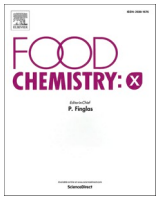
Food Chemistry: X 23 (2024) 101553

Contents lists available at ScienceDirect

Food Chemistry: X

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



Exploring microbial and moist-heat effects on Pu-erh tea volatiles and understanding the methoxybenzene formation mechanism using molecular sensory science

Tiehan Li [a,b], Yuming Wei [a,b], Mingxia Lu [a,b], Yida Wu [a,b], Yanqun Jiang [a,b], Han Ke [a,b], Aiju Shao [c], Jingming Ning [a,b,*]

[a] State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, 130 Changjiang West Road, Hefei, Anhui 230036, China
[b] School of Tea and Food Science and Technology, Anhui Agricultural University, 130 Changjiang West Road, Hefei 230036, China
[c] Menghai Tea Industry Co., Ltd., Yunnan 650000, China

ARTICLE INFO

Keywords:
Pu-erh tea
Gas chromatography-mass spectrometry
Microbial action
Aroma
Piling fermentation
Methoxybenzenes

ABSTRACT

Piling fermentation (PF) is crucial for Pu-erh tea aroma, yet its microbial and moist-heat impact on aroma quality is poorly understood. Solid-phase microextraction, solvent-assisted flavor evaporation, and gas chromatography–mass spectrometry were used to detected and analyses the samples of sun-green green tea, sterile PF and spontaneous PF. Microbiological action promotes the formation of stale aromas. Moist-heat action promotes the formation of plum-fragrance and sweet aroma. 20 microbial markers and 28 moist-heat markers were screened from 184 volatile components. Combining odor activity values and gas chromatography-olfactometry, 22 aroma-active compounds were screened (1,2,3-trimethoxybenzene, linalool, 1,2,4-trimethoxybenzene …), and analyzed during PF processing. Aroma omission and addition experiments verified its importance. Gallic acid addition experiments successfully verified that microorganisms are the main contributors to the synthesis of methoxybenzenes. Finally, *Blastobotrys, Rasamsonia*, and *Thermomyces* showed positive correlation with the synthesis of 1-ethyl-4-methoxybenzene, 1,2,4-trimethoxybenzene, 1,2,3-trimethoxybenzene, and 1,2-dimethoxybenzene. The formation mechanism of Pu-erh tea's aroma was clarified.

Exploring microbial and moist-heat effects on Pu-erh tea volatiles and understanding the methoxybenzene formation mechanism using molecular sensory science.

1. Introduction

Ripened Pu-erh tea is a unique post-fermented tea that is prepared from sun-dried green tea (SDGT) leaves using modern piling fermentation (PF) techniques (Cao et al., 2018; Wang et al., 2022; Wang et al., 2022; Li et al., 2022; Zhang, Zhang, Zhou, Ling, & Wan, 2013). The core of Pu-erh tea processing is PF, which is essential for developing the distinct qualities of ripened and raw Pu-erh tea (Li et al., 2023; Mo, Zhu, & Chen, 2008; Zhao et al., 2015). During PF, SDGT leaves undergo significant alterations caused by microbial and moist-heat action (Li et al., 2023; Akacha & Gargouri, 2015; Xie et al., 2020). In our previous study, we processed the same raw materials into sterile piling fermented (STPF) samples and spontaneous piling fermented (SPPF) samples and used omics-based analysis to detect nonvolatile components in tea leaves (Li, Zhang, et al., 2022; Li et al., 2023). Metabolomic analysis revealed 21 key metabolites were influenced by microorganisms. Conversely, 10 metabolites were influenced by moist-heat action (Li et al., 2023). Notably, the study also revealed that the degradation of flavanol and phospholipid metabolic pathways not only affected the taste of tea taste but also played a pivotal role in forming its aroma (Deng et al., 2021; Li et al., 2023). These transformations produce the key characteristics of Pu-erh tea, including a brownish-red infusion

Abbreviations: PF, piling fermentation; SDGT, sun-dried green tea; STPF samples, sterile PF; SPPF, spontaneous PF; SPME, solid-phase microextraction; SAFE, solvent-assisted flavor evaporation; GC–MS, gas chromatographs spectrometry; GC-O, GC–olfactometry; ROAVs, relative odor activity values; AEDA, aroma extract dilution analysis; FD, flavor dilution; GA, gallic acid.

* Corresponding author at: State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, 130 West Changjiang Rd., Hefei, Anhui 230036, China.

E-mail address: ningjm1998009@163.com (J. Ning).

https://doi.org/10.1016/j.fochx.2024.101553
Received 15 April 2024; Received in revised form 11 June 2024; Accepted 11 June 2024
Available online 13 June 2024

color, stale and mellow taste, and distinctive stale and fungal aroma (Zhang et al., 2022; Li et al., 2023; Deng et al., 2021). And relevant studies have indicated that the aroma of Pu-erh tea gradually transitions from floral and fruity to stale and woody during the PF process (Ma et al., 2022; Wang, Li, et al., 2022; Wang, Su, et al., 2022). However, the action mechanisms of microbial action and moist-heat action on the formation of the aroma of Pu-erh tea during PF are still unclear.

