npj | science of food

Article

Published in partnership with Beijing Technology and Business University & International Union of Food Science and Technology

https://doi.org/10.1038/s41538-025-00442-7

Mining of characteristic microbes and qualities in pickled and salted chili peppers through integrated analysis

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Fermentation techniques produce distinct microbes and qualities of various fermented chili peppers. However, comparative studies on chili peppers fermented using different techniques are limited. This study investigated the characteristic microbes and qualities of pickled and salted chili peppers through targeted and non-targeted analysis. The results revealed that *Lactiplantibacillus* and *Weissella* were the dominant microbes in pickled and salted chili peppers respectively. Additionally, pickled chilli peppers contained 6 key unique volatiles, with geraniol and *β*-myrcene showing significant positive correlation with *Lactiplantibacillus*. In contrast, salted chilli peppers contained 3 key unique volatiles, with no significant correlation with *Weissella*. Naringenin, trans-ferulic acid, and DL-*p*-hydroxyphenyllactic acid in pickled chili peppers exhibited significant positive correlation with *Lactiplantibacillus*. The weak correlation between *Weissella* and qualities in salted chili peppers may result from the inhibition of high-salt environment. This study provides insights for inoculated fermentation strategies to preserve microbial diversity and distinct flavors in different fermented chili peppers.

Chili pepper is an important part of the agricultural industry in Guizhou, and the largest chili pepper planting area is in Guizhou (>3070 km^2)[1]. Chili pepper is celebrated not only for their regional flavors but also for their nutritional benefits, making it a staple in the dietary habits of the population in Guizhou, where the per capita chili pepper consumption reaches 98.5 kg[2–4]. Natural fermentation is the predominant method for preserving and enhancing the sensory characteristics of chili peppers, which mainly includes two fermentation methods: pickling and salting[2,5,6]. Natural fermentation is influenced by the processing method with different fermentation conditions, which foster diverse microbial communities that play a crucial role in flavor and nutrition development, attributed to the biochemical transformations facilitated by microbes during the fermentation process[7,8]. Therefore, two kinds of fermented chili peppers have different qualities, with pickled chili peppers being predominantly sour and mainly used as a seasoning in Chinese Sichuan cuisine (e.g., pickled chili pepper beef), and salted chili peppers being predominantly salty and mainly used as a seasoning in Chinese Hunan cuisine (e.g., salted chili pepper fish head). Further, it is necessary to explore the main microbes and characteristic qualities of fermented chili peppers with different processing methods and to further differentiate between the two common types of fermented chili peppers (pickled and salted chili peppers), which can be better targeted at different fermented chili products for the creation of inoculated fermentation processing technology.

Recent advancements in multi-omics analysis have provided valuable insights into the complex interactions between microbial communities and the physicochemical properties of fermented foods, including microbiomes and metabolomes[9]. By employing next generation sequencing techniques, researchers can now elucidate the microbial diversity present during fermentation[6]. Furthermore, metabolomes enable the identification and quantification of volatile and non-volatile substances, providing a comprehensive understanding of the sensory attributes of fermented chili peppers[10]. Correlation analysis, inoculated fermentation experiments can further explore the role of dominant microbial metabolism in key flavor formation processes. Researches have studied that *Lactiplantibacillus*, *Weissella* and *Pediococcus* were the most abundant at the genus level in the fermented chili peppers from Guizhou provinces[3,6]. The key volatile and aroma-active substances of fermented chili peppers were identified as esters (e.g., methyl salicylate), alcohol (e.g., linalool, and phenylethyl alcohol), sour

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(e.g., n-Hexadecanoic acid) and so forth[6,11–13]. The non-volatile and nutritional substances of fermented chili peppers often contained elevated levels of bioactive substances, including antioxidants and vitamins, which contributed to health benefits such as improving digestion and enhancing immune response[14,15]. Additionally, flavonoids, crucial plant constituents exhibiting antioxidant activity, also could reduce the oxidative browning of chili pepper in low oxygen and acid fermentation environment, and make the fermented chili pepper maintain good color[16].

Due to different varieties of chili peppers and different fermentation environments caused by processing techniques, the dominant microbes in fermented chili peppers are different, thus forming a variety of fermented chili products with various flavors, such as pickled chili peppers, salted chili peppers, fermented chili sauce, zao chili peppers, kaili red sour soup and zha chili peppers. However, the existing studies only choose the fermented chili pepper processed by one technique as the experimental sample to explore its microbes and fermentation qualities, and there is a lack of research on the impact of different processing techniques on the formation of characteristic microbes and flavor profiles of the same chili pepper variety, which is not conducive to exploring the influence of environmental factors on microbial communities and fermentation quality. Therefore, this paper focused on two major types of fermented chili peppers - pickled and salted chili peppers. In addition, the time for fermented maturation of fermented chili peppers is not uniform in Guizhou, usually fermenting and maturing in 30 or 45 days. Therefore, in this paper, pickled and salted chili peppers with two-time endpoints of 30 and 45 days were selected as experimental samples, and the fermented chili peppers with better fermentation degree were further selected for investigation. Next generation sequencing was employed to analyze the dominant microbes. Additionally, ion chromatography (IC), high performance liquid chromatography (HPLC), and other techniques were used to determine targeted indicators, including total acid content (TA), pH, salt content, monosaccharides, organic acids, nitrites, and biogenic amines. High-resolution chromatography mass spectrometry was applied for non-targeted screening of volatile and non-volatile substances to identify novel and characteristic volatiles and non-volatiles. Ultimately, this study provides a comprehensive summary of the dominant microbes and characteristic qualities of pickled and salted chili peppers, providing valuable insights for future studies on the inoculated fermentation of these two common fermented chili peppers.

Results and discussion

Microbial analysis of dominant microbes

Figure 1a, b showed the microbial composition of pickled and salted chili peppers at genus level on days 30 and 45. Four non-fermentative microbes—*Raoultella*, *Serratia*, *Pseudomonas*, and *Pectobacterium*, constituted a significant portion of the microbial community in these products, originating from the planting environment of the chili pepper raw materials[2,17,18]. No significant differences were observed in these microbes between days 30 and 45, regardless of the fermentation process. However, *Pseudomonas* decreased in abundance in pickled chili peppers on day 45, likely due to its intolerance to high acidity[12].

Lactic acid bacteria (LAB) were common fermented microbe. In pickled chili peppers, *Lactiplantibacillus* exhibited the highest relative abundance, comprising 28.00% on day 30 and 42.99% on day 45. Other LAB, including *Weissella*, *Lactococcus*, and *Pediococcus*, accounted for 4.37% and 1.58%, 7.98% and 0.37%, 2.05% and 4.96% on day 30 and 45, respectively. A similar composition was observed in zha-chili, a regionally fermented chili pepper from Guizhou[19]. Additionally, the percentages of *Weissella* and *Lactococcus* in pickled chili peppers decreased over fermentation time, likely due to their intolerance to low pH, as noted in studies of fermented chili sauce from Northeast China[20,21]. *Weissella* was the dominant microbe in salted chili peppers, with relative abundances of 4.11% and 7.22% on days 30 and 45, while *Lactococcus* accounted for 1.42% and 2.78%, respectively, aligning with previous research[22]. However, the abundance of LAB in salted chili peppers was lower than pickled ones, which may be attributed to the high salt content used during the salted process, which was not conducive to the growth of LAB[22,23].

In summary, the proportion of LAB on day 45 was significantly higher than that on day 30, suggesting that a fermentation period of 45 days is optimal for the production of fermented chili peppers. Besides, as shown in Fig. 1c, d, the dominant LAB identified was *Lactiplantibacillus*, which exhibited acid tolerance in pickled chili peppers, and *Weissella*, which showed salt tolerance in salted chili peppers.

