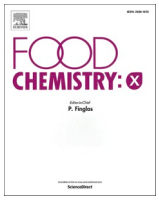
Food Chemistry: X 22 (2024) 101516

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Determination of the variations in the metabolic profiles and bacterial communities during traditional craftsmanship Liupao tea processing

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ARTICLE INFO

Keywords:
Traditional craftsmanship
TC-Liupao tea
Sensory quality
Non-targeted metabolomics
Bacterial communities

ABSTRACT

In this study, the metabolic profiles of traditional craftsmanship (TC) Liupao tea presented great changes at different processing stages. The contents of flavonoids and their glycosides generally exhibited a continuing downward trend, resulting in the sensory quality of TC-Liupao tea gradually improved. However, the taste of TC-Liupao tea faded when piling exceeded 12 h, as a result of the excessive degradation of some key flavor substances. Therefore, it could be deduced that piling for 10 h might be optimum for the quality formation of TC-Liupao tea. *Sphingomonas*, *Acrobacter*, *Microbacterium*, and *Methylobacterium* were the dominant bacteria during piling. The correlation analysis between differential metabolites and bacteria showed that only *Sphingomonas* and *Massilia* were significantly correlated to metabolites, demonstrating that the bacteria had less effect on the transformation of metabolites. Thus, the metabolic structure change during the process of TC-Liupao tea might be mainly attributed to the high temperature and humidity environment.

1. Introduction

Tea is now one of the three most consumed non-alcoholic beverages worldwide due to its excellent organoleptic properties and health benefits, mainly divided into two categories: fermented and non-fermented (Lin et al., 2021). As the only post-fermented tea among the fermented teas, the addition of exogenous microorganisms has uniquely improved the quality of dark tea. Nowadays, dark tea has become the second largest tea category after green tea in China, the total output of which is rising year by year (Cheng et al., 2021). Liupao tea is a typical Chinese dark tea named after its place of origin, Liupao Town in Guangxi Zhuang Autonomous Region. Liupao tea boasts a long consumption history spanning 1500 years, which has been introduced to Hong Kong, Macao, and many other countries in Southeast Asia via the Maritime Silk Road (Mao, Wei, Teng, Huang, & Xia, 2017). Presently, it has been regarded as a Chinese geographical indication product and gained increasing attention, owing to its unparalleled sensory characteristics of vibrant redness, thickness, aging aroma, and purity, significantly different from black tea and green tea (Huang et al., 2023). Moreover, Liupao tea has been reported to have multitudinous bioactive compounds, which lay the substance foundation for its various health benefits, such as antioxidant, anti-obesity, anti-diabetes, antihy-perlipidemic effect, anti-aging effects, improvements inmetabolic syndrome-related diseases, protection againstorganic damage, and regulation of intestinal microbiotaflora (Feng et al., 2023).

According to the difference in post-fermentation method, Liupao tea could be classified as traditional craftsmanship Liupao tea (TC-Liupao tea) and modern craftsmanship Liupao tea (MC-Liupao tea) (Feng et al., 2023). Traditional craftsmanship is an indispensable method in the development process of Liupao tea, which is of great significance. The combination of traditional craftsmanship and long-term age enriches the flavor of the tea, resulting in Liupao tea with a unique betel nut aroma. Moreover, TC-Liupao tea has a higher production efficiency through a quick piling (about 10 h) instead of long-term pile fermentation of modern craftsmanship. Meanwhile, the shorter piling treatment also allows TC-Liupao tea to retain more flavorful substances, resulting in a

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https://doi.org/10.1016/j.fochx.2024.101516
Received 19 February 2024; Received in revised form 15 May 2024; Accepted 27 May 2024
Available online 29 May 2024

more mellow and lighter taste. In addition, due to the oxidative polymerization of polyphenols, TC-Liupao tea possesses various health benefits on antioxidant, hypoglycemic, hypolipidemic and cardiovascular disease prevention (Qin et al., 2021). Considering the improved efficiency in Liupao tea production, an in-depth exploration of the variations in the substances and microorganisms responsible for the quality of TC-Liupao tea is of great importance for the extensive application of its standardized and intelligent production. Up to now, most of studies have focused on the pile-fermentation process of MC-Liupao tea, with comparatively less attention on the changes in the quality during traditional craftsmanship. It is often believed that the functional activities and unique flavor characteristics of TC-Liupao tea are closely associated with the chemical composition and microorganism succession, so a systematic and comprehensive study of their changes during TC-Liupao tea processing has a strong guiding significance for improving its quality.

In recent years, a variety of analytical methods have been employed to assess the quality of tea. Among them, analyzing the main chemical compositions including catechins, gallic acid, caffeine, etc., is the most widely used method in the quality evaluation of tea (Gu et al., 2020; Liu et al., 2021). However, it is not sufficient to analyze the unique flavor of TC-Liupao tea only depending on the main compounds, which are in fact related to the interaction of hundreds of active substances. Non-targeted metabolomics with the advantages of high sensitivity and broad coverage can be regarded as a powerful tool to analyze the small molecule metabolites in tea, which has been widely used for the quality assessment of tea (Wen et al., 2023). Based on non-targeted metabolomics the researchers successfully screened out the main flavor markers of dark tea with different fermentation treatments (Chen et al., 2023; Wang, Teng, Huang, Wei, & Xia, 2023). As is well-known, different from the other types of tea, microorganisms play a vital role in the transformation of chemical components in dark tea (Wu et al., 2023). High-throughput sequencing can provide insights into the composition, activities, and dynamics of a wide range of bacteria and elucidate bacterial influences on flavor metabolism (Reuter, Spacek, & Snyder, 2015). Based on high-throughput sequencing technology, researchers found the predominant bacterial genera of Fuzhuan tea, Pu-erh tea, and Liupao tea, and elaborated on their effects on the flavor and quality of dark tea (Li et al., 2019; Li et al., 2022; Wang et al., 2021). These bacterial genera are mainly involved in the metabolism of flavor compounds (tea polysaccharides, flavonoids, polyphenols, etc.) in dark tea fermentation. Since the corresponding chemical compositional changes and bacterial communities' succession during the processing of TC-Liupao tea have not been elucidated, the production still relies on sensory judgment rather than chemical indicators to determine its quality. Therefore, it is important to explore the correlation between the non-volatile substances and their bacterial communities in the TC-Liupao tea and elucidate their effects on quality generation.

This work aims to: (1) systematically investigate the non-volatile metabolites and screen out differential substances during the processing of TC-Liupao tea; (2) reveal the change of bacterial communities during processing and the effect of key bacteria on the quality of TC-Liupao tea; (3) analyze the correlation between metabolites and bacteria to clarify the flavor basis of TC-Liupao tea taste quality. This study will open a new route for a comprehensive understanding of TC-Liupao tea processing and the interaction between the bacterial community and non-volatile metabolites, providing a theoretical foundation for further improvement of TC-Liupao tea quality.

2. Materials and methods

2.1. Chemicals

Standards of catechin (C, ≥98%)、epicatechin (EC, ≥98%)、gallocatechin (GC, ≥98%)、epigallocatechin (EGC, ≥98%)、catechin gallate (CG, ≥98%)、epicatechin gallate (ECG, ≥98%)、gallocatechin gallate (GCG, ≥98%)、epigallocatechin gallate (EGCG, ≥98%)、gallic acid (GC, ≥98%)、caffeine (CAF, ≥98%)、theobromine (TB, ≥98%) and theophylline (TP, ≥98%) were purchased from Chengdu Refmedic Technology Co., LTD (Chengdu, China). LC-MS grade methanol was purchased from Honeywell China Co., Ltd. (Shanghai, China) and HPLC grade acetonitrile was purchased from Shanghai Ampere Scientific Instruments Co., Ltd. (Shanghai, China). Ultrapure water was used throughout the experiment.

2.2. Sampling and pretreatment

The TC-Liupao tea samples were produced by Guangxi Research Institute of Tea Science (Guilin, China). Fresh tea leaves from Lingyun Baihao cultivar with one bud and two or three leaves were collected in June 2022 and processed into TC-Liupao tea by traditional craftsmanship including the steps of spreading, fixation, rolling, drying and piling.

Firstly, the fresh leaves were spread in the withering tank to reduce the moisture (70% - 75%). Then, the withered teas were stir-fried to remove the grass flavors. Next, the tea leaves were rolled with a machine for around 15 min. Subsequently, the tea leaves were dried at 200 °C for about 5 min to lose moisture (56% - 61%). When the tea temperature dropped to 50 °C, it was wrapped with gauze into a bamboo basket, keeping the environment temperature 22–26 °C and the humidity 65% - 70%. The tea leaves were piling for about 12 h. The samples were collected at the eleven different stages for each batch: the fresh leaves (FL), spreading (S), fixation (F), rolling (R), drying (D), piling for 2 h (P2), 4 h (P4), 6 h (P6), 8 h (P8), 10 h (P10) and 12 h (P12). The samples were roasted until fully dry and sealed for sensory evaluation and metabolic analysis. Besides, the collected parallel samples during piling process were stored at −80 °C for the subsequent microbiological assay.