Aroma is a crucial quality characteristic of Pu-erh tea and extensive research has been conducted to explore its aromatic profile. Various methods such as sensory evaluations, solid-phase microextraction (SPME) (Chen et al., 2017; Lv et al., 2012;Wang, Li, et al., 2022 ; Wang, Su, et al., 2022), solvent-assisted flavor evaporation (SAFE) (Zhu, Niu, & Xiao, 2022), stir bar sorptive extraction (SBSE) (Wang et al., 2020), gas chromatography–mass spectrometry (GC–MS) (Feng et al., 2019; Rong et al., 2023; Shi, Zhu, Zhang, Lin, & Lv, 2019), gas chromatography-–olfactometry (GC-O) (Lv et al., 2012), odor activity values (OAV) (Yang, Song, Wang, & Jing, 2019), and other analysis methods have been employed (Zhai, Zhang, Granvogl, Ho, & Wan, 2022). Reportedly, >1000 volatile compounds have been detected in Pu-erh tea (Wang, Li, et al., 2022; Wang, Su, et al., 2022). In 2012, Lv et al. used SPME combined with GC–O to identify key compounds that contribute to the unique flavor of Pu-erh tea, including linalool oxides, 1,2,3-trimethoxybenzene, and β-ionone (Lv et al., 2012). In 2016–2018, Cao et al. reported that microbial activity is vital for the formation of the aroma of dark tea (Cao et al., 2018; Lv, Zhang, Shi, & Lin, 2016; Wang, Li, et al., 2022; Wang, Su, et al., 2022). In 2019, Pang et al. explored the aroma differences between raw and ripened Pu-erh teas using GC-O, aroma extract dilution analysis (AEDA), and OAV and identified potential odor-active markers. Their results highlighted the significance of specific compounds such as 1,2,4-trimethoxybenzene, linalool, and 1,2,3-trimethoxy-5-methylbenzene as potential odor-active markers for distinguishing between raw and ripe Pu-erh teas (Pang et al., 2019). In 2021, the study of Deng et al. point out that β-damascenone, 1,2,4-trimethoxybenzene, and (*E,E*)-2,4-nonadienal were the main components of the aroma of Pu-erh tea (Deng et al., 2021). In 2022, Ma et al. prepared five typical methoxy-phenolic and major flavor compounds with different concentrations based on the analysis of Pu-erh tea samples. They reported that gallic acid (GA) at low concentrations ($\leq$4 mg/g) enhanced the release of 3,4-dimethoxytoluene, 1,2-dimethoxybenzene, and 1,2,3-trimethoxy-5-methyl-benzene (Lv et al., 2012; Ma et al., 2022). However, the environmental factors of the process, process parameters, etc. are ignored in the simulation experiments; therefore, they may not be able to be used to explain the formation mechanism of GA. Therefore, exploring the reasons for the formation of Pu-erh tea aroma is very essential.

In summary, we found that methoxybenzene is the key to the formation of Pu-erh tea flavor, and the GA is the key precursor for the formation of aged aroma substances. However, there are many other reasons affecting the formation of stale aroma substances, such as fermentation length, temperature, humidity, microorganisms and so on. Therefore, this study aimed to reveal the effects of microbial and moist-heat actions on volatile metabolites and the formation mechanism of stale aroma substances in Pu-erh tea during the PF process. In this study, SDGT was processed into Pu-erh tea through spontaneous PF and sterile PF. This study uses aroma extraction methods such as SPME and SAFE combined with GC–MS to detect and analyze the aroma components of SPPF and STPF samples processed from SDGT. The objectives of this study were to (1) reveal the effects of microbial and moist-heat actions on volatile metabolites; (2) to elucidate the contribution of the reaction mechanism of the aroma precursor GA to the stale aroma substances. (3) The reasons for the formation of the characteristic flavor of Pu-erh tea. (4) Establishing microbial correlations with aroma components. This study will, for the first time, elucidate the respective contributions of microbial activity and moist heat to aroma during the fermentation process of Pu-erh tea. Furthermore, the reasons for the formation of the characteristic aroma of Pu-erh tea will be further analyzed. This study will provide a theoretical basis for the formation mechanism of Pu-erh tea aroma quality.

2. Materials and methods

2.1. Sample preparation and chemical material

Sample preparation: The raw material of SDGT was provided and processed by Menghai Tea Factory, consistent with the samples used in our previous study (Li, Zhang, et al., 2022; Li et al., 2023) (Fig. 1a). Our first batch of samples was taken on 2021.10; samples for the simulation validation experiment were collected on 2023.6, and the grade of material selected for the second sampling was the same as the first. In brief, PF was carried out in a fermentation room with controlled temperature (17 °C–22.5 °C) and humidity (65%–86%). The tea pile was turned weekly to maintain homogeneity and regulate the temperature until the tea reached the desired reddish-brown color and flavor. The PF process lasted 48 days. SPPF-processed samples were prepared traditionally with SDGT, while STPF-processed samples were sterilized in glass jars and supplemented with sterile water. Both sets of samples underwent PF after grouping. One SDGT sample and five samples each of SPPF-processed (SPPF-1 [7 days], SPPF-2 [14 days], SPPF-3 [22 days], SPPF-4 [32 days], and SPPF-5 [38 days]) and STPF-processed (SPPF-1 [7 days], SPPF-2 [14 days], SPPF-3 [22 days], SPPF-4 [32 days], and SPPF-5 [38 days]) were collected and stored at −80 °C for later use.

Chemical material: 1,2,3-trimethoxybenzene, benzeneacetaldehyde, 1-Octen-3-ol, 1,2,4-trimethoxybenzene, (*E*)-β-ionone, Linalool, (*E, E*)-2,4-heptadienal, β-damascenone, and 1,2-dimethoxy-benzene were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). All odorant standards had a purity exceeding 95% for GC analysis. Ethyl decanoate (purity >99%) was obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). n-Alkanes (C7-C40) were sourced from Sigma-Aldrich Fluka Co., Ltd. (St. Louis, MO, USA). Sodium chloride (NaCl) and anhydrous sodium sulfate (Na2SO4) were procured from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dichloromethane (purity >99%) was purchased from Tedia Co., Inc. (Fairfield, OH, USA), and ethanol (purity >99%) was sourced from Yongda Chemical Reagent Co., Ltd. (Tianjing, China). Deionized water was obtained from a Milli-Q water purification system (Millipore Inc., Billerica, MA, USA).

2.2. Traditional sensory evaluation

Six professionals from the quality supervision department of Menghai Tea Factory (Xishuangbanna, Yunnan, China). The samples were blind-coded with random numbers. A total of 3 g of tea leaves was infused into 150 mL of boiled, purified water in separate white porcelain cups and maintained for 5 min. And poured into a white porcelain bowl to describe the aroma profile of tea. The aforementioned professionals provided a report of their sensory evaluations (GB/T, 2017).