Salt, water activity, pH, TA

The salt content, water activity (aw), pH and TA content reflected the fermentation environment. As Fig. 2a shown, the salt content did not change significantly between days 30 and 45 for both pickled and salted chili peppers, as salt was not utilized by microbes during fermentation[24]. Notably, the salt content in salted chili peppers (20.45 g/100 g) was nearly 4 times than that of pickled chili peppers (5.28 g/100 g) on days 45. Because salted chili peppers were preserved primarily through salt, while pickled ones relied on both salt and acid, necessitating a higher addition of salt in salted processing. Further, the aw of salted chili peppers was lower than that of pickled chili peppers due to the higher salt content, measured at 0.785 ± 0.01 on days 45[6]. And as shown in Fig. 2b, the pH on days 45 of pickled and salted chili peppers was 3.71 and 4.49, respectively. As shown in Fig. 2e, the observed decrease in pH over time in pickled chili peppers could be attributed to the acid production by *Lactiplantibacillus* during fermentation, consistent with findings in Chinese pickled radishes[25]. The TA in pickled chili peppers was nearly six times higher than that in salted chili peppers, mainly due to the addition of acid during the pickling process and *Lactiplantibacillus* fermentation.

Overall, the salt content, aw, and TA content were stable between days 30 and 45, indicating a stable fermented environment over this period. However, the pH of pickled chili peppers fermented for 45 days was lower than those fermented for 30 days, reflecting the effects of *Lactiplantibacillus* fermentation. Pickled chili peppers had higher acid content and more pronounced sourness, while salted chili peppers had higher salt content and more salty taste.

Monosaccharides and organic acids

As shown in Fig. 2c, the galactose, glucose and fructose contents of both pickled and salted chili peppers at day 45 were lower than those at day 30 because of their provision of energy for microbial fermentation and continued utilization by *Lactiplantibacillus* and *Weissella*[26].

Eleven common organic acids were analyzed, including acetic acid, chlorogenic acid, D-(-)-quinic acid, citric acid, caffeic acid, ascorbic acid, aconitic acid, malic acid, L-lactic acid, vanillic acid and succinic acid. Among these organic acids, the content of acetic acid, D-(-)-quinic acid, citric acid, ascorbic acid, malic acid, L-lactic acid and succinic acid were higher than the other four organic acid, which were derived from materials or prduced byfermentation. Furthermore, the levels of four organic acids, acetic acid, citric acid, L-lactic acid and succinic acid, were significantly higher in pickled chili peppers, contributing to their enhanced sourness[27], of which L-lactic acid and acetic acid were only detected in pickled chili peppers. Thus, as shown in Fig. 2e, the higher contents of four organic acids in pickled chili peppers were attributed to the exogenous addition of citric and acetic acids, as well as acid production by *Lactiplantibacillus* through both homo- and hetero-fermentation processes[28].

In summary, glucose and fructose were the main sources of energy required for microbial fermentation. Besides, glucose and fructose were the main sources of sweetness in fermented chili peppers. Pickled chili peppers had a greater variety and content of organic acids, with L-lactic, acetic, citric and succinic acids being the main characteristic acidic substances.

Nitrite and biogenic amines

Figure 2d presented indicators related to safety during fermentation, including nitrite and nine biogenic amines. The nitrite content in pickled and salted chili peppers was ranged from 0.22-0.28 mg/kg, well below the

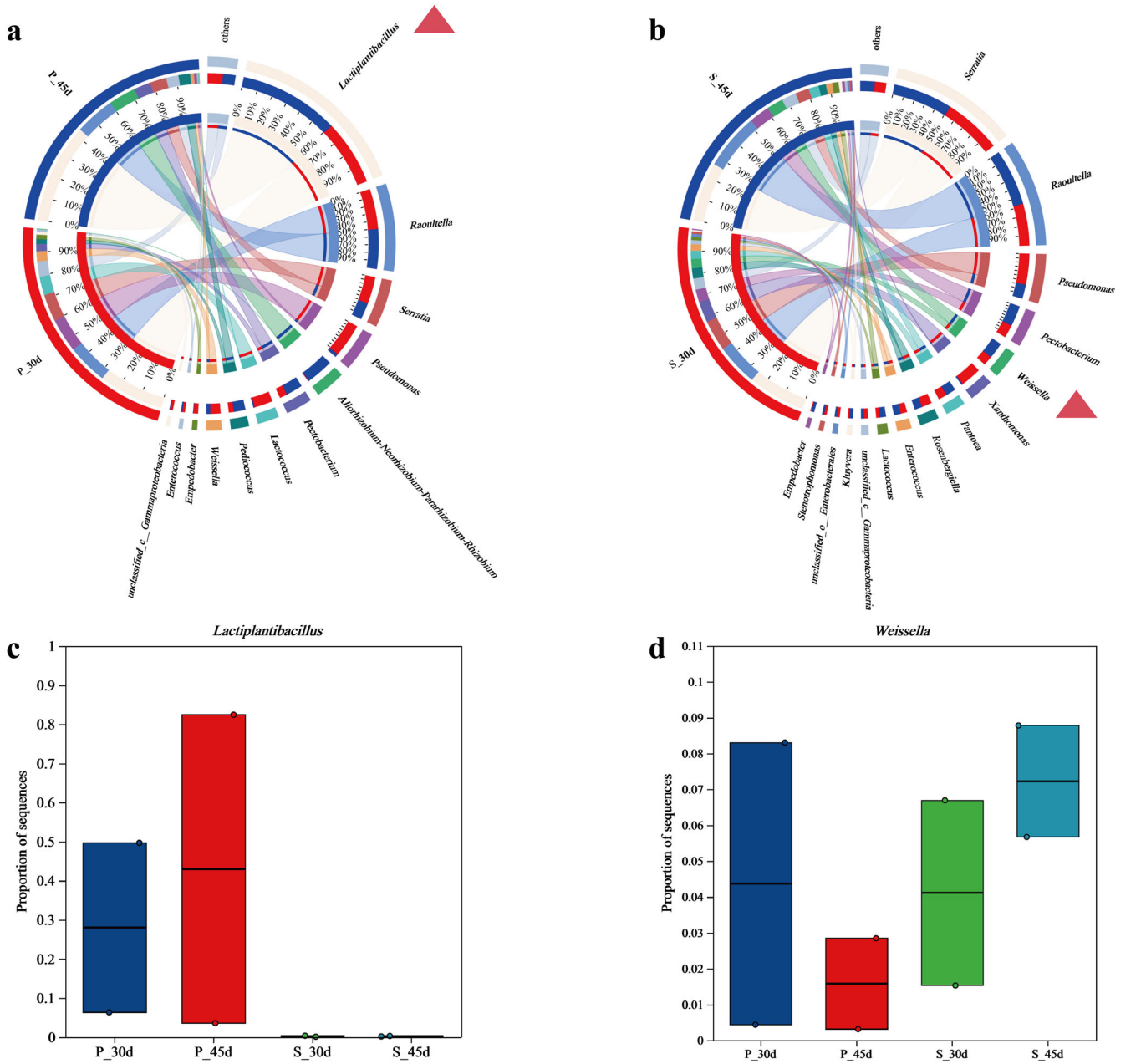

Fig. 1 | Microbial composition at genus level and statistical analysis of *Lactiplantibacillus* and *Weissella* in pickled and salted chili peppers. Microbial composition at genus level of pickled (**a**) and salted chili peppers (**b**); the one-way ANOVA on genus level of *Lactiplantibacillus* (**c**) and *Weissella* (**d**). (P_30 d, P_45 d: pickled chili peppers at 30 days and 45 days; S_30 d, S_45 d: salted chili peppers at 30 days and 45 days).

limit of 20 mg/kg set by the Chinese national standard[29]. Tryptamine, phenylethylamine, and histamine were not detected. In pickled chili peppers, the content of putrescine, cadaverine, octopamine, tyramine, spermidine and spermine was approximately 6–15 mg/kg on day 30, decreasing to 0.7–2.6 mg/kg by day 45. In salted chili peppers, the content of putrescine, cadaverine, tyramine, spermidine, and spermine was around 0.2–4 mg/kg on day 30 and remained at about 0.5–4 mg/kg on day 45. Overall, the content of biogenic amines was significantly lower than the 750 mg/kg threshold[30], indicating that both pickled and salted chili peppers met the safety standards for food consumption.