2.3. Sensory evaluation and color difference analysis of TC-Liupao tea

The sensory evaluation of TC-Liupao tea was described and scored by a sensory panel consisting of three well-trained panelists from the Guangxi Research Institute of Tea Science according to the national methodology of sensory evaluation of tea (GB/T 23776–2018). 3.0 g of each tea sample was brewed with 150 mL of freshly boiled water for 5 min, and then tea infusion was cooled to room temperature for the sensory evaluation, including appearance, infusion color, taste, bottom leaf and aroma. The color difference analysis was carried out using a CS-821 N spectrophotometer (FigSpec Technology Co., Ltd., Hangzhou, China), and the corresponding values of L*, a* and b* for each infusion indicated brightness, red-green degree, yellow-blue degree, respectively. The overall color difference was denoted by ΔE, as follows (Zhu et al., 2023):

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (1)$$

2.4. Chemical composition analysis

Each tea sample at different stages was well dispersed and milled into fine powder (100 mesh). 0.2000 g tea powder was placed into a 10 mL centrifuge tube, and then added 5 mL of methanol-water (70:30, *v*/v). The tube was kept in a water bath at 70 °C for 10 min and then centrifuged at 5000 rpm for 10 min. Repeated the above process twice and the supernatant was diluted to 10 mL by methanol-water (70:30, v/v) for quantitative analysis.

All high-performance liquid chromatography HPLC analysis were performed on a Prominence-i LC-2030C (Shimadzu, Japan) equipped with a ChromCore AQ-C18 (4.6 × 250 mm, 5 μm). The 5% acetonitrile and 80% acetonitrile solutions were prepared as mobile phases A and B, respectively. The chromatographic elution was 0.00–20.00 min, 100% - 100% A; 20.01–55.00 min, 100% - 75% A; 55.01–65.00 min, 100% A. The column temperature was kept at 35 °C. A 20 μL aliquot of the sample

solution was injected into the separation system and delivered at a flow rate of 0.8 mL min^{-1}. The detection wavelength was 278 nm.

2.5. Metabolomics analysis

The tea powder (0.0200 g) was accurately weighed and mixed with 1 mL methanol-water solution (70:30, *v*/v), and then subjected to ultrasonic extraction at room temperature for 15 min. The extract was centrifuged at 5000 rpm for 10 min. The analysis was performed in duplicate, and each extract was filtered through a 0.22 μm millipore filter and stored at −20 °C before metabolomics analysis. Quality control samples (QC) were prepared by mixing an equal amount of sample extract and inserted for every eight samples to test the stability and reproducibility of the instrument state and data.

The LC-QTOF/MS system was equipped with a Shimadzu HPLC (LC-20 A) and a Sciex QTOF/MS (TripleTOF 5600+), and LC separation was achieved on a Waters HSS T3 column (2.1 mm × 150 mm, 3.5 μm). The mass spectrometer was operated in both positive and negative ionization modes over a full-scan range of 50–1000 *m/z*. In the positive ion mode, formic acid (0.1%, *v*/v) and pure acetonitrile were used as mobile phases A and B, respectively. The mobile phase for the negative ion mode consisted of 5 mM ammonium formate (A) and pure acetonitrile (B). The gradient elution for the system was as follows: 0.00–3.00 min, 1% B; 3.01–24.00 min, 1% - 100% B; 24.01–32.00 min, 100% B, 32.01–37.00 min, 1% B. The source voltage and collision energy for the positive ion mode were set to 5500 V and 30 V, and 4500 V and − 30 V for the negative ion mode, respectively.

The raw data were converted to the ABF format using AnalysisBaseFileConverter and processed by MS-DIAL software (version 4.36), then subjected to perform noising filtering, peak identification, overlapped peak analysis, peak alignment, and peak filling. The pre-processed results such as the retention time, mass-to-charge ratio and peak area, etc. were summarized in a data matrix. Thereafter, metabolites were identified based on publicly available databases (e.g., MassBank, LipidBlast, and MetaboBase) and standard databases from chemical standards (including retention time, mass accuracy, and MS/MS fragmentation spectra) with an identification score cutoff of 80% and accurate mass tolerance of 0.05 Da for MS1 and 0.10 Da for MS2, respectively. The raw data obtained from positive and negative ions were all processed as described above and combined for further statistical analysis.

2.6. Bacterial analysis

After genomic DNA extraction according to the DNA extraction kit, the concentration and purity were measured using the NanoDrop One (Thermo Fisher Scientific, MA, USA). The V4 regions of the bacteria were amplified by using the primer 515F and 806R (Invitrogen, CA, USA). PCR amplification was performed in a total volume of 50 μL reaction mixture containing 25 μL 2× Premix Taq (Takara Biotechnology Co., Ltd., Dalian, China), 1 μL each primer (10 μM), and 3 μL DNA (20 ng μL^{-1}) template and nuclease-free water to adjust the volume. The reaction mixture was amplified by thermocycling: 5 min at 94 °C for initialization, 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 52 °C, and 30 s extension at 72 °C, followed by 10 min final elongation at 72 °C. The PCR instrument was BioRad S1000 (Bio-Rad Laboratory, CA, USA). The length and concentration of the PCR product were detected by 1% agarose gel electrophoresis. Samples with bright main strips between 290 and 310 bp could be used for further experiments. PCR products were mixed in equidensity ratios according to the GeneTools Analysis Software (version 4.03.05.0). Then, the mixture of PCR products was purified with an E.Z.N.A. Gel Extraction Kit (Omega, GA, USA). Sequencing libraries were generated using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA) following the manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA). At last, the library was sequenced on an Illumina Nova6000 platform and 250 bp paired-end reads were generated.

Paired-end clean reads were merged using usearch-fastq_mergepairs (version 10) according to the relationship of the overlap between the paired-end reads, and the resulting spliced sequences were the original Tags data (Raw Tags). Fastp (version 0.14.1) was resorted to control the quality of the raw data to obtain the paired-end clean tags. For each representative sequence, the silva database was used to annotate taxonomic information by usearch-sintax with the confidence threshold ≥0.8). Then the OTU taxonomy synthesis information table was obtained for the final analysis. R software (version 5.1.3) was employed for common and endemic species statistics and community composition analysis.

2.7. Statistical analysis

Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using SMICA-P (version 14.1) and the Metware cloud tools at https://cloud.metware.cn. The Variable Importance in Projection (VIP) was used to screen key differential marker metabolites. Data were evaluated using a significance level of $p = 0.05$ through one-way ANOVA by SPSS (version 26.0). Heatmap visualization and correlation analysis were performed using the OmicStudio tools at https://www.omicstudio.cn/tool.

3. Results

3.1. Color and sensory evaluation of TC-Liupao tea at different process stages

Color and flavor, as vital parameters for the quality assessment of tea, in general, can be markedly affected by different processing procedures. To investigate the effects of traditional craftsmanship treatment, the color and sensory properties of TC-Liupao tea samples were measured and their scores were exhibited in **Fig. S1, Table S1** and **Table S2**. As shown in **Table S1**, the color of tea infusion and bottom leaf of TC-Liupao tea samples gradually turned from green to yellow during traditional craftsmanship processing and lost their luster especially after piling. Combined with changes of chromatic aberration, the L* and a* values of tea infusion initially presented a sharp downtrend and then into a plateau after rolling, while the b* values were significantly increased, indicating that the brightness and red degree of tea infusion were decreased after rolling treatment and became more green and yellow. The chromatic aberration of tea infusion was basically consistent with the results of sensory evaluation. Moreover, the ΔE values between each processing stage and the initial stage of fresh leaves were calculated to verify the change trend of the total color difference. Apparently, the ΔE sharply increased ($p < 0.05$) before the rolling stage, whereas after that it showed no significant difference, further denoting that rolling might play a key role for the TC-Liupao tea infusion color formation. Besides, it was noteworthy that the b* during the overall piling procedure compara-bly improved only with slight fluctuations, resulting in noticeable yellow deepening of tea infusion rather than transforming into reddish-brown like most of dark tea through pile-fermentation (He et al., 2023), which could be viewed as one of the typical features of TC-Liupao tea.

From **Table S1**, it could be also observed that the aroma and taste started to change from the fixation procedure, the grassy aroma faded away and the bitter and astringent taste emerged, and the appearance of TC-Liupao tea became tight and sturdy and maintained after rolling. With the prolongation of piling duration, the intensity of bitterness and astringency gradually subsided and the tea infusion presented a mellow taste and a strong fragrance with higher acceptance. However, the scores of infusion color, aroma, taste and bottom leaf of TC-Liupao tea samples began to decline with 12 h piling, and the taste of TC-Liupao tea

became lighter in especial, which demonstrated that a lengthy piling duration (>10 h) was not desirable for the formation of TC-Liupao tea with high-quality.

3.2. Quantitation of main chemical components during TC-Liupao tea processing

The main characteristic components including gallic acid (GA), theobromine (TB), caffeine (CAF), theophylline (TP) and 8 monomers of catechins (C, EC, GC, EGC, CG, ECG, GCG and EGCG) were analyzed by HPLC to investigate the effect of traditional processing technology on the quality of TC-Liupao tea. One-way ANOVA analysis of variance using Duncan's post-hoc test revealed specific differences in chemical composition during different processing stages of TC-Liupao tea, as summarized in Table 1.