2.3. Quantitative descriptive analysis

A panel comprising 14 experts, consisting of 8 men and 6 women aged 22–30 years, conducted a sensory evaluation of three types of tea infusions: SDGT, SPPF-A, and STPF-A. The teas were prepared via the traditional sensory evaluation method. The panelists assessed the aroma profile attributes of the samples and selected 12 quality descriptors in common that best represented the SDGT, SPPF-A, and STPF-A infusions: sweet (like honey), sweet (like brown sugar), woody, refreshing, flora fruity, cooling, plum fragrance, sour, smoky, stale/musty, medicinal, and earthy. Standards corresponding to the 12 characteristic properties (sweet (like honey)- phenylacetaldehyde, sweet (like brown sugar)-corresponding physical object, woody- corresponding physical object, refreshing- hexanal, flora fruity- linalool, cooling- methyl salicylate, plum fragrance- corresponding physical object, sour- corresponding

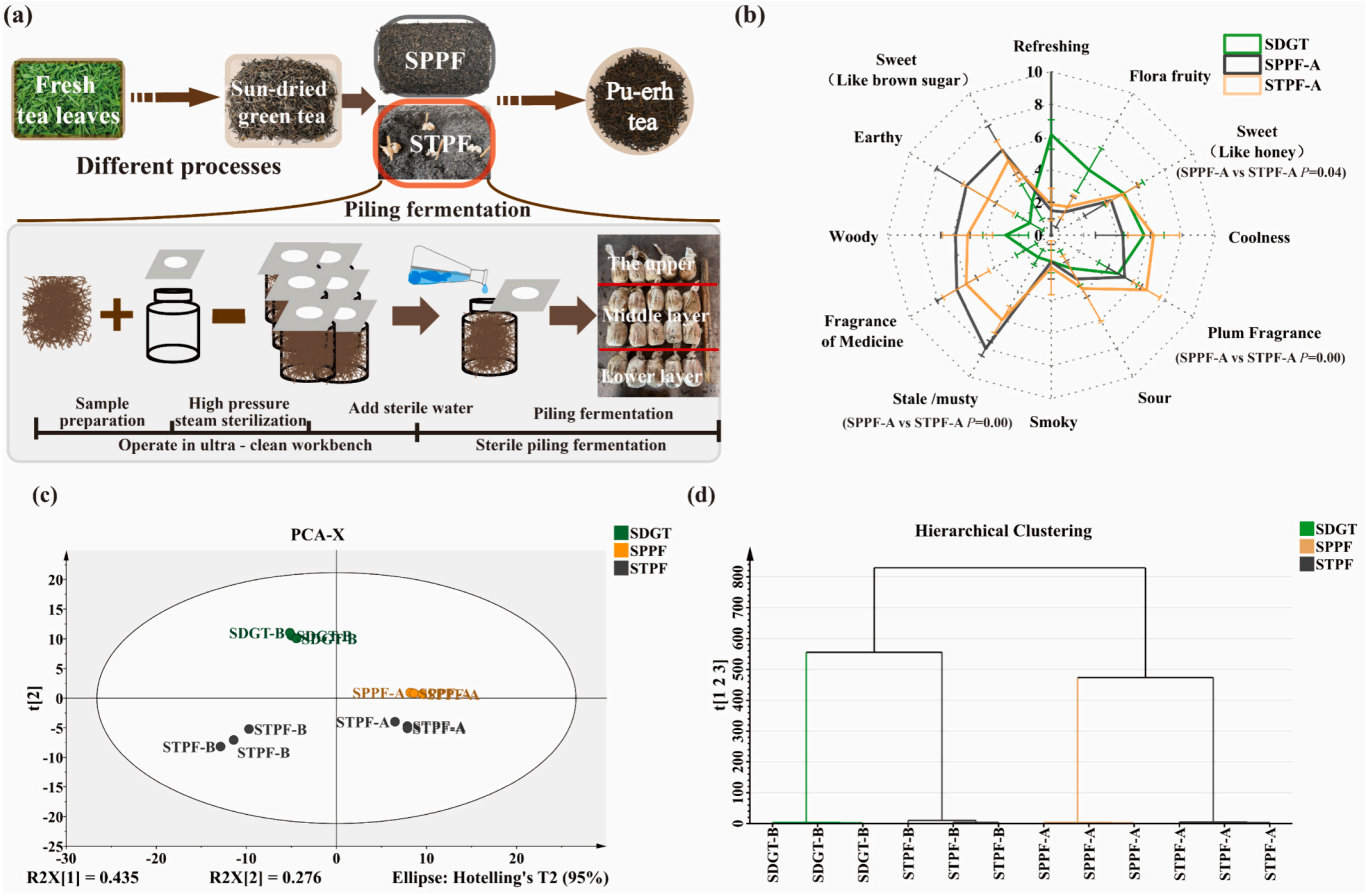


Fig. 1. Analyze the flowchart. (a) Schematic diagram of sample preparation (Li, Wu, et al., 2022; Li, Zhang, et al., 2022), (b) Demonstration of tea samples, (c) Comparative descriptive aroma profiles of different tea infusions, (d) Key aroma components in different samples.
Note: SDGT, sun-dried green tea; SPPF, spontaneous piling fermentation; and STPF, sterile piling fermentation. And SDGT-B, sun-dried green tea before piling fermentation; SPPF-A, spontaneous piling fermentation after piling fermentation; STPF-B, sterile piling fermentation before piling fermentation; STPF-A, sterile piling fermentation after piling fermentation.

physical object, smoky-corresponding physical object, stale/musty-1,2,3-Trimethoxybenzene, fragrance of medicine-corresponding physical object, and earthy-corresponding physical object) were employed as intensity references for the characteristic attributes. Furthermore, panelists used a 10-point scale to quantitatively express the strength of each descriptor, with scores ranging from "extremely strong" (8–10) to "strong" (6–8), "neutral" (4–6), "weak" (2–4), and "extremely weak" (0–2). This scale served as a precise tool to objectively quantify the sensory characteristics of the three tea infusions, offering valuable data for evaluating SDGT, SPPF-A, and STPF-A. Ethical permission, to conduct a human sensory study, was granted by our institution. Participants gave informed consent via the statement "I am aware that my responses are confidential, and I agree to participate in this sensory evaluation" where an affirmative reply was required to enter the sensory evaluation. They were able to withdraw from the sensory evaluation at any time without giving a reason. The tea products evaluated were safe for consumption. And "human sensory ethical inspection" was provided in the supplementary material.

2.4. Isolation of the volatiles

HS-SPME: In separate white porcelain cups, 6 g tea leaves were steeped in 120 mL boiled purified water for 5 min. Subsequently, 10 mL tea infusion was transferred into a headspace vial, to which 30 μL 10-μg/mL ethyl caprate solution was added as an internal standard along with 3.0 g NaCl. The vial was promptly sealed and allowed to equilibrate for 15 min. Following this, SPME fiber was introduced into the headspace vial for 60 min while the vial was placed in a water bath at 40 °C. This method followed the procedure detailed by Fang et al. and Wei et al. (Fang et al., 2019; Wei et al., 2022).