Furthermore, as shown in Fig. 2e, the content of biogenic amines in pickled chili peppers was higher than that in salted ones due to the higher percentage of LAB (*Lactiplantibacillus*) among microbial communities. Because studies reported that *Lactiplantibacillus* and *Pediococcus* were the main LAB which possessed the amino acid decarboxylase gene that was responsible for producing biogenic amines[20,31]. Some strains of *Lactiplantibacillus* and *Pediococcus* have been shown to degrade biogenic amines through the action of amine oxidase enzymes, as observed in wine and soy sauce[32]. Therefore, a 45-day fermentation was preferable, as it had lower content of biogenic amines compared to 30 days.

The composition of total volatile substances

As shown in Fig. 3a, b, the volatile substances of pickled and salted chili peppers fermented for 30 and 45 days were different. Further, the volatile substances of chili peppers fermented for 30 and 45 days were analyzed comparatively using the screening criteria of FC > 2 and $p < 0.05$, and it was found that chili peppers fermented for 45 days contained more volatile substances (as shown in the yellow highlighted part in the volcano diagram), especially the salted peppers. Therefore, in this paper, we chose the chili peppers fermented for 45 days as samples to further study their volatile

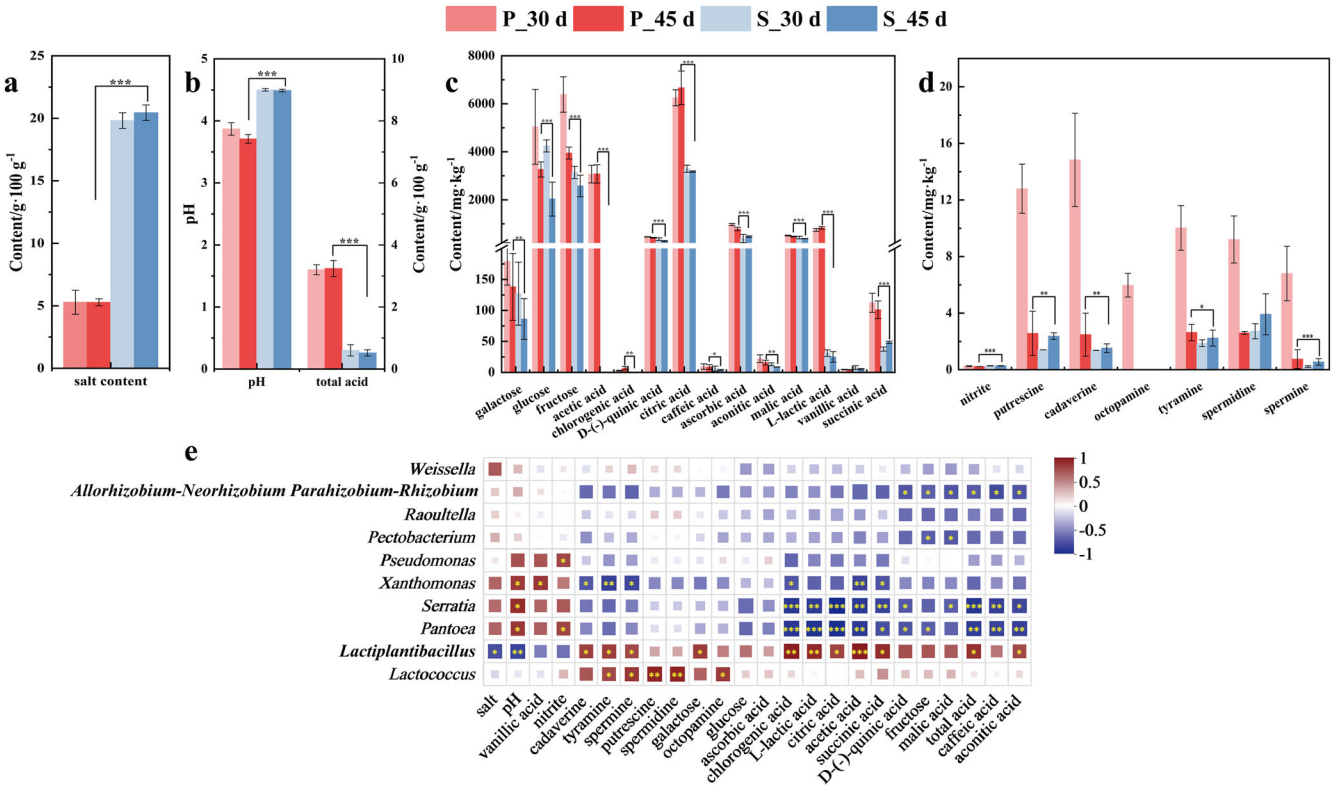

Fig. 2 | Physicochemical properties and microbial correlation analysis of pickled and salted chili peppers. Physicochemical properties through targeted analysis of pickled and salted chili peppers (**a**–**d**); correlation analysis with microbes (**e**). (P_30 d, P_45 d: pickled chili peppers at 30 days and 45 days; S_30 d, S_45 d: salted chili peppers at 30 days and 45 days). (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

substances. As Fig. 3c presented, there were eight types of volatile substances identified in pickled and salted chili peppers: ester, alkane, olefin, alcohol, phenol, ketone, aldehyde, and acid. In addition, as shown in Fig. 3d, esters and alcohols presented the highest content of all volatile substances. Previous studies have also reported that esters were the predominant volatile substances in fermented chili peppers[6,33].

The shared volatile substances in pickled and salted chili peppers

Among 64 volatile substances, a total of 29 volatile substances were detected in both pickled and salted chili peppers (as listed in Table S1). Among them, 7 volatile substances significantly contributed to the flavor of fermented chili peppers, as indicated by their OAV > 1. These included methyl salicylate, methyl palmitate, linalool, phenethyl alcohol, benzyl alcohol, *β*-ionone, and (*Z*)-*β*-ocimene, which have been frequently identified as key volatile substances in both fermented and fresh chili peppers[11,34]. Furthermore, as shown in Figs. 3e and 3f, among 7 volatile substances, none was found to be significantly correlated with *Lactiplantibacillus* and *Weissella*, so these volatile substances may be related to raw materials of fresh chili peppers. These substances imparted a range of flavors, including peppermint, citrus, honey, woody, and floral notes[11,34–36].

The unique volatile substances in pickled and salted chili peppers

As Table 1 shown, there were 20 volatile substances only detected in pickled chili peppers, which could regard as the unique volatile substances of pickled chili peppers. And there were 6 volatile substances with OAV > 1 that could be used as key unique volatile substances in pickled chili peppers, and they were ethyl 2-hydroxybenzoate (OAV = 59.95), dihydro-*β*-ionone (OAV = 77), guaiacol (OAV = 245.83), 2-methoxy-4-methylphenol (OAV = 14.67), *β*-myrcene (OAV = 79.17) and geraniol (OAV = 303.64), providing sweet and fruity[37], violet and citrus[38], smoky and woody[39], strong lilac[40], and spicy aroma[11]. Among them, two of the terpenoids, geraniol and *β*-myrcene, were significantly and positively correlated with *Lactiplantibacillus*, because *Lactiplantibacillus* produced acids such as L-lactic acid during fermentation, which could damage plant cells and lead to the release of terpenoids. *Lactiplantibacillus* possessed terpene synthases, involved in terpene biosynthesis and biochemical reactions[41].

As Table 2 shown, 25 volatile substances were exclusively found only in salted chili peppers, which could be regarded as the unique volatile substances of salted chili peppers. Among them, a total of 3 volatile substances of OAV > 1, 4-ethylphenol, 4-ethyl-2-methoxyphenol, and 2-methoxy-4-vinylphenol, were found to provide strong phenolic, sweet vanilla and lightly fruity aroma[42–44]. However, as Fig. 3f shown, no substance in salted chili peppers was found to be significantly and positively correlated with *Weissella*. This suggested that the changes in volatile substances in salted chili peppers were mainly related to salting and weakly related to LAB fermentation. After chopping and salting, a large amount of phenolic acids, tyrosine, and other substances in chili peppers were released, which may be metabolized and converted into 4-ethylphenol, 4-ethyl-2-methoxyphenol, and 2-methoxy-4-vinylphenol through enzymatic reaction[45]. In addition, due to the strong salt tolerance of yeast, the above three substances may also be obtained through fermentation with salt tolerant yeast[46].