As important polyphenol nutrients with rich biological activities in tea, in general, gallated catechins exhibit more bitter and astringent than non-gallated catechins, and the bitterness and astringency of epicatechins are more intense than non-epicatechins at the same level (Xu et al., 2018). It could be obviously observed that the abundances of epicatechins were overall higher than those of non-epicatechins. And the contents of non-epicatechins such as GC, CG and GCG, significantly increased during piling procedure, whereas the concentration of epicatechins notably decreased compared to fresh leaves, especially after 10 h pile. Among the four detected gallated catechins, the EGCG and ECG abundances were relatively higher, the concentrations of which fluctuated and changed repeatedly with the minimum and maximum values located at the fixation and P4 stages, respectively. Comparably, the contents of CG and GCG were generally low with minimum concentrations of 1.12 ± 0.00 mg g^{-1} and 1.08 ± 0.01 mg g^{-1}, respectively, but showed a relatively large increase during the piling stage. Similar to the gallated epicatechins, the concentrations of total catechins (TCs) fluctuated during the overall TC-Liupao tea process, and reached the maximum values after 4 h pile, and then gradually declined to 165.85 ± 0.20 mg g^{-1}, but in reverse increased to 172.05 ± 0.45 mg g^{-1} when the piling time exceeded ten hours.

GA is another important chemical component in TC-Liupao tea, which increased during the piling procedure with the highest content at the P10 stage approximately double that of fresh leaves. The common alkaloids in TC-Liupao tea include CAF, TB and TP, which not only contribute to allaying tiredness but also have a stimulating effect on the central nervous system (Fernández, López, Pablos, González, & Martín, 2003). By comparison, the levels of CAF were far higher than the other two components and presented a similar change trend with catechins. The concentrations of TP remained relatively stable throughout, however, the contents of TB slightly decreased during piling, with its highest value present in fresh leaves (3.50 ± 0.04 mg g^{-1}). In general, the content of catechins and alkaloids in dark tea was reported to show a relatively great decrease after fermentation (Shi et al., 2021). Compared with before-piling, despite the content of total catechins (TCs) and total alkaloids (TAs) in P10 declined, but by a very small margin, approximately 0.31% and 1.25%, respectively, which allowed more flavor compounds to be retained in TC-Liupao tea, providing a rich material foundation for its flavor transformation during storage. In fact, the substance changes in the traditional craftsmanship treatment of TC-Liupao tea are dynamic and complicated, it is not enough to judge its influence on the quality of TC-Liubao tea only according to the analysis of these main ingredients. Therefore, the non-targeted metabolomic analysis was employed to explore the biochemical change in TC-Liupao tea with traditional processing technology from a broad range of non-volatile compounds aspects.

3.3. Metabolomic profiling of different process stages

To better reflect the changes in the quality of TC-Liupao tea during the processing, the non-targeted metabolomic analysis based on LC-QTOF/MS was performed on all samples from different process stages. As displayed in **Fig. S2**, the total ion chromatograms of the QC samples were basically consistent, indicating that the data recorded in this study had good stability and reproducibility. After peak extraction and alignment, 376 non-volatile metabolites were identified based on positive and negative ion modes.

The supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was first used to observe the general tendency in the non-

Table 1
Content of main chemical components among TC-Liupao tea during processing.

Content (mg g^{-1})	FL	S	F	R	D	P2	P4	P6	P8	P10	P12
GA	0.16 ± 0.00^{a}	0.25 ± 0.00^{b}	0.25 ± 0.00^{b}	0.28 ± 0.00^{f}	0.26 ± 0.00^{c}	0.27 ± 0.00^{e}	0.30 ± 0.00^{g}	0.28 ± 0.00^{f}	0.27 ± 0.00^{d}	0.31 ± .00^{h}	0.31 ± 0.00^{h}
TB	3.50 ± 0.04^{h}	2.31 ± 0.02^{bc}	3.19 ± 0.02^{g}	2.25 ± 0.01^{a}	2.28 ± 0.02^{ab}	2.32 ± 0.00^{bc}	2.28 ± 0.01^{ab}	2.50 ± 0.01^{e}	2.38 ± 0.01^{d}	2.32 ± 0.02^{c}	2.57 ± 0.03^{f}
TP	2.19 ± 0.01^{ef}	1.90 ± 0.01^{a}	1.97 ± 0.02^{b}	2.37 ± 0.01^{h}	2.01 ± 0.02^{c}	2.17 ± 0.02^{de}	2.22 ± 0.01^{f}	2.03 ± 0.02^{c}	2.14 ± 0.02^{d}	2.19 ± 0.02^{ef}	2.31 ± 0.04^{g}
CAF	35.64 ± 0.26^{a}	38.37 ± 0.25^{d}	35.57 ± 0.40^{a}	39.32 ± 0.08^{e}	37.93 ± 0.18^{c}	39.18 ± 0.09^{e}	40.70 ± 0.03^{f}	37.92 ± 0.10^{c}	38.57 ± 0.11^{d}	37.49 ± 0.14^{b}	39.06 ± 0.34^{e}
C	10.92 ± 0.13^{h}	9.75 ± 0.06^{c}	9.33 ± 0.06^{b}	10.37 ± 0.02^{f}	10.15 ± 0.03^{e}	10.50 ± 0.02^{g}	10.45 ± 0.02^{fg}	8.69 ± 0.02^{a}	10.00 ± 0.05^{d}	10.00 ± 0.01^{d}	10.36 ± 0.13^{f}
EC	17.91 ± 0.23^{h}	15.24 ± 0.05^{bc}	15.32 ± 0.08^{c}	16.11 ± 0.10^{e}	15.08 ± 0.05^{b}	17.23 ± 0.04^{g}	16.16 ± 0.02^{e}	14.73 ± 0.08^{a}	15.69 ± 0.28^{d}	15.68 ± 0.06^{d}	16.59 ± 0.15^{f}
GC	12.66 ± 0.11^{b}	12.00 ± 0.12^{a}	12.68 ± 0.02^{b}	15.19 ± 0.03^{g}	13.24 ± 0.02^{c}	13.35 ± 0.06^{c}	13.68 ± 0.05^{d}	12.69 ± 0.06^{b}	13.34 ± 0.03^{c}	14.00 ± 0.04^{e}	14.19 ± 0.09^{f}
EGC	22.79 ± 0.16^{h}	17.12 ± 0.12^{a}	19.25 ± 0.06^{f}	19.43 ± 0.03^{g}	17.11 ± 0.05^{a}	18.62 ± 0.02^{d}	18.83 ± 0.07^{e}	18.20 ± 0.06^{c}	17.83 ± 0.12^{b}	18.57 ± 0.04^{d}	18.94 ± 0.09^{e}
CG	1.12 ± 0.00^{a}	1.20 ± 0.01^{b}	1.32 ± 0.01^{c}	1.48 ± 0.01^{e}	1.44 ± 0.01^{d}	1.60 ± 0.01^{h}	1.67 ± 0.01^{i}	1.55 ± 0.01^{g}	1.55 ± 0.02^{g}	1.59 ± 0.01^{h}	1.51 ± 0.02^{f}
ECG	38.47 ± 0.31^{c}	40.85 ± 0.14^{e}	34.36 ± 0.30^{a}	38.64 ± 0.18^{c}	40.20 ± 0.18^{d}	41.09 ± 0.09^{e}	43.59 ± 0.11^{f}	36.30 ± 0.07^{b}	40.86 ± 0.17^{e}	38.76 ± 0.15^{c}	40.30 ± 0.28^{d}
GCG	1.45 ± 0.02^{b}	1.08 ± 0.01^{a}	1.61 ± 0.01^{c}	1.95 ± 0.02^{f}	1.85 ± 0.03^{de}	2.06 ± 0.03^{h}	2.02 ± 0.01^{g}	1.89 ± 0.01^{e}	1.98 ± 0.02^{fg}	2.06 ± 0.01^{h}	1.83 ± 0.05^{d}
EGCG	56.89 ± 0.59^{de}	55.15 ± 0.50^{b}	54.37 ± 0.20^{a}	57.8 ± 0.40^{fg}	57.32 ± 0.65^{e}	55.79 ± 0.10^{c}	60.54 ± 0.05^{h}	60.06 ± 0.14^{h}	57.06 ± 0.07^{ef}	56.41 ± 0.07^{d}	58.34 ± 0.33^{g}
TC	172.74 ± 1.20^{h}	161.26 ± 1.01^{b}	156.36 ± 0.01^{a}	171.62 ± 0.15^{fg}	166.37 ± 0.62^{d}	170.83 ± 0.34^{f}	176.64 ± 0.24^{i}	163.78 ± 0.35^{c}	168.05 ± 0.69^{e}	165.85 ± 0.2^{d}	172.05 ± 0.45^{gh}
TA	44.02 ± 0.31^{b}	45.06 ± 0.28^{c}	42.96 ± 0.39^{a}	46.85 ± 0.07^{e}	44.92 ± 0.21^{c}	46.55 ± 0.10^{e}	47.83 ± 0.04^{f}	45.11 ± 0.15^{c}	45.74 ± 0.13^{d}	44.36 ± 0.15^{b}	46.64 ± 0.43^{e}

volatile metabolites of TC-Liupao tea during processing. As provided in Fig. 1A, the samples before and after piling were clearly separated along the x-axis of the score plot, which implied that there presented significant difference in metabolic composition before and after piling. In other words, the piling procedure was responsible for chemical transformation that might cause a great influence on sensory quality of TC-Liupao tea. In order to further clarify the primary differential changes on the metabolites induced by piling, the data performed by $\log_2$ and center normalization were subjected to the supervised OPLS-DA analysis with the samples divided into two groups (before-piling and after-piling), as plotted in Fig. 1B. It could be found that the before-piling samples were positioned on the right side of the diagram, while the after-piling samples were on the left side of the diagram, indicating a distinct separation between these different processing groups. And the differential compounds were screened in terms of the criterion of $p < 0.01$ combined with VIP > 1.5. A total of 62 key differential compounds were screened out between the before-piling and after-piling groups, consisting of 27 flavonoids and their glycosides, 10 terpenoids, 3 lignins, 3 alkaloids, 3 phenols, 2 coumarins, 2 lipids, 2 esters, 1 organic acid, 1 steroid and 8 other or unknown substances. To better observe the changes of differential metabolites, the heatmap visualization analysis of the 54 identified differential metabolites was carried out with the combination of hierarchical clustering, the data were pretreated by $\log_{10}$ and *Z*-score normalization before analysis. It could be achieved from Fig. 2A that most of differential compounds were characterized by a large decrease after piling, particularly during the later period of piling where most of flavonoids attached their lowest content levels, such as quercetin-3,7-O-alpha-L-dirhamnopyranoside, myricetin-3-O-galactoside, etc., which was in agreement with previous studies on the changes in metabolite profiles during Fu brick tea fermentation (Xiao et al., 2022). Moreover, to uncover the dominant chemical transformations that resulted in the decrease of the non-volatile metabolites, the piling process has been further investigated in detail.