SAFE: Overall, 5 μL 16.67-μg/L ethyl caprate was introduced into 150 mL tea infusion, following the procedure in the HS-SPME extraction method. This solution was subsequently processed using the SAFE apparatus at 40 °C under a vacuum of 10^{-3} Pa. After thorough mixing, SAFE distillation was performed thrice using 30 mL dichloromethane for extraction. The resulting extracts were dehydrated using anhydrous sodium sulfate and concentrated to 100 μL using nitrogen in a water bath at 25 °C. This method aligns with the approach employed by Huang et al. and Zhang et al. (Huang et al., 2022; Zhang et al., 2023; Zhang et al., 2023).

2.5. Gas chromatography-mass spectrometry (GC–MS) analysis

A gas chromatograph, the 8890A model, equipped with a 5977B mass spectrometer (Agilent, USA), was employed in this study. And The separation of volatile compounds was achieved using an HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent) and DB-FFAP column (30 m × 0.25 mm, 0.25-μm film thickness) with a programmed oven temperature. Helium with a purity exceeding 99.99% served as the carrier gas. SPME analysis was performed in the spitless injection mode. The GC oven temperature was programmed as follows: beginning at 40 °C (held for 5 min) and then increased to 50 °C at 4 °C/min, 180 °C at 4 °C/min, and 280 °C at 20 °C/min (held for 5 min). For SAFE analysis, the temperature ranged from 40 °C (held for 5 min) to 70 °C at 8 °C/min, then

increased to 100 °C at 8 °C/min, 200 °C at 4 °C/min, and finally to 280 °C at 10 °C/min (held for 5 min). The mass selective detector operated in positive electron-ionization mode with a mass scan range of *m/z* 30–350 at 70 eV. The volatiles compounds were identified by comparing MS data with the National Institute of Standards and Technology database and using retention indices (RIs) determined via n-alkanes C_7–C_{40} (Wei et al., 2024). The aroma-active compounds were obtained via GC–MS/O and AEDA. The GC oven temperature and the conditions for the mass selective detector remained consistent with those used in GC–MS. To comprehensively assess odorants, the AEDA values of the headspace SPME and SAFE distillate were analyzed by three trained assessors using GC–O (Huang et al., 2022). For SPME, aroma activity was gradually diluted, with the split ratio adjusted (1:1, 3:1, 7:1, …) until no odor was detectable at the sniffing port. This information was used to calculate the flavor dilution (FD) factor. Furthermore, the SAFE distillate was diluted stepwise at a 1:1 ratio (with dichloromethane) and examined using GC–O, progressing from high to low concentrations until no odor was perceptible at the sniffing port. This process assigned an FD factor to each identified aroma compound, which indicated the last dilution step where the odor was detectable.

2.6. Quantitative analysis and calculation of relative odor activity values (ROAVs)

Quantitative analysis relied on the internal and external standard method, with ethanol serving as the solvent for the standard method. Standard curves were established for nine aroma-active compounds by analyzing a range of known standard compound concentrations and the concentrations of the aroma-active compounds detected in the present study were determined by applying these standard curves. When authentic standards were unavailable, the content of volatile compounds was estimated using the standard curve for ethyl caprate. To calculate ROAVs, the concentration of each aroma-active compound was compared with its odor threshold (OT) value in water. These OT values were sourced from “Odour Thresholds: Compilations of Odour Threshold Values in Air, Water, and Other Media" (second enlarged and revised edition) and https://www.leibniz-lsb.de. Additionally, aroma-active compounds with ROAVs of $\geq$1 were considered potential contributors to the aroma profile of the tea (Liao et al., 2020).

2.7. Aroma addition experiment and omission experiment

Aroma addition experimental and methods refer to our team's previous studies. (Huang et al., 2022). In this study, we used this method mainly to verify the causes of SPPF processing leading to stale flavor characteristics. In the same methodology as in Section 2.3, 14 trained panelists assessed omitted and added models by triangle test (Yang et al., 2019). In brief, in the aroma addition experiment, one key aroma substance was added to the tea broth according to the tested concentration each time. In the aroma omission experiment, the five aroma substances were added to the tea broth in accordance with the tested concentration of the remaining four aroma substances in the missing one aroma substance at a time. All of them were analyzed by sensory description for the evaluation of aroma changes.

2.8. Simulation laboratory for the addition of methoxybenzene

In our experiment, in order to verify the integrity of this conclusion, we designed a simulation experiment in an industrial PF workshop. During regular PF, we packed SPPF samples (100 g + 2 g GA), STPF samples (100 g), and STPF samples (100 g + 2 g GA) into glass containers and sealed them with aseptic film. Following the same procedure as above, after 38 days, we removed the final samples. Details are provided in the sample preparation section. The preparation procedure is consistent with the sample preparation details in 2.1.

2.9. Fungal sample collection and detection

First, we weighed 25 g of tea samples and added 250 mL of sterile phosphate buffer solution (PH = 7.3–7.5) in a 500 mL sterile conical flask. After that, we sonicated the membrane for 10 min, then centrifuged it (8000* g, 4 °C, 10 min); finally, we passed it through a 500 mL vacuum filtration system with 0.22 μm. Finally, the membrane is collected and placed in a freezing tube.

DNA extraction: The DNA was extracted with the TGuide S96 Magnetic Soil /Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to manufacturer instructions. The DNA concentration of the samples was measured with the Qubit dsDNA HS Assay Kit and Qubit 4.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, USA). Amplicon sequencing was conducted using the 338F (5’-ACTCCTACGGGAGGCAGCA-3′) and 806R (5’-GGACTACHVGGGTWTCTAAT-3′) universal primer set to amplify the V3-V4 region of the 16S rRNA gene from genomic DNA extracted from each sample. Both forward and reverse primers were tagged with sample-specific Illumina index sequences to facilitate deep sequencing. The PCR was carried out in a total reaction volume of 10 μL, including 5–50 ng of DNA template, 0.3 μL of each Vn F and Vn R primer (10 μM each), 5 μL of KOD FX Neo Buffer, 2 μL of dNTP mix (2 mM each), 0.2 μL of KOD FX Neo polymerase, and ddH2O up to 10 μL. PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 40 s, with a final extension step at 72 °C for 7 min. Purification of PCR amplicons was performed using Agencourt AMPure XP Beads (Beckman Coulter, Indianapolis, IN), and quantification was conducted using the Qubit dsDNA HS Assay Kit and Qubit 4.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, USA). Subsequently, amplicons were pooled in equal amounts for library construction, and Illumina NovaSeq 6000 (Illumina, Santiago CA, USA) was utilized for sequencing.