The composition of total non-volatile substances

As shown in Fig. 4a, b, the non-volatile substances of chili peppers fermented for 30 and 45 days were analyzed comparatively using the screening criteria of FC > 2 and $p < 0.05$, and it was found that there was no difference in non-volatile substances between 30 and 45 days fermented chili peppers. In keeping with the study of volatile substances, we selected 45-day fermentation samples for analysis of non-volatile substances.

As Fig. 4c presented, there were nine types of non-volatile substances identified in pickled and salted chili peppers: phenylpropanoids and polyketides, organic acids and their derivatives, organoheterocyclic substances, organic nitrogen substances, nucleosides, nucleotides and their analogues, organic oxygen substances, benzenoids, lipids and lipid-like molecules and homogeneous. Phenylpropanoids and polyketides were the most non-volatile substances, which contributed to the

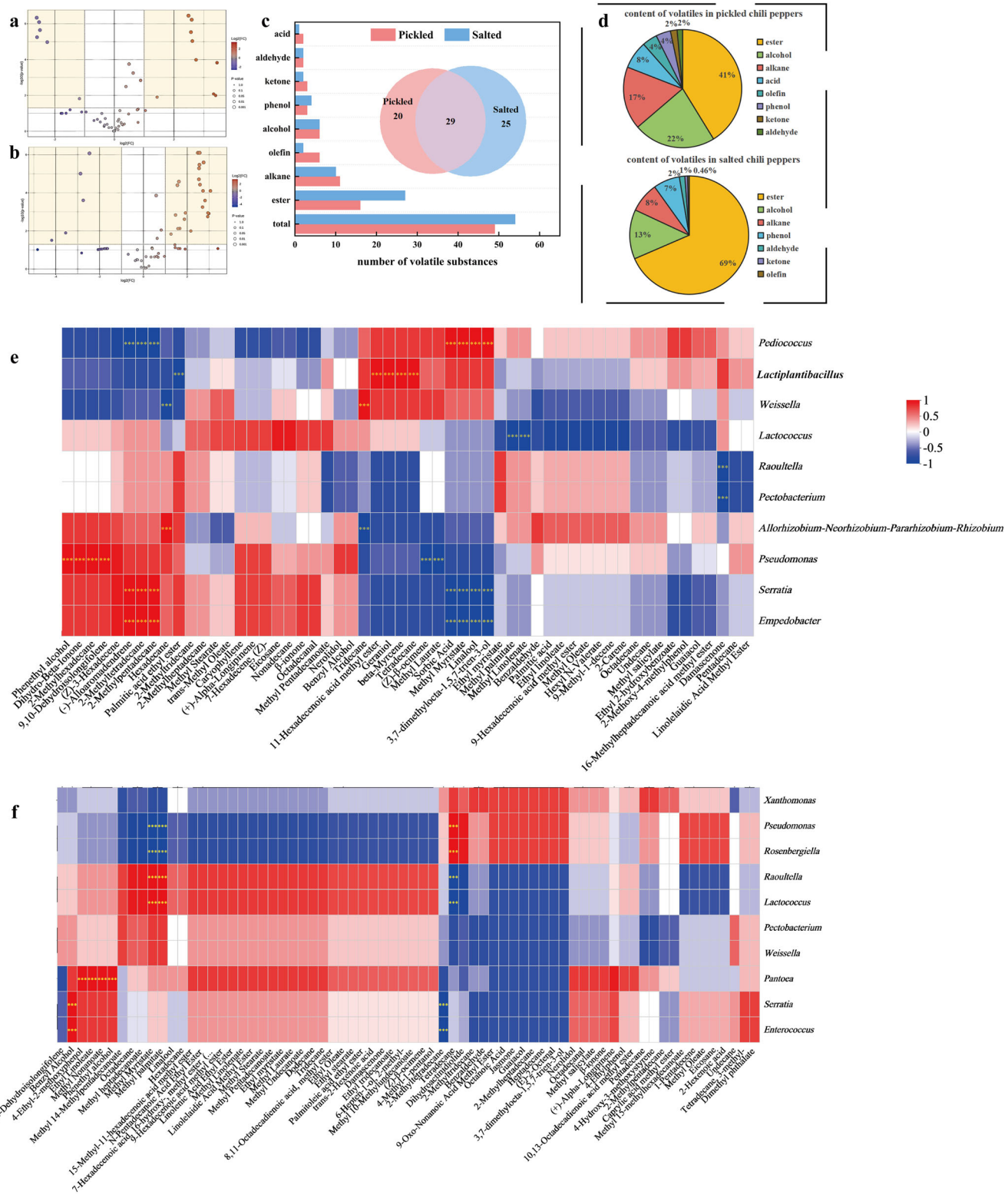

Fig. 3 | Volatile substances of pickled and salted chili peppers at 30 and 45 days and their correlation with microbes. Volatile substances of pickled and salted chili peppers at 30 and 45 days: analysis of differences by volcano plots (FC > 2, $p < 0.05$) (**a**, in pickled ones; **b**, in salted ones); composition and classification of volatile substances (**c**, **d**); correlation analysis with microbes (**e**, in pickled ones; **f**, in salted ones). (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

properties of antioxidant activity[47]. The organic acids and derivatives in chili peppers mainly consisted with amino acids, peptides and their analogues, which were responsible for the umami flavor and nutrition[34]. As for the other kinds of substances with less abundance in chili peppers, they were closely related to life activities.

The novel non-volatile substances in fermented chili peppers

As shown in Fig. 5, 11 non-volatile substances were detected for the first time in fermented chili peppers. Among them, 6 substances belonged to phenylpropanoids and polyketides, including 4-*O*-*β*-D-glucosyl-4-coumaric acid, apigenin 6,8-digalactoside, isorhamnetin 3-galactoside, 3-phenyllactic

Table 1 | The qualitative and semi-quantitative results of volatile substances only in pickled chili peppers

Classification	substances	Formula	CAS	Concentration (mg/kg)	OT (mg/kg)	OAV
Ester (6)	Ethyl 2-hydroxybenzoate	$C_9H_{10}O_3$	118-61-6	0.256 ± 0.034	0.084	59.95
	11-Hexadecenoic acid methyl ester	$C_{17}H_{32}O_2$	132170-71-9	0.123 ± 0.098	/	/
	Palmitic acid ethyl ester	$C_{18}H_{36}O_2$	628-97-7	0.567 ± 0.060	2	< 1
	Hexyl N-valerate	$C_{11}H_{22}O_2$	1117-59-5	0.170 ± 0.099	/	/
	16-Methylheptadecanoic acid methyl ester	$C_{19}H_{38}O_2$	5129-61-3	0.098 ± 0.039	/	/
	Methyl oleate	$C_{19}H_{36}O_2$	112-62-9	0.177 ± 0.022	/	/
Olefin (5)	*β*-Myrcene	$C_{10}H_{16}$	123-35-3	0.095 ± 0.041	0.0012	79.17
	9-Methyl-1-decene	$C_{11}H_{22}$	61142-78-7	0.370 ± 0.225	/	/
	(*Z*)-3-Hexadecene	$C_{16}H_{32}$	34303-81-6	0.057 ± 0.075	/	/
	(-)-Alloaromadendrene	$C_{15}H_{24}$	25246-27-9	0.173 ± 0.082	/	/
	2-Carene	$C_{10}H_{16}$	554-61-0	0.141 ± 0.018	/	/
Alkane (2)	2-Methylhexadecane	$C_{17}H_{36}$	1560-92-5	0.234 ± 0.055	/	/
	2-Methylheptadecane	$C_{18}H_{38}$	1560-89-0	0.080 ± 0.046	/	/
Acid (2)	Sorbic acid	$C_6H_8O_2$	110-44-1	0.230 ± 0.022	/	/
	Palmitic acid	$C_{16}H_{32}O_2$	57-10-3	1.528 ± 0.328	/	/
Phenol (2)	Guaiacol	$C_7H_8O_2$	90-05-1	0.118 ± 0.035	0.00048	245.83
	2-Methoxy-4-methylphenol	$C_8H_{10}O_2$	93-51-6	0.308 ± 0.038	0.021	14.67
Alcohol (2)	3,7-Dimethylocta-1,5,7-trien-3-ol	$C_{10}H_{16}O$	29957-43-5	0.537 ± 0.193	/	/
	Geraniol	$C_{10}H_{18}O$	106-24-1	0.334 ± 0.158	0.0011	303.64
Ketone (1)	Dihydro-*β*-Ionone	$C_{13}H_{22}O$	17283-81-7	0.077 ± 0.043	0.001	77

OT odor thresholds in water.
OAV odor activity value.

acid, DL-*p*-hydroxyphenyllactic acid and 6"-malonylgenistin, which were generally proven to have antioxidant and anti-inflammatory properties.