Similarly, the OPLS-DA score plots provided that the P2, P4, P6 and P8 were clustered together, while they were clearly separated from P10 and P12 along the x-axis, the P10 group further from the P2–8 cluster than the P12 group (Fig. 1C), which suggested that the metabolic components in TC-Liupao tea kept transforming with the increase of piling time, and piling for 10 h might be an important turning point for the formation of TC-Liupao tea quality. Subsequently, for in-depth insight into the variation of metabolites during the piling procedure, the OPLS-DA model based on the samples which were redivided into three groups (P2-P8, P10 and P12), was constructed to succinctly screen piling discriminatory metabolites. The cross-validation with 200 permutation tests manifested that the OPLS-DA models were reliable (**Fig. S3**). A total of 10 potential marker metabolites could be screened and differentially accumulated with different piling times according to the criterion of VIP > 1.5 and $p < 0.01$, including 3 flavonoids and their glycosides (robinin, quercetin-3,7-O-alpha-L-dirhamnopyranoside, apigenin 6,8-di-glucopyranoside), 3 organic acids (2-hydroxy-3-

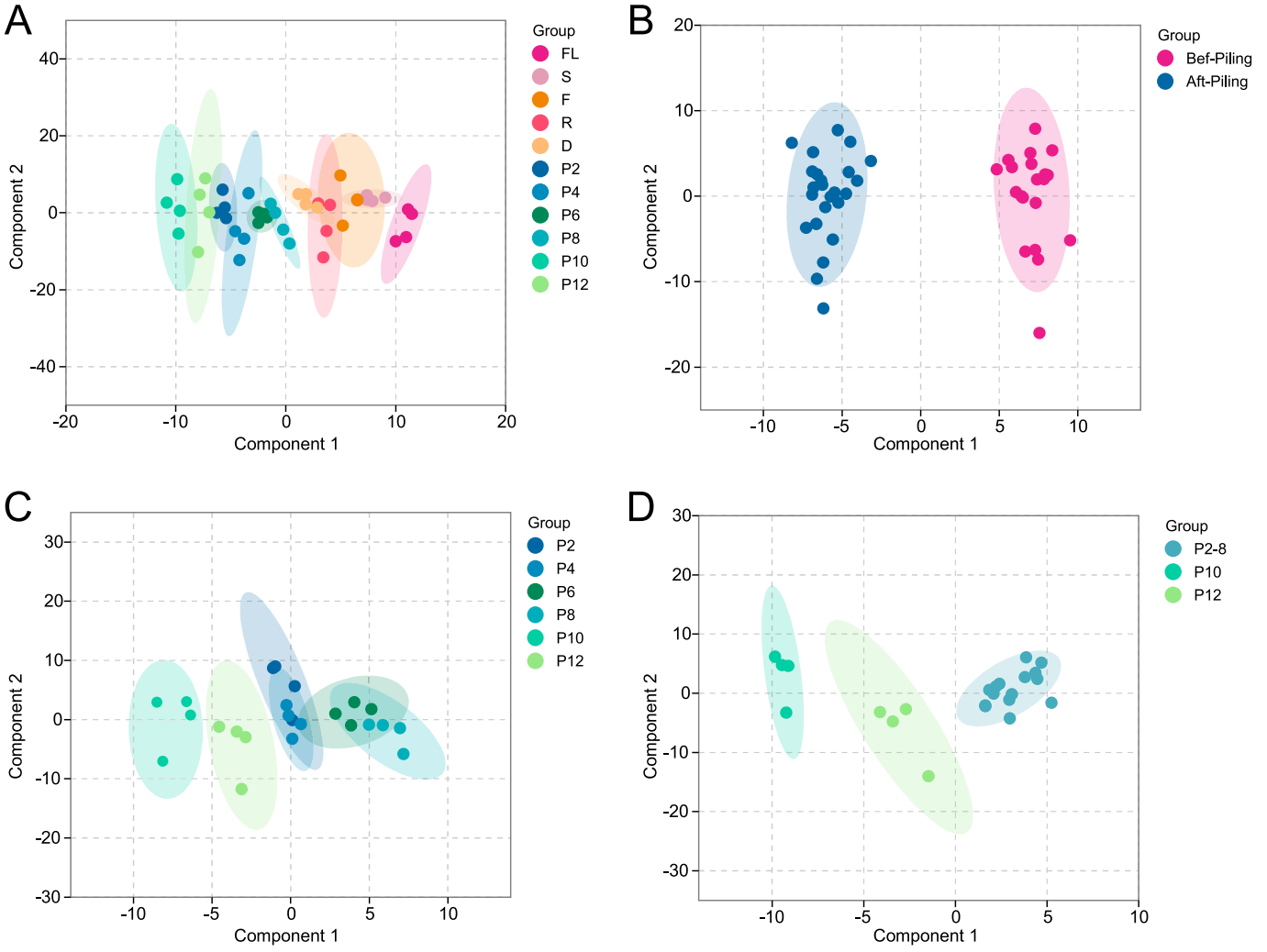

Fig. 1. OPLS-DA score plots: (A) based on all samples at different processing stages. (B) based on before-piling and after-piling samples. (C) based on the samples during the piling procedure. (D) based on three groups (P2-P8, P10 and P12).

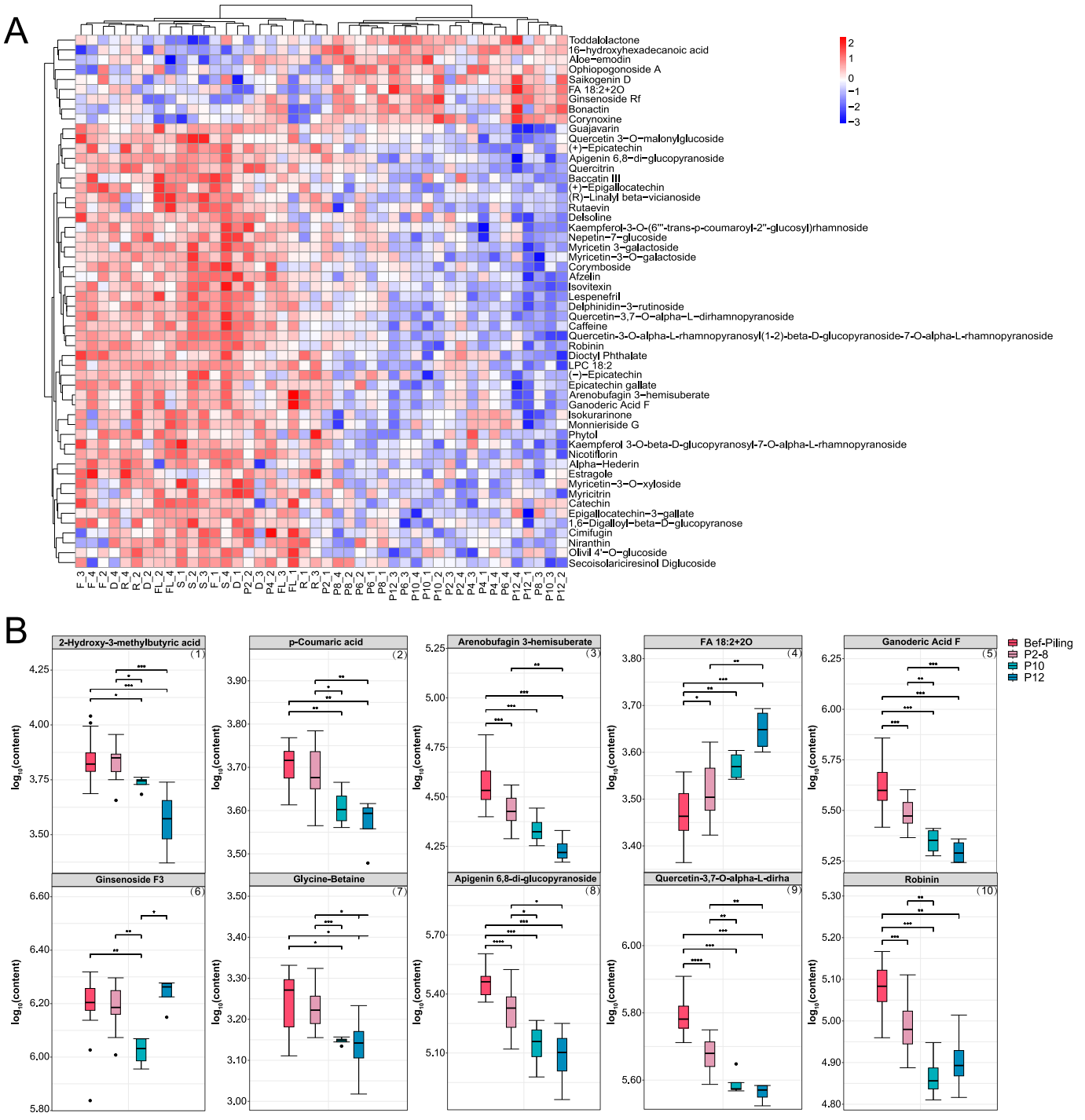

Fig. 2. (A) Heatmap of 54 differential compounds at different processing stages. (B) The boxplots of 10 differential metabolites after different piling times.