2.10. Statistical analysis

Analysis software such as SPSS (version 25; IBM, Armonk, NY, USA), TBtools (https://github.com/CJ-Chen/TBtools/releases), SIMCA-P v. 14.1 (Umetrics, Umea, Sweden) were used in this study. Taxonomy annotation relied on the Naive Bayes classifier in QIIME2 with the SILVA database (release 132), set at a confidence threshold of 70% (Bolyen et al., 2019; Quast et al., 2013). All experiments were conducted in triplicate.

3. Results and discussion

3.1. Aroma profile of Pu-erh tea samples

The aroma attributes of the samples were described by a trained evaluation team using traditional sensory review methods. A total of 12 aroma attributes were eventually screened based on the frequency of occurrence of various descriptors. Secondly, to further assess the aroma differences among samples, the results of the aroma profile description analysis are shown in Fig. 1b, and the samples (SDGT, SPPF-A, and STPF-A) showed their distinctive aroma profiles. For the SPPF sample evaluation, sweet (such as brown sugar), earthy, woody, fragrance of medicine, and stale scored the highest among the attributes. In contrast, STPF scored highest in plum fragrance, coolness, and sweet (such as honey) odor attributes, and intensity was higher than SPPF samples. For SDGT samples, refreshing, sweet, and floral and fruity aroma attributes scored highest. The analysis of variance revealed that stale (musty), plum fragrance, and sweet (like honey) were significantly different between SPPF and STPF. The results suggest that microbial action plays a significant role in producing the aroma attributes of Pu-erh tea's stale (musty) aroma. The moist-heat effect mainly contributed to the flavor characteristics of plum fragrance, and sweet (like honey) of Pu-erh tea. Molecular sensory science technology has been applied to investigate

the formation mechanism of Pu-erh tea stale flavor.

3.2. Identification and analysis of key aroma components in Pu-erh tea

3.2.1. Overall aroma profile analysis of volatile compounds

As shown in Table S1, based on the different aroma extraction methods (HS-SPME and SAFE) combined with the GC–MS detection technique. A total of 184 volatile compounds were identified by MS and RI. Each compound was incorporated into the website (dscentscompany.com/index.html). In general, the primary identified aroma components include 12 methoxy-phenolic compounds, 32 alcohols, 28 aldehydes, 36 ketones, 15 esters, 5 heterocyclic compounds, 11 hydrocarbons, 8 organic acids, 5 nitrogenous molecules, and other volatile compounds (Table S1). The content variations among different samples are shown in Table S2, expressed as mean content and range. In order to observe the differences between the samples further, we analyzed 184 metabolites by PCA and HCA (Fig. 1c and d). From Fig. 1c and d, we can see that SDGT-B and STPF-B were clustered together, whereas the samples of SPPF-A and STPF-A were clustered together. This suggests that changes in microorganisms, temperature, and humidity during PF have a strong influence on the aroma and quality of Pu-erh tea. From the HCA analysis, the STPF and SPPF samples were completely separated when the even mile distance was <480, which indicated that the microbial effect on the aroma of Pu-erh tea was very significant (Fig. 1d).

The contents of different aroma components in different samples were analyzed in Fig. S1, Fig. 2, and Table S3. As shown in Fig. S1a, esters and aldehydes were significantly higher in SDGT samples than in the fermented SPPF and STPF samples. In previous studies, it was pointed out that aldehyde compounds are the main contributors to the "green", "grassy", and "fatty" scents of Pu-erh tea (Yang et al., 2019). In the SDGT samples, hexanal (fruity), 2-furfural (sweet), (*E*)-2-heptenal (green fruit flavor), (*E*)-2-hexenal, (*E*)-2-methyl-2-butenal, (*E*)-2-decenal, (*E, E*)-2,4-heptadienal, and benzaldehyde with high content were detected. Furthermore, the esters mainly included methyl stearate, hexanoic acid methyl ester, methyl hexadecanoate, benzeneacetic acid, methyl ester (floral), butanoic acid, and 2-methyl methyl ester (Fig. 2). Heterocyclic and hydrocarbon compounds were significantly higher in the STPF-A (Fig. S1a). In Fig. S1b, the results showed that alcohols, ketones, and methoxy-phenolic compounds were significantly higher in SPPF samples than in others. Combined with the analysis in Figs. 2 and S1, we found that methoxy-phenolic compounds (1,2,4-trimethoxybenzene, 1,2,3-trimethoxybenzene, 1,2,3,4-tetramethoxybenzene, 1-methoxy-4-methylbenzene, 2,3-dimethoxyphenol, and others) were produced mainly by microbial action, and thus contributed majorly to the characterization of the stale aroma of Pu-erh tea (Wang, Li, et al., 2022; Wang, Su, et al., 2022). The primary reason for this phenomenon is that polyphenols with antioxidant activity are oxidatively degraded during the fermentation process, resulting in the generation of methoxybenzenes (Li et al., 2023; Zhang, Li, et al., 2023; Zhang, Xia, et al., 2023). However, the content of alcohols is second only to that of methoxyphenols, which mainly provide Pu-erh tea with aroma attributes such as "floral" and "sweet" (Lv et al., 2012). In this study, the main alcohol components were floral pyranoid, 4-methylphenol, 2-phenylethanol, 2-methoxybenzyl alcohol *E*-citral (geranial), linalool, alpha-terpineol, (*E*)-linalool oxide (furanoid), 1-butanol, 3-methyl-2-methylbutan-1-ol, and (*Z*)-linalool oxide (furanoid) (Fig. 2). Secondly, ketones are the main contributors to the "woodsy" (Wang, Li, et al., 2022; Wang, Su, et al., 2022). Herein, acetophenone isophorone, 2,5-pyrrolidinedione, 1-ethyl-lavender, 3-hydroxy-2-butanone, 4-methylene isophorone, dehydromevalonic lactone, 2(3 h)-furanone, dihydro-5-methyl, and dimethyl sulfone aroma components were detected (Fig. 2), which are consistent with the results of previous studies (Wang, Li, et al., 2022; Wang, Su, et al., 2022). Based on these results, it can be found that the effects of SPPF and STPF on the quality of Pu-erh tea are substantial and distinct, with microbes playing a major role as well (Xu, Wei, Li, Weng, & Wei, 2022).