Besides, 4 substances belonged to peptides, including Ne, Ne, Ne trimethyllysine, 1-methyl-L-*α*-aspartyl-L-phenylalanate, Gly-Leu and isoleucylisoleucine, which may enhance the nutritional value, and had a positive impact on the flavor and taste of fermented chili peppers[34]. For example, Ne, Ne, Ne trimethyllysine is a derivative of lysine that mainly participates in regulating neurotransmitters, lipid metabolism, and immune function[48–50]. 1-Methyl-L-*α*-aspartyl-L-phenylalanate is a methyl ester of the dipeptide of the amino acid aspartic acid and phenylalanine[51], that may improve cognitive function[52]. Gly-Leu is a dipeptide of glycine and leucine, which have antioxidant and anti-inflammatory properties to improve the freshness and antioxidant capacity of chili peppers[53]. Isoleucylisoleucine is a dipeptide composed of isoleucine, which is used to promote the production of glucose to provide immediate energy for the body, so it may have anti fatigue and enhanced exercise endurance effects[54].

One of the remaining substances, acetylcholine, belonged to organonitrogen, which is a neurotransmitter that prevents Alzheimer's disease[55].

The shared non-volatile substances in pickled and salted chili peppers

Among the 62 non-volatile substances, a total of 59 shared nonvolatile substances were detected in pickled and salted chili peppers (as listed in Table S2), i.e., the non-volatile substances in pickled and salted chili peppers were almost identical, and it was possible that the non-volatile substances were less affected by the fermentation process, and were mainly related to the varieties of the raw materials of fermented chili peppers. Furthermore, as shown in Fig. 4d, there were two non-volatile substances with significantly positive correlation with *Lactiplantibacillus*, namely naringenin and trans-ferulic acid, which could be regarded as characteristic non-volatile substances of pickled chili peppers fermented with *Lactiplantibacillus*. It has been shown that *Lactiplantibacillus* could produce glycosidic bond cleavage enzymes that broke down naringenin-7-*O*-rutinoside into naringenin, which had higher bioavailability[56]. Similarly, *Lactiplantibacillus* was able to convert polyphenols into active phenolic metabolites such as trans-ferulic acid[57].

The unique non-volatile substances in pickled chili peppers

As Table S2 shown, there were three non-volatile substances only detected in pickled chili peppers, namely 3-phenyllactic acid, DL-*p*-hydroxyphenyllactic acid and acetycholine. 3-Phenyllactic acid and DL-*p*-hydroxyphenyllactic acid are aromatic and phenolic acids that result from the catabolism of phenylalanine by *Lactiplantibacillus*[58–60]. Research indicated that *Lactiplantibacillus* can produce acetylcholine through esterification, enhancing its biological activity[61]. What's more, DL-*p*-hydroxyphenyllactic acid had significantly positive correlation with *Lactiplantibacillus*, which could be regarded as characteristic non-volatile substance of pickled chili peppers fermented with *Lactiplantibacillus*. However, as Fig. 4e shown, there was no non-volatile substance with positive correlation with *Weissella*.

Antioxidant activities of pickled and salted chili peppers

The DPPH values of pickled chili peppers fermented for 30 and 45 days were 2.16 ± 0.09 mmol/kg and 2.00 ± 0.03 mmol/kg, respectively, while those of salted chili peppers were 2.07 ± 0.22 mmol/kg and 1.93 ± 0.05 mmol/kg, respectively. Therefore, it was found that there was no significant difference in DPPH values between pickled and salted chili peppers ($p > 0.05$). In addition, the FRAP values of pickled chili peppers fermented for 30 and 45 days were 14.65 ± 0.58 mmol/kg and 12.96 ± 2.10 mmol/kg, respectively, and the FRAP values of salted chili peppers were 7.91 ± 0.45 mmol/kg and 6.44 ± 0.37 mmol/kg, respectively. Therefore, it was found that the FRAP values of pickled chili peppers were significantly higher than those of salted chili peppers ($p < 0.001$).

Table 2 | The qualitative and semi-quantitative results of volatile substances only in salted chili peppers

Classification	substances	Formula	CAS	Concentration (mg/kg)	OT (mg/kg)	OAV
Ester (17)	Methyl 10-methylundecanoate	$C_{13}H_{26}O_2$	5129-56-6	0.200 ± 0.039	/	/
	Methyl nonanoate	$C_{10}H_{20}O_2$	1731-84-6	0.067 ± 0.029	/	/
	Caprylic acid methyl ester	$C_9H_{18}O_2$	111-11-5	0.065 ± 0.021	0.2	< 1
	Methyl undecanoate	$C_{12}H_{24}O_2$	1731-86-8	0.050 ± 0.015	/	/
	Ethyl tridecanoate	$C_{15}H_{30}O_2$	28267-29-0	0.053 ± 0.022	/	/
	Methyl stearate	$C_{19}H_{38}O_2$	112-61-8	0.227 ± 0.133	/	/
	Linolenic acid ethyl ester	$C_{20}H_{34}O_2$	1191-41-9	0.170 ± 0.054	/	/
	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	5129-60-2	0.041 ± 0.048	/	/
	7-Hexadecenoic acid, 16-hydroxy-, methyl ester, (*Z*)-	$C_{17}H_{32}O_3$	88785-30-2	0.197 ± 0.063	/	/
	N-Pentadecanoic acid ethyl ester	$C_{17}H_{34}O_2$	41114-00-5	0.137 ± 0.046	/	/
	Palmitoleic acid ethyl ester	$C_{18}H_{34}O_2$	56219-10-4	0.032 ± 0.014	/	/
	Methyl heptadecanoate	$C_{18}H_{36}O_2$	1731-92-6	0.075 ± 0.023	/	/
	15-Methyl-11-hexadecenoic acid methyl ester	$C_{18}H_{34}O_2$	55044-54-7	0.087 ± 0.027	/	/
	Ethyl stearate	$C_{20}H_{40}O_2$	111-61-5	0.062 ± 0.010	/	/
	Ethyl oleate	$C_{20}H_{38}O_2$	111-62-6	0.063 ± 0.016	/	/
	10,13-Octadecadienoic acid methyl ester	$C_{19}H_{34}O_2$	56554-62-2	0.010 ± 0.012	/	/
	8,11-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	18287-22-4	0.029 ± 0.005	/	/
Phenol (3)	4-Ethylphenol	$C_8H_{10}O$	123-07-9	0.222 ± 0.270	0.013	17.08
	4-Ethyl-2-methoxyphenol	$C_9H_{12}O_2$	2785-89-9	0.589 ± 0.611	0.0044	133.86
	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	7786-61-0	0.153 ± 0.135	0.003	51
Alcohol (2)	4-Methyl-1-pentanol	$C_6H_{14}O$	626-89-1	0.094 ± 0.027	0.82	< 1
	6-Hepten-1-ol, 2-methyl-	$C_8H_{16}O$	67133-86-2	0.029 ± 0.004	/	/
Alkane (1)	Dodecane	$C_{12}H_{26}$	112-40-3	0.058 ± 0.011	10	< 1
Acid(1)	trans-2-Hexenoic acid	$C_6H_{10}O_2$	13419-69-7	0.140 ± 0.073	/	/
Olefin (1)	(+)-α-Longipinene	$C_{15}H_{24}$	5989-08-2	0.020 ± 0.023	/	/

OT odor thresholds in water.
OAV odor activity value.