methylbutyric acid, p-coumaric acid, FA 18:2 + 2O), 2 terpenoids (ganoderic acid F and ginsenoside F3), 1 alkaloid (glycine-betaine) and 1 steroid (Arenobufagin 3-hemisuberate). As exhibited in Fig. 2B, the box plots of these ten metabolites were established to identify their variation at different piling stages. Compared to the P2-P8 cluster, the contents of seven differential metabolites showed a continuous downward trend, but the abundance of FA 18:2 + 2O constantly increased with the extension of the piling time. Meanwhile, it was worth noting that the content of no matter robinin or ginsenoside F3 during piling tended to decrease in the first 10 h, however a relatively great increase was found after piling 12 h. This phenomenon might provide a reasonable explanation for the P12 group more approaching to the P2–8 cluster in the score plots of OPLS-DA and once again confirmed that P10 was a time node of great concern in the formation of characteristic flavor of TC-Liupao tea.

3.4. Analysis of bacterial diversity of TC-Liupao tea after different piling times

For better understanding, regulating and ensuring the efficiency of the piling procedure, it is necessary to investigate the effect of bacterial communities on metabolites during piling. High-throughput sequencing of the 16S rDNA region yielded a total of 427,769 valid sequences from the piling procedure. In total, 895 bacterial operational taxonomic units

(OTUs) were identified, belonging to 10 phyla, 18 classes, 48 orders, 69 families and 96 genera. The Chao1 and Shannon indexes of each sample are commonly applied to evaluate the richness and diversity of the bacterial community, respectively. As displayed in **Fig. S4**, the Chao 1 indexes had two slight increases at P4 and P10, whereas at the corresponding stages, the Shannon indexes were slightly decreased, implying that the bacterial succession at P4 and P10 during the overall piling period might play a vital role on the chemical transformation of metabolites in TC-Liupao tea. Herein, *Proteobacteria* was the most important phylum in all piling samples, accounting for 69.60–84.40% of the total sequence number, followed by *Actinobacteria* (6.19–19.50%) and *Bacteroidetes* (7.29–10.86%), which was consistent with most of the traditional fermented foods (Bahule et al., 2024). In addition, the UpSet plot was employed to extract common and specific bacterial communities at the genus level (Fig. 3A). It could be obtained that there were 37 shared bacterial genera during the piling procedure and the number of specific bacterial genera showed a trend of first decreasing and then increasing. Furthermore, it could be yielded from Fig. 3B that *Sphingomonas* (14.28–42.76%), *Achromobacter* (1.74–27.27%), *Microbacterium* (5.97–19.14%) and *Methlobacterium* (5.86–14.11%) were the main bacterial genera (Fig. 3B). The abundance of *Sphingomonas* was generally higher during the piling process, but the lowest of 14.28% in P10. The *Achromobacter* abundance began to increase in the later period of piling. Compared with P2, the abundance of *Acronobacter* after piling 10 h increased from 6.43% to 48.64%, becoming the dominant bacterium of P10. However, Li et al. observed that *Aspergillus* was predominant throughout the entire manufacturing process of Fu brick tea (Li et al., 2017). Zhao et al. found that *Lactobacillus* maintained a dominant position during the fermentation process of Chi-flavor type Baijiu (Zhao et al., 2022). Both phenomena were different from the findings of this work, indicating that there might exist the significant differences in microbial succession between short-term and long-term fermentation processes. The abundances of *Microbacterium* and *Methlobacterium* were stable at the early piling stage, however, both of them decreased to 5.97% and 5.86% in P10, respectively. The abundance of *Ralstonia* (0.60–4.90%), *1174–901-12* (0.61–1.24%), *Aureimonas* (0.47–0.73%), *Luteibacter* (0.90–1.67%) and *Bosea* (0.30–0.66%) were observed to show a downward trend throughout the piling procedure.

Random forest as a powerful machine learning method could be used to screen the biomarker in terms of the contribution of each variable. Here, the entire bacteria genera were employed for random forest modeling, and a scatter diagram was established by the variable importance (**Fig. S5**). It could be obviously obtained for 10 bacteria genera with variable importance >1, such as *Deinococcus*, *Brevibacterium*, *Massilia*, *Mucilaginibacter*, *Methylobacterium*, *Curtobacterium*, *Cupriavidus*, *Sphingomonas*, *Terriglobus* and *Kocuria*. To analyze the

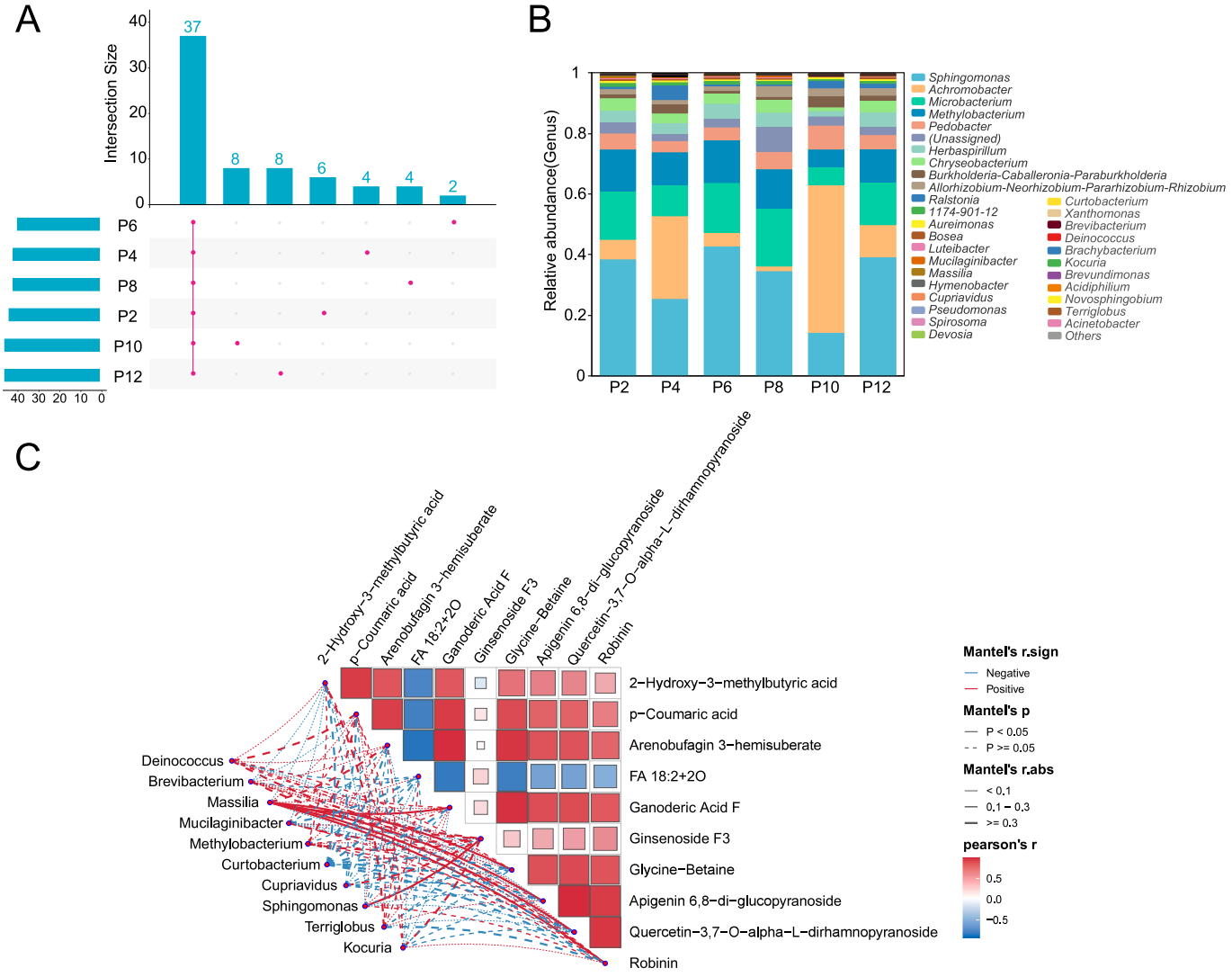

Fig. 3. (A) UpSet diagram based on bacterial genus level in piling samples. (B) Bacterial structure bar plot at the genus level. (C) Correlation analysis between differential metabolites and bacterial genera.

correlation between bacterial genera and differential metabolites at the piling stage, mantel test analysis was employed for drawing a correlation diagram of the bacterial genera (variable importance >1) and differential metabolites (Fig. 3C). *Sphingomonas* was significantly ($p < 0.05$) positively correlated with ginsenoside F3 and *Massilia* was significantly ($p < 0.05$) proportional to ganoderic acid F, glycine-betaine, apigenin 6,8-di-glucopyranoside, quercetin-3,7-O-alpha-L-dirhamnopyranoside and robinin. In contrast, the other bacterial genera have no significant correlations ($p > 0.05$).