3.2.2. Screening of key aroma metabolites

In order to visualize the overall change pattern of volatile components under SPPF and STPF by microbial action and hygrothermal action, k-mean trend analysis was used to screen key aroma-active compounds (Zhang et al., 2021). As shown in Fig. S2, 20 similar metabolic profiles were analyzed. The results showed that profiles 13, 14, and 16 enriched a total of 16 volatile compounds that were significantly upregulated by microbial effects under SPPF. Secondly, we found that 28 volatile compounds were enriched on profile 15, which were

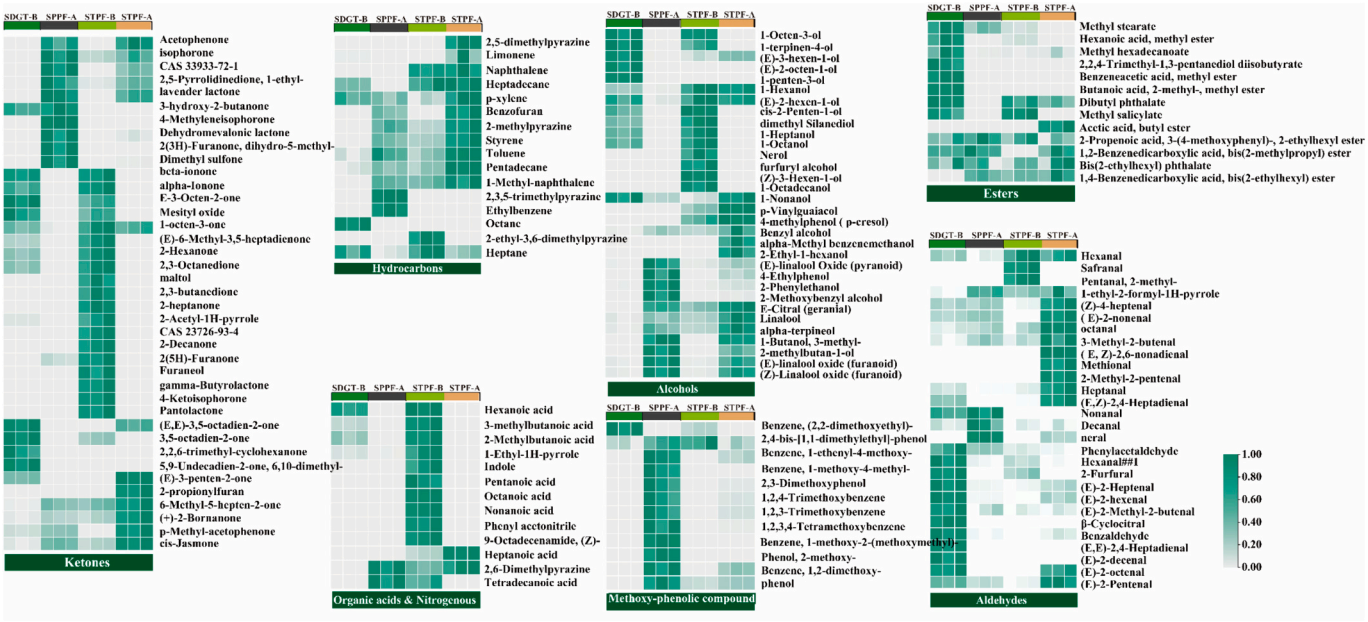


Fig. 2. Heatmap resulting from the change in aroma composition under different process conditions.
Note: sun-dried green tea before piling fermentation (SDGT-B); sterile piling fermentation before piling fermentation (STPF-B); spontaneous piling fermentation after (SPPF-A); and sterile piling fermentation after piling fermentation (STPF-A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significantly upregulated by the synergistic effects of microbes and moist-heat. Additionally, 28 volatile compounds were affected by moist-heat effects (profiles 6, 10, 17, and 19).

The Sunrise chart was used to visualize the aroma's active components. As shown in Fig. 3, the main aroma-active compounds that are only subjected to microbial action are floral and honey-like 2-phenylethanol, soapy and citrus-like decanal, earthy 2,3,5-trimethyl pyrazine, nutty 1-methoxy-4-methyl-benzene, gammon-like 2-methoxy-phenol, etc. Secondly, we found that the metabolites affected by synergism in Fig. S1 consisted mainly of alcohols, methoxybenzenes, and ketones. However, in combination with Fig. 2, we can find that the six methoxybenzenes produced by synergistic interactions (mainly the stale-flavored 1,2,3,4-tetramethoxybenzene, 1,2,4-trimethoxybenzene, 1,2,3-trimethoxybenzene, 1-ethenyl-4-methoxy-benzene, 1,2-dimethoxy-benzene, and 2,3-dimethoxyphenol) were primarily affected by microbial action, whereas moist-heat effect on them was very slight (microbial action is roughly seven times more important than hygrothermal action, as shown in Table S2). We found that some woody cis-jasmone, 4-ethylphenol, linalool, cool isophorone, as well as floral (*Z*)-linalool oxide (furanoid), alpha-terpene alcohols (flowery, citrus-like), (*E*) linalool oxide (furanoid) were synthesized in a synergistic effect. However, we can also observe from Fig. S1 that alcohols were significantly affected by microbial actions, higher than moist-heat action by about 3-fold. Among the 28 metabolites affected by the action of moist-heat, it mainly contains nine floral and fruity aromatic substances (benzyl alcohol, 2-ethyl-1-hexanol, linalool, geraniol, 2-methyl-2-pentenal, 3-methyl-2-butenal, acetic acid, styrene, methyl 2-furoate, etc.), as well as sweet-smelling alpha-methyl benzenemethanol, baking-smelling 2,5-dimethylpyrazine, medicinal-smelling 1-methyl-napphthalene, cucumber-flavored (*E,Z*)-2,6-nonadienal, nutty-smelling 2,5-dimethylpyrazine, nutty-smelling naphthalene, and other aromatic substances. The present study indicated a decrease in esters and aldehydes and an increase in ketones, alkanes, and alcohols after microbial PF. In contrast, previous studies showed a decrease in alcohols and an increase in esters (Deng et al., 2021). Further evidence will be sought in the future. Previous research has pointed out that methoxy-phenolic compounds, alcohols, ketones, and aldehydes are active substances that contribute to the quality of Pu-erh tea; however, these studies could not clearly indicate how these substances are transformed and which factors have an effect on them (Wang, Li, et al., 2022; Wang, Su, et al., 2022). This study could more clearly point out the effect of the PF environment on metabolites based on the comparative experiments of SPPF and STPF. Therefore, in the latter section, we performed simulations in order to verify the synthesis mechanism of methoxybenzenes influenced by microorganisms.