Rumpf et al. [62] found that DPPH values indicated the antioxidant activity of hydrophobic substances, and the FRAP values indicated the antioxidant activity of hydrophilic substances. Therefore, fermented chili peppers had a high content of hydrophilic antioxidant substances, such as phenylpropanoids and polyketides. What's more, it indicated that the content of hydrophilic antioxidant substances in pickled chili peppers was higher than that in salted chili peppers. The results in 2.3 and 2.11 found that the substances with high content in pickled chili included citric acid, acetic acid, succinic acid, L-lactic acid, 3-phenyllactic acid, DL-*p*-hydroxyphenyllactic acid and acetycholine, which all had certain antioxidant activity as hydrophilic substances. This may be the reason why pickled chili peppers had better antioxidant activity related to FRAP.

Methods

Samples collection

As shown in Fig. 6, approximately 13 tons of chili peppers were fermented in a brine solution. The 38-ton fermentation system, comprising both chili peppers and brine, included 85 kg of salt, 0.5 kg of sodium metabisulfite, 6 kg of citric acid, 4 kg of acetic acid, and 3 kg of calcium chloride per ton to maintain adequate salinity and acidity. The fermentation pool was then sealed with a plastic sheet and sticks for either 30 or 45 days. During the initial 7 days, the brine was circulated using a machine to ensure thorough mixing of the additives with the water. Additionally, salted chili peppers were prepared by mixing chopped chili peppers with salt (8:2, *w*/*w*) and fermenting them in a three-layer nylon bag for either 30 or 45 days. This bag consisted of an innermost plastic layer for food contact, a black plastic layer in the middle for UV blocking, and a coarse woven layer on the outside for dust protection, oxygen-proofing, and insulation.

In this study, we collected pickled chili pepper samples (P_30 d/45 d) and salted chili pepper samples (S_30 d/45 d) after 30 and 45 days of fermentation. And we selected three pools of pickled chili peppers and three bags of salted chili peppers as biological replicates. The samples were collected using sterile sampling bags, with a portion of the samples immediately used for water activity, salt content, pH, and TA determination after collection, and a portion of the samples stored in a refrigerator at −80 °C for the determination of remaining indicators.

Aw and salt content

The determination of aw was conducted using a fast aw meter (HD-4, Huake Instrument Co., Wuxi, China). The method for determining salt content adhered to research of Peng et al. [6], with revisions made to the sample pre-treatment procedure. Specifically, 5 g of crushed samples (accurate to 0.01 g) were placed in a 100 mL stoppered cuvette, followed by the addition of 50 mL of hot water at 70 °C. The mixture was sonicated for 20 minutes, allowed to cool to room temperature (20–25 °C), then diluted with water to a final volume of 100 mL and shaken thoroughly. Subsequently, 5 mL of the test solution was pipetted into a 250 ml triangular flask, to which 50 ml of water and 1 mL of 10% potassium chromate solution were added. While shaking, drops of silver nitrate standard titration solution (0.1 mol/L) were added until the color

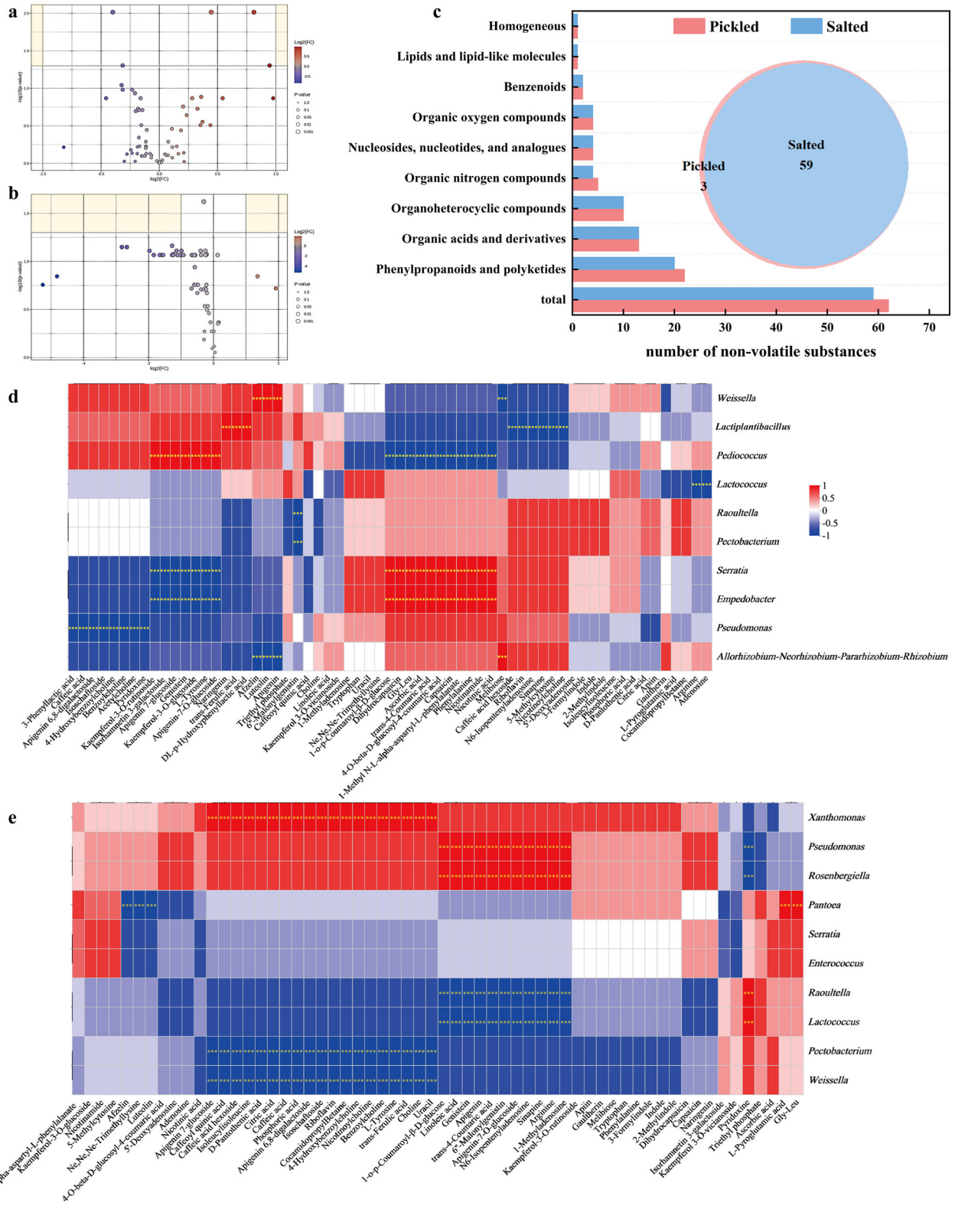

Fig. 4 | Non-volatile substances of pickled and salted chili peppers at 30 and 45 days and their correlation with microbes. Non-volatile substances of pickled and salted chili peppers at 30 and 45 days: analysis of differences by volcano plots (FC > 2, $p < 0.05$) (**a**, in pickled ones; **b**, in salted ones); composition and classification of non-volatile substances (**c**); correlation analysis with microbes (**d**, in pickled ones; **e**, in salted ones). (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

changed from yellow to orange, maintaining the color for 1 min without fading. The volume of silver nitrate standard titration solution consumed was recorded. Each sample underwent three biological replicates and two extraction replicates.

pH and TA

The pH was determined following the method of Wen et al. [63]. The juice from the crushed sample was extracted using three layers of gauze, and the pH electrode (PHS-3E, Shanghai Yidian Scientific