4. Discussion

The sensory quality of TC-Liupao tea was highly associated with flavor substances, which were greatly impacted by the bacterial communities during the piling process. In this study, the variations of non-volatile metabolites and bacterial communities of TC-Liupao tea were clarified throughout the process by using non-targeted metabolomics, high-throughput sequencing technology and multivariate statistical analysis.

The results showed that all sensory attributes of TC-Liupao tea were significantly improved after piling. The infusion color gradually turned yellow, which might be attributed to the conversion of catechins into theaflavin and the oxidation of chlorophyll under high temperature and humidity conditions (Li et al., 2022). Although TC-Liupao tea was endowed with a strong and mellow flavor through piling, the sensory evaluation revealed that the flavor was muted when the piling time exceeded ten hours. The reason for this phenomenon was probably due to the excessive decomposition of the flavorful substances.

The increase of total catechins (TCs) content in the early stage of piling was caused by the hydrolyzation of polyphenols dominated by proanthocyanidins (Zhu et al., 2002). Subsequently, EGCG as the most abundant gallated epicatechin, transformed into non-ester catechins and GA, while non-ester catechins have further participated in oxidative polymerization to form theaflavins or degradation into phenolic acids (An et al., 2021), resulting in the infusion color turning into bright yellow. Herein, the enzymatic polymerization of non-ester catechins generally involved catalase and oxidase, both of which might be secreted by *Sphingomonas* (Zhao et al., 2008). In addition, the increase of the GA content during piling was not only related to the hydrolysis of the ester catechins, but also might be associated with the hydroly-sis of tannin acid. The microorganisms in the piling samples, such as *Achromobacter* and *Microbacterium*, could release tannase to hydrolyze gallic tannin, generating GA during piling (Bajpai & Patil, 1997). The *Achromobacter* abundance began to increase in the later period of piling. Compared with P2, the abundance of *Acronobacter* after piling 10 h increased from 6.43% to 48.64%, becoming the dominant bacterium of P10, which rationally explained the highest GA content in the P10 samples. Unlike other dark teas, the abundance of GA in the late periods of piling was still maintained high, probably owing to that the shorter piling treatment prevented the formation of GA derivatives (Hu et al., 2021). A similar trend could be found for CAF, the level of which increased slightly during the piling process. It might be attributed to the microbial action, highly consistent with the previous findings yielded by Zhang et al. They found that the CAF content of ripened Pu-erh Tea increased after fermentation with the aid of microorganisms (Zhang, Li, Ma, & Tu, 2011). However, the content of TB decreased after piling, possibly because it was considered as a precursor for the CAF synthesis in the presence of microorganisms (Wang, Wan, Hu, & Pan, 2008). And the levels of monomeric catechins of Liupao tea produced by modern craftsmanship treatment were reported to decrease significantly by >89% (Wang et al., 2023). But the levels of TCs, TAs and GA in TC-Liupao tea have been not changed on a large scale throughout the traditional process. The reasons for these tendencies might be as follows: the short piling time induced the transformation of substances incomplete, and the high moisture of samples before piling was not conducive to the degradation of substances.

Catechins and flavonoid glycosides were reported to be the main contributors for the bitterness and astringency of tea (Ye et al., 2022). At the early processing stages (before piling), the tea samples exhibited a relatively intense bitter and astringent taste due to the higher contents of catechins and flavonoid glycosides. With the process entering into the piling stage, the levels of catechins and flavonoid glycosides of the piling samples were lower than those of the early processing samples as a whole, as a result, the bitter and astringent taste of tea samples was reduced by a large margin. In addition, some of flavonoid glycosides showed a continuing downward trend in concentration during piling, leading to the sensory quality of TC-Liupao tea gradually improved, such as kaempferol glycoside (kaempferol-3-O-(6‴-trans-p-coumaroyl-2″-glucosyl)rhamnoside, kaempferol 3-O-beta-D-glucopyranosyl-7-O-alpha-L-rhamnopyranoside), myricetin glycoside (myricetin 3-O-galactoside, myricetin 3-O-xyloside), quercetin glycoside (quercetin 3-O-malonylglucoside, quercetin 3,7-O-alpha-L-dirhamnopyranoside) and apigenin 6,8-di-glucopyranoside, as presented in Fig. 2A and Fig. 4A. Specifically, the apigenin 6,8-di-glucopyranoside and quercetin 3,7-O-alpha L-dirhamnopyranoside decreased by 33.12% and 18.10% in P10 compared to the P2–8 cluster, respectively, which might be due to that the glycosidic bond of flavonoid glycosides was easily broken under higher temperature and humidity conditions. Moreover, *Algoriphagus* as a bacterial genus specific to P10 samples, could secrete various glycoside hydrolases, glycosyltransferases and polysaccharide lyases with the capacity for polysaccharide degradation (Alegado et al., 2011), becoming another important factor for the obvious reduction of flavonoids and their glycosides especially in later stages of piling. Meanwhile, Wang et al. found that *Sphingomonas* was a functional dominant microorganism in the processing of Liupao tea, which could effectively less flavonoid glycoside contents and thus reduce the bitterness and astringency, which was consistent with the phenomenon in the previous study (Wang et al., 2021). Notably, the taste of TC-Liupao tea faded when the piling time reached 12 h, which might be correlated to the excessive degradation of flavor substances (quercetin-3,7-O-alpha-L-dirhamnopyranoside, apigenin 6,8-di-glucopyranoside, 2-hydroxy-3-methylbutyric acid and p-coumaric acid). Meanwhile, it had unfavorable effects for the remodeling of TC-Liupao tea quality in the later aging process.

Additionally, glycine as one of the flavor amino acids has a unique sweet taste and can moderate the acidity and bitterness of tea (Razak, Begum, Viswanath, & Rajagopal, 2017). As plotted in Fig. 2B and Fig. 4B, glycine could transform into glycine-betaine through fully *N*-methyl substituted and the contents of glycine-betaine reached a minimum value in P10, but then slightly increased after piling 12 h. In contrast, the P10 samples had relatively higher level of glycine, which was consistent with the previous study that the prolonged period of yellowing caused the increase of glycine-betaine content, hindering the accumulation of flavorful amino acids (Wei et al., 2021). The relatively high accumulation of glycine in P10 produced taste improvement of TC-Liupao tea. It could therefore be deduced that piling for ten hours might be probably the optimal solution to ensure the quality for TC-Liupao tea. Besides, it should be pointed out that there existed small amounts of metabolites with the contents increasing during the piling procedure. The majority of these metabolites were terpenoids, related to the biosynthesis of terpenoids by microbial actions, and a similar phenomenon was observed in Qingzhuan tea (Cheng et al., 2021) that the contents of 6 terpenoids showed increasing trends during the 20-year aging process. Some species of *Sphingomonas* have been proven to secrete glucosidase to promote the bioconversion of ginsenosides (Choi et al., 2010). In addition, *Sphingomonas* was found to be the dominant bacterial genus in the fermentation of Liupao tea to facilitate the synthesis of terpenoids (Pan et al., 2023). Thus, the increase of terpenoids during piling was potentially related to the action of the dominant bacterial genus of *Sphingomonas*. The coumarin metabolites were lactones formed from the esterification of p-coumaric acid (Kosuge & Conn, 1959), so the increase in toddalolactone might be accompanied by the decrease of p-coumaric acid, as described in Fig. 2B and Fig. 4C. Thereby, the acidity

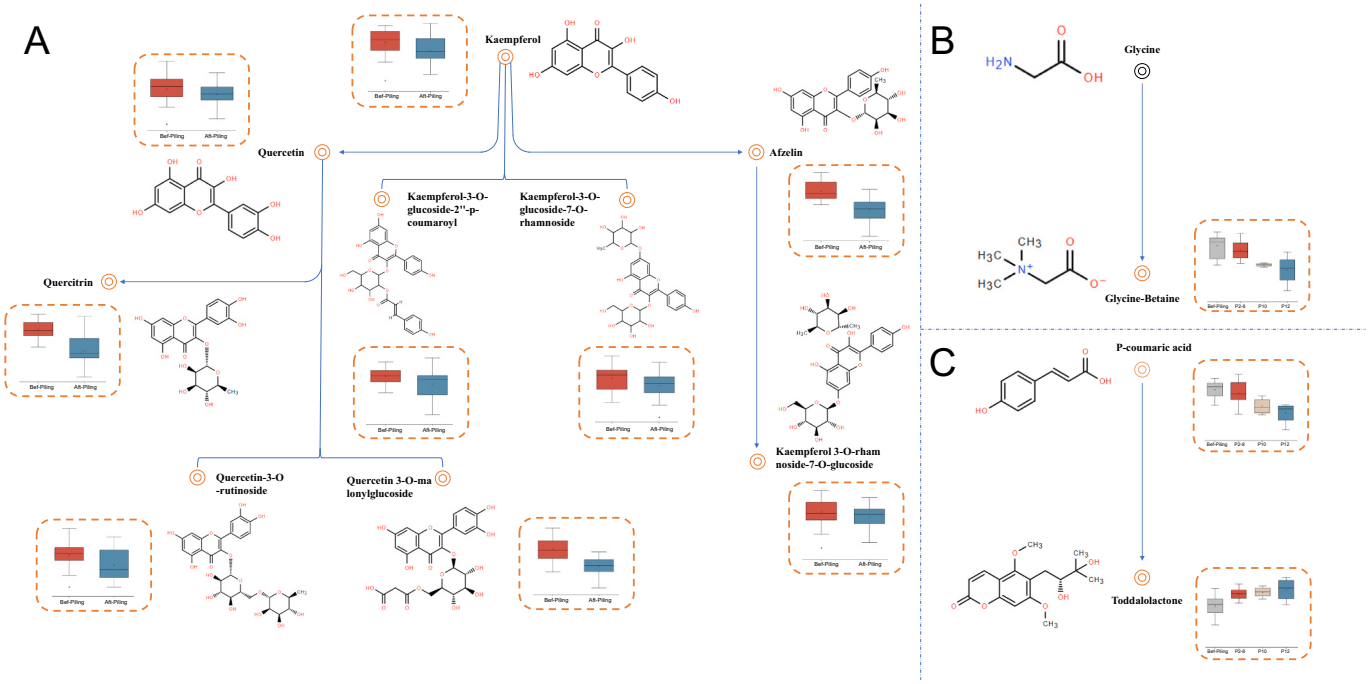

Fig. 4. The primary differential metabolites and their metabolic pathways. (A) Flavone and flavonol biosynthesis. (B) Glycine metabolism. (C) P-coumaric acid metabolism.

