentrations. In general, salt concentration has a significant influence on the quality of pickled peppers, and the quality of pickled peppers fermented with low salt concentration is better. Our work provided insights into the role of key microbial communities involved in the formation of the distinctive flavor of pickled peppers with different salt concentrations. The results may be helpful for the development of high-quality pickled pepper products. Further studies should deeply focus on the underlying mechanism of core functional microbial communities affecting the formation of characteristic flavors of pickled peppers with different salt concentrations during fermentation.

CRediT authorship contribution statement

Jianbo Tang: Resources, Project administration, Methodology, Investigation, Funding acquisition. **Xiaomeng Wu:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization. **Du Lv:** Writing – original draft, Visualization, Software. **Shan Huang:** Writing – original draft, Investigation, Formal analysis. **Yu Zhang:** Writing – review & editing, Software, Methodology. **Fanhua Kong:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization.

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.2024.101594.

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