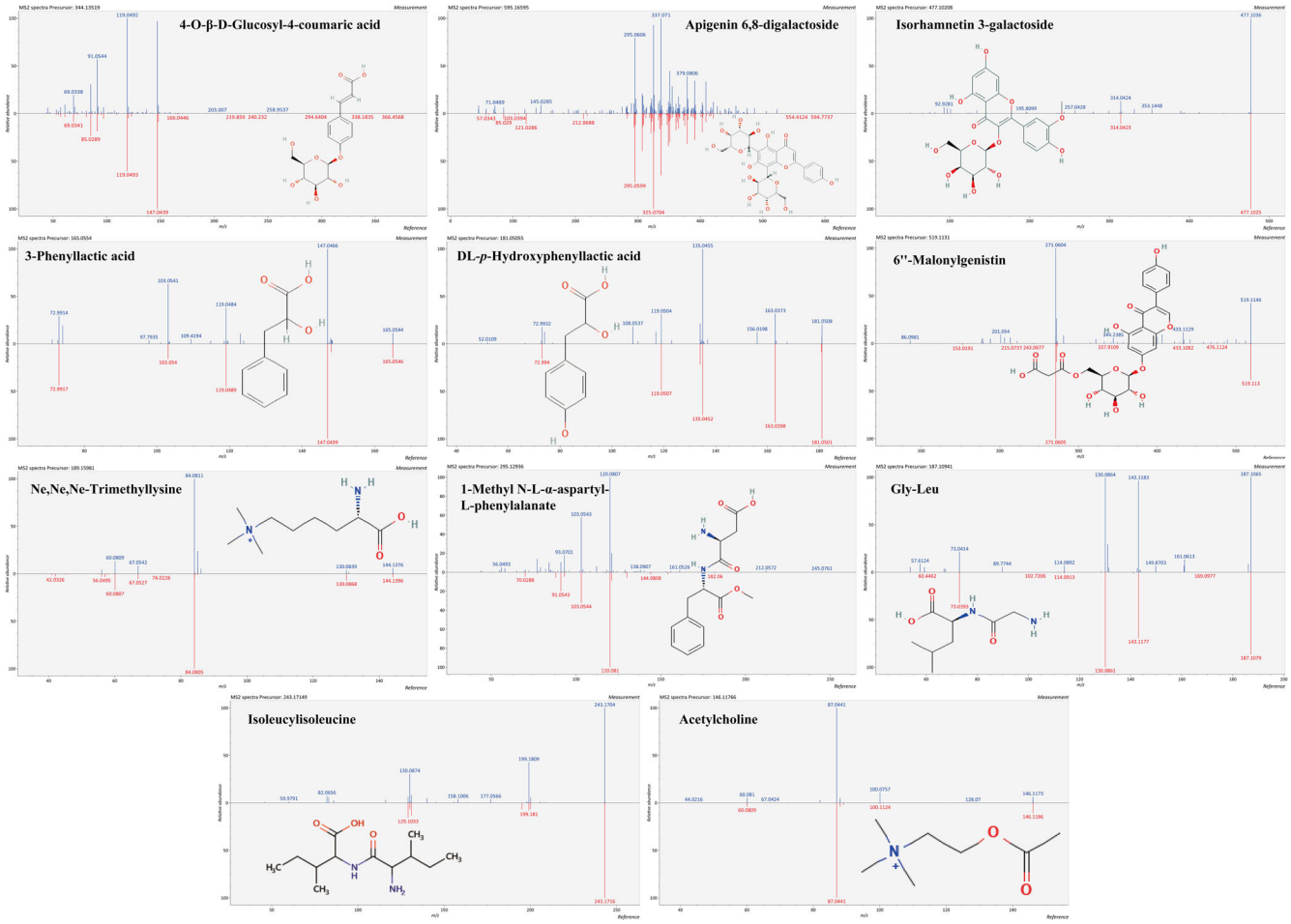

Fig. 5 | Mirror maps and structural formulas of eleven novel non-volatile substances in pickled and salted chili peppers.

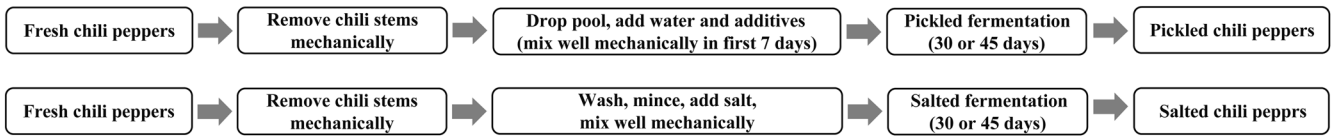

Fig. 6 | Natural fermentation processing of pickled and salted chili peppers in Guizhou, China.

Instrument Co., Shanghai, China) was immersed in the specimen for measurement.

TA was determined using the acid-base indicator titration method. A 10 g sample (accurate to 0.01 g) was weighed into a 250 mL volumetric flask, followed by the addition of 50 mL of water at 80 °C. The mixture was thoroughly mixed and then placed in a boiling water bath for 20 minutes, with shaking 2–3 times to ensure complete dissolution of all organic acids in the solution. After cooling to room temperature (20–25 °C), the solution was diluted to 250 mL with water and shaken well. Subsequently, 10 mL of the test solution was pipetted into a 250 mL triangular flask, to which 50 mL of de-carbonated purified water was added, along with 3 drops (10 g/L) of phenolphthalein indicator. The solution was titrated with 0.1 mol/L sodium hydroxide standard titrimetric solution until a slight red color persisted for 30 s without fading, and the volume of the 0.1 mol/L sodium hydroxide titrimetric solution consumed was recorded. Each sample was analyzed with three biological replicates and two extraction replicates.

Monosaccharides

A sample weighing 2.50 g (accurate to 0.01 g) was placed into a 50 mL plastic centrifuge tube, to which 25 mL of ultrapure water was accurately added. The mixture was subjected to ultrasonic treatment for 30 min at 20 °C, followed by centrifugation at 12,000 × g for 15 minutes at 20 °C. The supernatant was diluted ten-fold and then filtered through the 0.22 μm aqueous PES membrane. Each sample included three biological replicates and two extraction replicates.

IC (ICS 3000 +, ThermoFisher Scientific, USA) method for monosaccharides determination was based on the research of Wang et al. [64].

Organic acids

Organic acids content was determined following the method of Wen et al. [65] with modifications. Ground samples were prepared using a freeze grinder (Ikea Co, Sweden) with liquid nitrogen. Approximately 1 g (accurate to 0.0001 g) of ground samples were transferred into a 5 mL centrifuge tube. Extraction was performed by adding 3 mL of 70% methanol solution (*v*/*v*) followed by ultrasonic treatment for 10 min. Subsequently, the samples were centrifuged at 10,000 × g and 4 °C for 10 minutes. The supernatant was collected and the residue was subjected to two additional extractions under the same conditions. All extracts were combined, diluted to 10 mL with 70% methanol (*v*/*v*), and filtered through a 0.22 μm nylon membrane prior to analysis. A blank test was

conducted using the same procedure, and data processing was conducted according to reference.

Organic acids were analyzed using ultra-high performance liquid chromatography-tandem triple quadruple mass spectrometry (UPLC-QqQ-MS/MS) (Waters Xevo TQ-S, Waters Corporation, Massachusetts, USA) equipped with an HSS T3 column (1.8 μm, 2.1 × 150 mm, ACQUITY UPLC® HSS T3, Waters Corporation, Massachusetts, USA). The UPLC conditions included a column temperature of 30 °C, a flow rate of 0.2 mL/min, and an injection volume of 1 μL. Mobile phase A was 0.1% formic acid in water (*v/v*), and mobile phase B was methanol. The gradient elution conditions were as follows: 0–0.5 min, 2% B; 0.5–4 min, 2–35% B; 4–8 min, 35–50% B; 8–12 min, 50–2% B; 12–16 min 2% B. MS/MS analysis employed electrospray ionization (ESI) in negative ion mode and multiple reaction monitoring (MRM) mode. Optimal daughter ions, cone voltage, and collision energy were determined using standards via intellistart. The desolvation gas temperature was maintained at 550 °C with a gas flow of 1000 L/h, and capillary voltage set at 3.0 kV.

Nitrite and biogenic amine

Nitrite determination was conducted in accordance with the Chinese agriculture industry standards[66]. Each sample included three biological replicates and two extraction replicates.