3.2.3. *Screening of key aroma-active compounds*

The contribution of aroma-active compounds to tea aroma is not only affected by their concentrations but also by ROAVs thresholds as the thresholds of aroma-active compounds in water are mostly dominant, considering the characteristics of tea broth and water (Chen et al., 2017). Combining the GC–MS/O and AEDA results of the concentrated aroma distillates, 22 of 184 compounds had ROAVs >1. Therefore, they were identified as aroma-active compounds that contributed to the aroma of tea. The ROAVs of the aromatic compounds ranged from 1 to 128 (Table 1). The aroma-active compounds contributing to the aroma of SDGT in descending order were as follows: floral β-phytolone (ROAVs = 176, FD = 32) and floral (*E*, *E*)-2,4-heptadienal (ROAVs = 167, FD = 32), fruity methyl 2-methylbutanoate (ROAVs = 28, FD < 8), fish-like (*Z*)-4-heptenal (ROAVs = 13, FD < 8), floral linalool (ROAVs = 11, FD = 32), mushroom-like 1-octen-3-one (ROAVs = 10, FD < 8), mushroom-like 1-octen-3-ol (ROAVs = 9, FD = 32, and flowery and honey-like phenylacetaldehyde (ROAVs = 2, FD = 8). Following SPPF, the aroma-active compounds in the samples were mainly stale 1,2,3-trimethoxybenzene (ROAVs = 1238, FD = 128), stale 1,2-dimethoxybenzene (ROAVs = 81, FD = 32), stale 1,2,4-trimethoxybenzene (ROAVs = 69, FD = 64), stale 1,2,3,4-tetramethoxybenzene (ROAVs = 38, FD < 8), stale pyranoid (ROAVs = 12, FD = 8), and floral and honey-like 2,3-dimethoxyphenol (ROAVs = 2, FD < 8). This was followed by floral linalool (ROAVs = 32, FD = 32), fish-like (*Z*)-4-heptenal (ROAVs = 14, FD < 8), mushroom-like 1-octen-3-one (ROAVs = 3, FD < 8), floral 2-phenylethanol (ROAVs = 6, FD = 8), and flowery phenylacetaldehyde (ROAVs = 1, FD < 8). Finally, we also found small amounts of the stale aromatics 1,2,3-trimethoxybenzene (ROAVs = 132, FD = 32), 1,2-

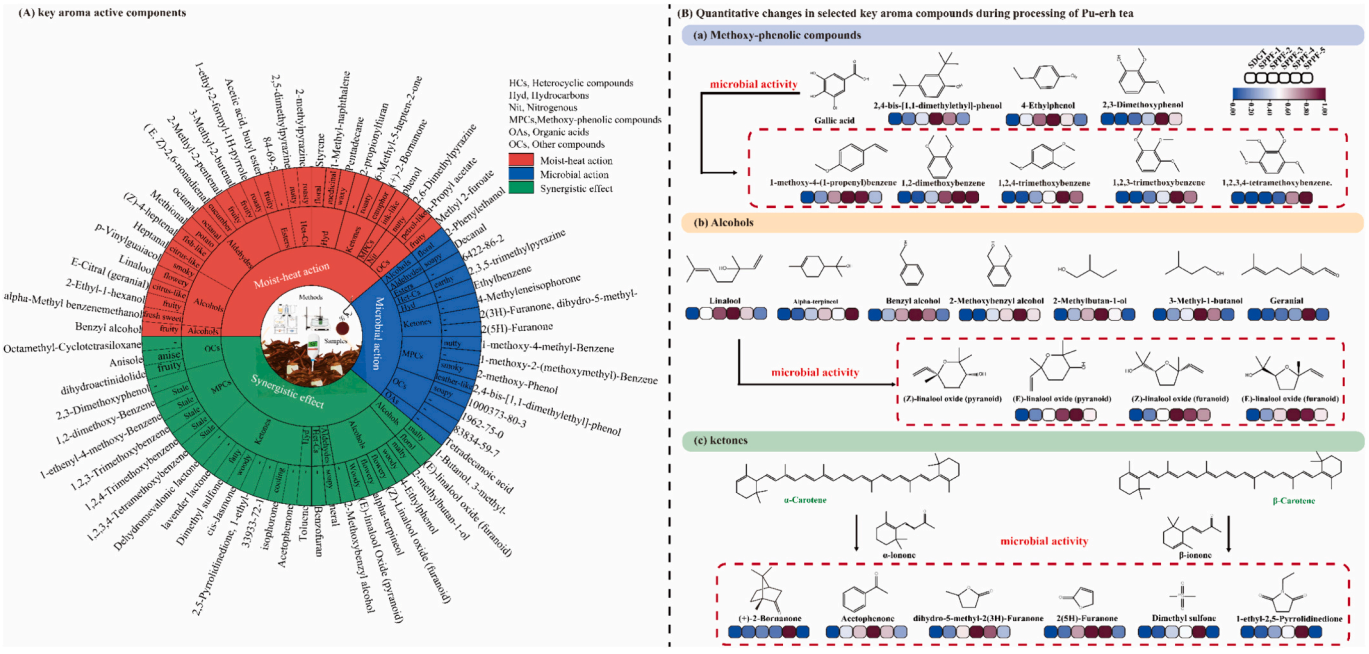


Fig. 3. (a) Sunburst chart of clustering of key aroma active components under the action of heat and moisture and microorganisms and (b) Content changes and pathway analysis of selected key aroma compounds during Pu-erh tea processing.
Note: sun-dried green tea (SDGT); spontaneous piling fermentation (SPPF). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
OAV Concentrations, OTs in Water, and relative odor activity values (ROAVs) of Odorants in Tea Infusions.