of TC-Liupao tea was reduced, and the mellow flavor could be gradually improved. Fatty acids were important aroma components in tea (Chen et al., 2022), and the increase of FA 18:2 + 2O content favored the improvement of the aroma of TC-Liupao tea.

In this work, correlation analysis was used to explore the association between bacterial communities and metabolic components. However, except for *Sphingomonas* and *Massilia*, other bacteria showed a weak correlation with the key metabolites. This phenomenon was significantly different from other traditional fermented foods (Qian et al., 2023), most of which were subjected to a relatively longer fermentation period. Thus, it could be preliminarily inferred that the relatively shorter piling time was an important reason for the weak correlation between bacteria and metabolites in TC-Liupao tea. In other words, the metabolic structure change during the process of TC-Liupao tea might be mainly attributed to the high temperature and humidity environment.

5. Conclusion

In the work, the sensory, metabolic characteristics and bacterial community of TC-Liupao tea were found with significant variances throughout the processing stages. The results of sensory evaluation revealed significant improvement in infusion color, aroma, and taste after piling. The differential metabolites between the before-piling and after-piling samples were mainly flavonoids and their glycosides, and the degradation of metabolites was the dominant trend during the piling procedure. Meanwhile, a series of reactions such as terpenoid biosynthesis and esterification of p-coumaric acid occurred at the piling stages. Moreover, when the piling time reached 12 h, the excessive degradation of metabolites caused the quality decrease of TC-Liupao tea, thereby piling 10 h could be regarded as the optimal fermentation period for TC-Liupao tea. In addition, *Sphingomonas*, *Achromobacter*, *Microbacterium*, and *Methylobacterium* were the main bacterial genera. Based on the pearson correlation analysis, it was found that *Sphingomonas* and *Massilia* were associated with flavonoid glycosides and terpenoids through secreting extracellular enzymes, potentially improving the tea quality.

CRediT authorship contribution statement

Huahong Liu: Writing – original draft, Visualization, Data curation. **Yingyi Huang:** Writing – original draft, Formal analysis. **Zhusheng Liu:** Supervision, Funding acquisition. **Yuelan Pang:** Methodology, Conceptualization. **Chun Yang:** Resources. **Min Li:** Visualization, Data curation. **Qianhua Wu:** Formal analysis. **Jinfang Nie:** Writing – review & editing, Validation, Investigation, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work.

Data availability

Data will be made available on request.

Acknowledgement

This research was financially supported by the Guangxi Innovation Driven De-velopment Special Fund Project of China (No. GuikeAA20302018-9), Guangxi Science and Technology Major Program (No. GuikeAA23023006), National Natural Science Foundation of China (Grant No. 21874032 and 21765007), Guangxi Scholarship Fund of Guangxi Education Department (No. Guijiaoren-2019-5), National Modern Agricultural Industry Technology System Guangxi Innovation Team Tea Processing Position (nycytxgxcxtd-18-03).

Ethical statement

No ethical permission was required for this work but the authors confirm that the appropriate protocols for protecting the rights and privacy of all participants were utilized during the execution of the research, with no coercion to participate, full disclosure of study requirements and risks. Participants provided verbal informed consent

prior to taking part in the study. There will be no release of participant data without their knowledge, and they have the ability to withdraw from the study at any time.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101516.

References

Alegado, R. A., Ferriera, S., Nusbaum, C., Young, S. K., Zeng, Q., Imamovic, A., … King, N. (2011). Complete genome sequence of Algoriphagus sp. PR1, bacterial prey of a colony-forming choanoflagellate. *Journal of Bacteriology, 193*(6), 1485–1486. https://doi.org/10.1128/jb.01421-10

An, T., Chen, M., Zu, Z., Chen, Q., Lu, H., Yue, P., & Gao, X. (2021). Untargeted and targeted metabolomics reveal changes in the chemical constituents of instant dark tea during liquid-state fermentation by Eurotium cristatum. *Food Research International, 148*, Article 110623. https://doi.org/10.1016/j.foodres.2021.110623

Bahule, C. E., da Silva Martins, L. H., Chaúque, B. J. M., Trindade, F., Herrera, H., da Costa, I. R. C., … Lopes, A. S. (2024). Metaproteomics revealing microbial diversity and activity in the spontaneous fermentation of maize dough. *Food Chemistry, 435*, Article 137457. https://doi.org/10.1016/j.foodchem.2023.137457

Bajpai, B., & Patil, S. (1997). Induction of tannin acyl hydrolase (EC 3.1. 1.20) activity in some members of fungi imperfecti. *Enzyme and Microbial Technology, 20*(8), 612–614. https://doi.org/10.1016/s0141-0229(96)00206-2

Chen, M., Zu, Z., Shen, S., An, T., Zhang, H., Lu, H., Fu, M., Wen, Y., Chen, Q., & Gao, X. (2023). Dynamic changes in the metabolite profile and taste characteristics of loose-leaf dark tea during solid-state fermentation by Eurotium cristatum. *Lwt, 176*, Article 114528. https://doi.org/10.1016/j.lwt.2023.114528

Chen, Q., Zhu, Y., Liu, Y., Liu, Y., Dong, C., Lin, Z., & Teng, J. (2022). Black tea aroma formation during the fermentation period. *Food Chemistry, 374*, Article 131640. https://doi.org/10.1016/j.foodchem.2021.131640

Cheng, L., Wang, Y., Zhang, J., Xu, L., Zhou, H., Wei, K., Peng, L., Zhang, J., Liu, Z., & Wei, X. (2021). Integration of non-targeted metabolomics and E-tongue evaluation reveals the chemical variation and taste characteristics of five typical dark teas. *Lwt, 150*, Article 111875. https://doi.org/10.1016/j.lwt.2021.111875

Cheng, L., Wang, Y., Zhang, J., Zhu, J., Liu, P., Xu, L., Wei, K., Zhou, H., Peng, L., & Zhang, J. (2021). Dynamic changes of metabolic profile and taste quality during the long-term aging of Qingzhuan tea: The impact of storage age. *Food Chemistry, 359*, Article 129953. https://doi.org/10.1016/j.foodchem.2021.129953

Choi, T., Liu, Q., Yang, J., Sun, S., Kim, S., Yi, T., & Im, W. (2010). Sphingomonas ginsenosidimutans sp. nov., with ginsenoside converting activity. *The Journal of Microbiology, 48*, 760–766. https://doi.org/10.1007/s12275-010-0469-z

Feng, X., Chen, M., Song, H., Ma, S., Ou, C., Li, Z., Hu, H., Yang, Y., Zhou, S., & Pan, Y. (2023). A systemic review on Liubao tea: A time-honored dark tea with distinctive raw materials, process techniques, chemical profiles, and biological activities. *Comprehensive Reviews in Food Science and Food Safety, 22*(6), 5063–5085. https://doi.org/10.1111/1541-4337.13254

Fernández, P. L., López, A., Pablos, F., González, A. G., & Martín, M. J. (2003). The use of catechins and purine alkaloids as descriptors for the differentiation of tea beverages. *Microchimica Acta, 142*, 79–84. https://doi.org/10.1007/s00604-003-0002-8

Gu, H., Yin, X., Ma, Y., Wang, J., Yang, F., Sun, W., … Liu, Z. (2020). Differentiating grades of Xihu Longjing teas according to the contents of ten major components based on HPLC-DAD in combination with chemometrics. *Lwt, 130*, Article 109688. https://doi.org/10.1016/j.lwt.2020.109688

He, S., Deng, X., Han, Y., Gong, Z., Wang, J., Tao, X., Tong, H., & Chen, Y. (2023). Metabolites and metagenomic analysis reveals the quality of Pu-erh "tea head". *Food Chemistry, 429*, Article 136992. https://doi.org/10.1016/j.foodchem.2023.136992

Hu, S., He, C., Li, Y., Yu, Z., Chen, Y., Wang, Y., & Ni, D. (2021). Changes of fungal community and non-volatile metabolites during pile-fermentation of dark green tea. *Food Research International, 147*, Article 110472. https://doi.org/10.1016/j.foodres.2021.110472