For the determination of biogenic amines, the sample pre-treatment and Ultra-performance liquid chromatography (UPLC) (Acquity™ Ultra Performance LC, Waters Corporation, Massachusetts, USA) methods were revised according to Li et al. [67]. Weigh 3.00 g (accurate to 0.01 g) of the chili peppers sample into a 50 mL stoppered plastic centrifuge tube, then accurately add 15.00 mL of 0.1 mol/L HCl solution. Sonicate the mixture for 15 min at 20 °C, followed by centrifugation at 12,000 × g and 4 °C for 15 min. Take 1 mL of the supernatant or 1 mL of the biogenic amine standard solution in a 15 mL centrifuge tube. Next, add 200 μl of 2 mol/L NaOH to alkalinize the solution. Subsequently, add 300 μL of saturated sodium bicarbonate solution for buffering, followed by the addition of 1 mL of 10 mg/mL dansulfonyl chloride solution (dissolved in acetone). Vortex the mixture for 1 minute in the dark and incubate in a 40 °C water bath for 45 min. After incubation, add 100 μL of concentrated ammonia and place the mixture in a dark environment for 30 minutes to interrupt the reaction and remove excess dansulfonyl chloride. The solution was then subjected to nitrogen blowing to reduce the volume to approximately 1–1.5 mL. Finally, add acetonitrile to reach a final volume of 2 mL and filter through a 0.22 μm organic filter membrane (PTFE) before proceeding to UPLC analysis. The UPLC conditions were as follows: the column was an EC-C18 (100 mm × 3.0 mm, 2.7 μm) from Agilent (California, USA), with a column temperature set at 30 °C and a detection wavelength of 254 nm. The sampling volume was 10 μL, and the flow rate was maintained at 0.4 mL/min. The mobile phases consisted of 5 mM ammonium acetate solution (solvent A) and acetonitrile (solvent B), with the elution gradient as follows: 0–5 min, 40% A; 5–12 min, 40–25% A; 12–20 min, 25–5% A; 20–22 min, 5% A; 22–22.1 min, 5–40% A; and 22.1–25 min, 40% A. The concentration of the standard solution, all solution configurations, and final calculations were referenced according to Li et al. [67]. Each sample included three biological replicates and two extraction replicates.

Volatile substances analysis by GC-MS

The volatile substances were identified and quantified using gas chromatography mass spectrometry (GCMS) with reference to the method of Peng et al. [6], with pre-treatment protocols adapted from our research: 0.5 g (accurate to 0.0001 g) of the sample was weighed into a 20 mL glass vial, followed by the addition of 20 μL of 2-methyl-3-heptanone (10^4-fold dilution) as an internal standard. Each sample consisted of three biological replicates and two extraction replicates.

Non-volatile substances analysis by HPLC-qTOF-MS

Chili peppers were crushed using a Freeze Grinder (Ikea Co, Sweden) after being frozen with liquid nitrogen. A weight of 3 g (accurate to 0.0001 g) of chili pepper powder was measured and mixed with 15 mL of methanol-water solution (7:3, *v/v*), followed by vortexing. The mixture was allowed to stand in an ice bath for 12 h. Subsequently, the solution was centrifuged at 8000 rpm and 4 °C for 10 min, and the supernatant was filtered through a 0.22 μm nylon membrane into a liquid-phase vial, which was then stored at −80 °C for injection. Quality control (QC) samples were prepared by combining all the sample solutions to be tested in equal proportions. Each sample was subjected to three biological replicates and three extraction replicates to collect parent ion information.

Each of the 10 QC samples was fragmented at varying ion fragmentation energies to obtain daughter ion information. The samples were then analyzed using an Agilent 1290 HPLC system (Agilent, Santa Clara, CA, USA) coupled with a Triple TOF G6560B system (Agilent, Santa Clara, CA, USA) featuring an electrospray ionization (ESI) source in both positive and negative modes. In ESI+ and ESI- modes, mobile phases A and B consisted of 0.1% formic acid in water and acetonitrile, respectively. The injection volume was 2 μL. Chromatographic separation was achieved with a BEH C18 column (2.1 × 100 mm, 2.7 μm; Waters Pacific Pte. Ltd., Singapore) at a flow rate of 0.3 mL/min, using the following gradient: 0-2 min, 98% A; 2–2.50 min, 98–82% A; 2.50–6 min, 82–70% A; 6–9.50 min, 70–46% A; 9.50–14.00 min, 46–18% A; 14.00–15.00 min, 18–1% A; 15–15.10 min, 1–98% A; and 15.10–18.00 min, 98% A. The acquired mass range was set to 100–3000 Da for both TOF and product ion scans. The accumulation time was 0.5 s for TOF scans and 0.25 s for product ion scans. The ion fragmentation energies for MS/MS were set at 10, 20, 40, and 60 eV, respectively. The capillary voltage was maintained at 3000 V, the nozzle voltage at 1500 V, and the fragmentor voltage at 380 V. The gas parameters were as follows: gas temperature at 325 °C, nebulizer pressure at 35 psi, gas flow at 7 L/min, sheath gas flow at 11 L/min, and sheath gas temperature at 350 °C. The injection sequence included blanks (ultrapure water), background controls (methanol:water = 7:3, *v/v*), and three injections of QC samples at the beginning of the sequence to ensure system stability and repeatability. Subsequently, samples were injected randomly into the system to minimize signal drift effects, with a QC sample placed after every six samples. To ensure mass accuracy, automated mass calibration using the Agilent reference solution was performed in real time.

Microbial diversity by next generation sequencing

The study of microbial diversity was referred from Peng et al. [6], and the pretreatment of samples was revised. In a sterile environment, chili pepper samples were cut and mixed. Approximately 1.0 g of chili peppers was weighed into sterile tubes and subsequently stored in a −80 °C refrigerator. Each sample included three biological replicates. The samples were then transported to Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) on dry ice.

Determination of antioxidant activity

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and Ferric ion reducing antioxidant power (FRAP) of fermented chili peppers samples were evaluated using DPPH kit (A153-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and FRAP kit (S0116, Beyotime Biotechnology, Shanghai, China), and the pretreatment of samples was revised. A weight of 0.1 g (accurate to 0.0001 g) of chili pepper samples was measured and mixed with 1.5 mL of methanol-water solution (8:2, *v/v*). The results were expressed as trolox equivalents mmol/kg.

Data processing, statistical analysis, and visualization

For the analysis of volatile substances using GC-MS, raw data collection and identification were deconvoluted with Agilent MassHunter Unknown software. Substance screening was conducted with the NIST14 mass spectrum database, applying a quality match score threshold of > 60%. The mean concentration of each volatile substance was semi-quantitatively determined based on the peak area relative to that of the internal standard. For non-volatile substances analyzed by HPLC-qTOF-MS, peak extraction, alignment, and correction were executed using MS-DIAL version

4.36 software. The parameters were configured as follows: MS1 and MS2 tolerances were set at 0.01 Da and 0.05 Da, respectively, for identification settings. Retention time tolerance and MS1 tolerance for peak alignment were established at 0.05 min and 0.015 Da; a peak was considered valid if it had a detection rate exceeding 50% in at least one group, and the signal-to-noise (S/N) ratio (maximum in the sample/mean in the blank sample) was set to 5. The exported MS1 peak list was further analyzed using Microsoft Office Excel 2020 (Microsoft Corporation, USA) to exclude MS1 features with a relative standard deviation (RSD) greater than 30% in the quality control (QC) group[68]. substance identification was facilitated by MS-FINDER version 3.50, MassHunter PCDL version B.07.00, and LibraryViewTM version 1.1. For 16S rRNA sequencing, data were analyzed using online tools from Majorbio. Data analysis was performed using MetaboAnalyst version 4.0 and SIMCA 14.1. Mean values with relative standard deviation (RSD) were calculated, and statistical analyses were conducted with Microsoft Office Excel 2020 (Microsoft Corporation, USA). The significance of differences was assessed using SPSS® 20.0 software (Chicago, Illinois, USA).

Data availability

No datasets were generated or analysed during the current study.

Received: 2 February 2025; Accepted: 3 May 2025;
Published online: 17 May 2025

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Acknowledgements

This work was supported by Special National Key Research and Development Plan (2021YFD1600100), China Agriculture Research System of MOF and MARA, Professor Workstation of China Agricultural University and Guisanhong Food Company, and the 2115 Talent Development Program of China Agricultural University.

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Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41538-025-00442-7.

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