Huang, Y., Liu, H., Zhang, X., Wu, Y., Liu, Z., Pang, Y., Liu, R., Yang, C., & Nie, J. (2023). Impact of storage time on non-volatile metabolites and fungal communities in Liupao tea using LC-MS based non-targeted metabolomics and high-throughput sequencing. *Food Research International, 174*, Article 113615. https://doi.org/10.1016/j.foodres.2023.113615

Kosuge, T., & Conn, E. E. (1959). The metabolism of aromatic compounds in higher plants: I. COUMARIN AND o-COUMARIC ACID. *Journal of Biological Chemistry, 234*(8), 2133–2137. https://doi.org/10.1016/S0021-9258(18)69879-5

Li, J., Wu, J., Xu, N., Yu, Y., Brake, J., Xu, R., & Wu, X. (2022). Dynamic evolution and correlation between microorganisms and metabolites during manufacturing process and storage of Pu-erh tea. *Lwt, 158*, Article 113128. https://doi.org/10.1016/j.lwt.2022.113128

Li, Q., Huang, J., Li, Y., Zhang, Y., Luo, Y., Chen, Y., Lin, H., Wang, K., & Liu, Z. (2017). Fungal community succession and major components change during manufacturing process of Fu brick tea. *Scientific Reports, 7*(1), 6947. https://doi.org/10.1038/s41598-017-07098-8

Li, Q., Li, Y., Luo, Y., Zhang, Y., Chen, Y., Lin, H., Wang, K., Huang, J., & Liu, Z. (2019). Shifts in diversity and function of the bacterial community during the manufacture of Fu brick tea. *Food Microbiology, 80*, 70–76. https://doi.org/10.1016/j.fm.2019.01.001

Li, Y., Hao, J., Zhou, J., He, C., Yu, Z., Chen, S., Chen, Y., & Ni, D. (2022). Pile-fermentation of dark tea: Conditions optimization and quality formation mechanism. *Lwt, 166*, Article 113753. https://doi.org/10.1016/j.lwt.2022.113753

Lin, F., Wei, X., Liu, H., Li, H., Xia, Y., Wu, D., … Gan, R. (2021). State-of-the-art review of dark tea: From chemistry to health benefits. *Trends in Food Science & Technology, 109*, 126–138. https://doi.org/10.1016/j.tifs.2021.01.030

Liu, K., Wang, L., Jiang, B., An, J., Nian, B., Wang, D., Chen, L., Ma, Y., Wang, X., & Fan, J. (2021). Effect of inoculation with Penicillium chrysogenum on chemical components and fungal communities in fermentation of Pu-erh tea. *Food Research International, 150*, Article 110748. https://doi.org/10.1016/j.foodres.2021.110748

Mao, Y., Wei, B., Teng, J., Huang, L., & Xia, N. (2017). Analyses of fungal community by Illumina MiSeq platforms and characterization of Eurotium species on Liupao tea, a distinctive post-fermented tea from China. *Food Research International, 99*, 641–649. https://doi.org/10.1016/j.foodres.2017.06.032

Pan, J., Wang, J., Teng, J., Huang, L., Wei, B., Xia, N., & Zhu, P. (2023). Deciphering the underlying core microorganisms and the marker compounds of Liupao tea during the pile-fermentation process. *Journal of the Science of Food and Agriculture, 104*(5), 2862–2875. https://doi.org/10.1002/jsfa.13177

Qian, M., Ruan, F., Zhao, W., Dong, H., Bai, W., Li, X., Huang, X., & Li, Y. (2023). The dynamics of physicochemical properties, microbial community, and flavor metabolites during the fermentation of semi-dry Hakka rice wine and traditional sweet rice wine. *Food Chemistry, 416*, Article 135844. https://doi.org/10.1016/j.foodchem.2023.135844

Qin, H., Huang, L., Teng, J., Wei, B., Xia, N., & Ye, Y. (2021). Purification, characterization, and bioactivity of Liupao tea polysaccharides before and after fermentation. *Food Chemistry, 353*, Article 129419. https://doi.org/10.1016/j.foodchem.2021.129419

Razak, M. A., Begum, P. S., Viswanath, B., & Rajagopal, S. (2017). Multifarious beneficial effect of nonessential amino acid, glycine: A review. *Oxidative Medicine and Cellular Longevity, 2017*, Article 171670. https://doi.org/10.1155/2017/1716701

Reuter, J. A., Spacek, D. V., & Snyder, M. P. (2015). High-throughput sequencing technologies. *Molecular Cell, 58*(4), 586–597. https://doi.org/10.1016/j.molcel.2015.05.004

Shi, J., Ma, W., Wang, C., Wu, W., Tian, J., Zhang, Y., Shi, Y., Wang, J., Peng, Q., & Lin, Z. (2021). Impact of various microbial-fermented methods on the chemical profile of dark tea using a single raw tea material. *Journal of Agricultural and Food Chemistry, 69*(14), 4210–4222. https://doi.org/10.1021/acs.jafc.1c00598

Wang, H., Teng, J., Huang, L., Wei, B., & Xia, N. (2023). Determination of the variations in the metabolic profile and sensory quality of Liupao tea during fermentation through UHPLC–HR–MS metabolomics. *Food Chemistry, 404*, Article 134773. https://doi.org/10.1016/j.foodchem.2022.134773

Wang, J., Zhang, J., Chen, Y., Yu, L., Teng, J., Xia, N., Wei, B., Xiao, S., & Huang, L. (2021). The relationship between microbial dynamics and dominant chemical components during Liupao tea processing. *Food Bioscience, 43*, Article 101315. https://doi.org/10.1016/j.fbio.2021.101315

Wang, X., Wan, X., Hu, S., & Pan, C. (2008). Study on the increase mechanism of the caffeine content during the fermentation of tea with microorganisms. *Food Chemistry, 107*(3), 1086–1091. https://doi.org/10.1016/j.foodchem.2007.09.023

Wei, Y., Li, T., Xu, S., Ni, T., Deng, W., & Ning, J. (2021). The profile of dynamic changes in yellow tea quality and chemical composition during yellowing process. *Lwt, 139*, Article 110792. https://doi.org/10.1016/j.lwt.2020.110792

Wen, M., Zhu, M., Han, Z., Ho, C. T., Granato, D., & Zhang, L. (2023). Comprehensive applications of metabolomics on tea science and technology: Opportunities, hurdles, and perspectives. *Comprehensive Reviews in Food Science and Food Safety, 22*(6), 4890–4924. https://doi.org/10.1111/1541-4337.13246

Wu, H., Zhao, H., Ding, J., Wang, Y., Hou, J., & Yang, L. (2023). Metabolites and microbial characteristics of Fu brick tea after natural fermentation. *Lwt, 181*, Article 114775. https://doi.org/10.1016/j.lwt.2023.114775

Xiao, Y., He, C., Chen, Y., Ho, C., Wu, X., Huang, Y., … Wang, Y. (2022). UPLC–QQQ–MS/MS-based widely targeted metabolomic analysis reveals the effect of solid-state fermentation with Eurotium cristatum on the dynamic changes in the metabolite profile of dark tea. *Food Chemistry, 378*, Article 131999. https://doi.org/10.1016/j.foodchem.2021.131999

Xu, Y., Zhang, Y., Chen, J., Wang, F., Du, Q., & Yin, J. (2018). Quantitative analyses of the bitterness and astringency of catechins from green tea. *Food Chemistry, 258*, 16–24. https://doi.org/10.1016/j.foodchem.2018.03.042

Ye, J., Ye, Y., Yin, J., Jin, J., Liang, Y., Liu, R., … Xu, Y. (2022). Bitterness and astringency of tea leaves and products: Formation mechanism and reducing strategies. *Trends in Food Science & Technology, 123*, 130–143. https://doi.org/10.1016/j.tifs.2022.02.031

Zhang, L., Li, N., Ma, Z., & Tu, P. (2011). Comparison of the chemical constituents of aged pu-erh tea, ripened pu-erh tea, and other teas using HPLC-DAD-ESI-MS n. *Journal of Agricultural and Food Chemistry, 59*(16), 8754–8760. https://doi.org/10.1021/jf2015733

Zhao, H., Wang, L., Ren, J., Li, Z., Li, M., & Gao, H. (2008). Isolation and characterization of phenanthrene-degrading strains Sphingomonas sp. ZP1 and Tistrella sp. ZP5. *Journal of Hazardous Materials, 152*(3), 1293–1300. https://doi.org/10.1016/j.jhazmat.2007.08.008

Zhao, W., Liang, Z., Qian, M., Li, X., Dong, H., Bai, W., Wei, Y., & He, S. (2022). Evolution of microbial communities during fermentation of chi-flavor type baijiu as

determined by high-throughput sequencing. *Lwt, 170*, Article 114102. https://doi.org/10.1016/j.lwt.2022.114102

Zhu, Q. Y., Holt, R. R., Lazarus, S. A., Ensunsa, J. L., Hammerstone, J. F., Schmitz, H. H., & Keen, C. L. (2002). Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. *Journal of Agricultural and Food Chemistry, 50*(6), 1700–1705. https://doi.org/10.1021/jf011228o

Zhu, R., Chen, Z., Lv, H., Pan, Y., Feng, X., Chen, G., Hu, W., Xu, T., Fan, F., & Gong, S. (2023). Another thread to uncover the aging mystery of white tea: Focusing on the natural nanoparticles in tea infusion. *Food Chemistry, 429*, Article 136838. https://doi.org/10.1016/j.foodchem.2023.136838