Name	Odor	Concentration (μg/Kg)				OT (μg/L)	ROAV[d] (AEDA, FD >8)			
		SDGT	SPPF	STPF-B	STPF-A		SDGT	SPPF	STPF-B	STPF-A
Linalool CAS 78–70-6	citrus-like, flowery	6.48 ± 0.99	18.63 ± 3.19	13.45 ± 0.9	40.6 ± 8.74	0.58[2,3,4]	11 (FD < 8)	32 (FD = 16)	23 (FD < 8)	70 (FD = 64)
1-Octen-3-ol CAS 3391-86-4	mushroom-like	14.24 ± 2.12	ND	13.42 ± 0.29	ND	1.5[4]	9 (FD = 32)	<1 (FD < 8)	9 (FD = 16)	<1 (FD < 8)
4-methylphenol (*p*-cresol) CAS 106–44-5	faecal, phenolic, horse stable-like	ND	ND	2.19 ± 0.63	4.13 ± 0.16	3.9[2]	<1 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)	1 (FD < 8)
2-Phenylethanol CAS 60–12-8	floral, honey-like	23.22 ± 5.27	883.54 ± 112.68	24.35 ± 6.71	91.28 ± 13.24	140[2]	<1 (FD < 8)	6 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)
(*E*)-linalool Oxide (pyranoid) CAS 39028–58-5	Stale	5.08 ± 0.35	69.25 ± 16.02	6.68 ± 1.34	22.42 ± 4.31	100[2,4]	<1 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)
(*Z*)-4-heptenal CAS 6728-31-0	fish-like, train oil-like	0.11 ± 0.01	0.12 ± 0.02	0.05 ± 0.01	0.3 ± 0.05	0.0087[1,2]	13 (FD < 8)	14 (FD < 8)	5 (FD < 8)	34 (FD < 8)
(*E, Z*)-2,6-nonadienal CAS 557–48-2	cucumber-like	ND	ND	ND	0.06 ± 0	0.0045[1, 2]	<1 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)	14 (FD = 8)
(*E, E*)-2,4-Heptadienal CAS 4313-03-5	fatty, flowery	5.35 ± 0.51	ND	ND	ND	0.032[2,3]	167 (FD = 32)	<1 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)
Phenylacetaldehyde CAS 122–78-1	flowery, honey-like	10.39 ± 3.86	5.2 ± 0.32	2.37 ± 0.32	1.5 ± 0.41	5.2[3]	2 (FD < 8)	1 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)
methyl 2-methylbutanoate CAS 868–57-5	Fruity	6.77 ± 0.34	ND	ND	ND	0.25[1, 2]	28 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)
(*E*)-*β*-damascenone CAS 23726–93-4	cooked apple-like	ND	ND	0.07 ± 0.02	ND	0.006[2,4]	<1 (FD < 8)	<1 (FD < 8)	12 (FD < 8)	<1 (FD < 8)
1-octen-3-one CAS 4312-99-6	mushroom-like	0.16 ± 0.02	0.05 ± 0	0.29 ± 0.07	0.2 ± 0.03	0.016[1, 2]	10 (FD < 8)	3 (FD < 8)	18 (FD < 8)	13 (FD < 8)
β-ionone CAS 79–77-6	flowery, violet-like	3.69 ± 0.34	ND	4.13 ± 0.7	ND	0.021[2,4]	176 (FD = 32)	<1 (FD < 8)	197 (FD = 32)	<1 (FD < 8)
1,2,3,4-Tetramethoxybenzene CAS 21450–56-6	Stale, musty	ND	24.34 ± 4.98	ND	0.51 ± 0.04	0.64[2,4]	<1 (FD < 8)	38 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)
1,2,4-Trimethoxybenzene CAS 135–77-3	Stale, musty	ND	211.54 ± 57.83	ND	17.42 ± 2.4	3.06[2,4]	<1 (FD < 8)	69 (FD = 64)	<1 (FD < 8)	6 (FD = 16)
1,2,3-Trimethoxybenzene CAS 634–36-6	Stale, musty	ND	928.48 ± 241.7	ND	99.06 ± 13.5	0.75[2,4]	<1 (FD < 8)	1238 (FD = 128)	<1 (FD < 8)	132 (FD = 64)
1,2-dimethoxybenzene CAS 91–16-7	Stale, musty	ND	299.39 ± 52.77	ND	95.07 ± 16.87	3.71[2,4]	<1 (FD < 8)	81 (FD = 32)	<1 (FD < 8)	26 (FD = 16)
2,3-Dimethoxyphenol CAS 5150-42-5	Stale, musty	ND	53.17 ± 13.81	ND	2.2 ± 0.25	29[2,4]	<1 (FD < 8)	2 (FD < 8)	<1 (FD < 8)	<1 (FD < 8)

Note: "SDGT-B", sun-dried green tea before piling fermentation; "STPF-B", sterile piling fermentation before piling fermentation; "SPPF-A", spontaneous piling fermentation after; and "STPF-A", sterile piling fermentation after piling fermentation; "ND", not detected. Odor thresholds (OT) in the water were referenced as Odor Thresholds: Compilations of Odor Threshold Values in air, water and other media (Edition 2011) [1], OTs in water from Zhai et al., 2022[2]; OTs in water from Leibniz-LSB@TUM Odorant Database [3], OTs in water from Pang et al., 2019[4]; OTs in water from Zhu et al. 2017[5].

dimethoxybenzene (ROAVs = 26, FD < 8), and 1,2,4-trimethoxybenzene (ROAVs = 6, FD = 8) in the samples of STPF under the action of moist-heat. Additionally, flowery linalool (ROAVs = 70, FD = 64), fish-like (*Z*)-4-heptenal (ROAVs = 34, FD < 8), and mushroom-like (ROAVs = 13, FD < 8) contributed two to four times more to the aroma of the teat than SPPF. Cucumber-like (*E, Z*)-2,6-nonadienal (ROAVs = 14, FD < 8) and phenolic 4-methylphenol (*p*-cresol; ROAVs = 1, FD = 8) contributed significantly to the aroma of the STPF samples only. The changing pattern of the aroma activity values of different key aromatic substances is consistent with the results of our analysis. To further verify the accuracy of our results, we quantified 22 target aroma-active compounds. Based on the difficulty of purchasing aroma specimens and analysis of the test results, nine odorants were finally quantified with an R^2 of >0.9900 (Table S4), thus confirming the reliability of our conclusion.

3.2.4. Aroma addition and aroma omission analysis

To further validate the contribution of the five key active compounds to the aroma of the stale aroma, aroma addition experiment and aroma omission experiment was conducted (Table S5). Sensory descriptive analyses combined with GC-O and ROAVs analyses showed that the aroma attributes of the stale aroma in Pu-erh ripe tea mainly originated

from microbial PF, and five key active substances were mainly screened (linalool, benzeneacetaldehyde, 1,2,3-trimethoxybenzene, 1,2,4-trimethoxybenzene, 1,2-dimethoxy-benzene). Aroma addition experiments showed that 1,2,3-trimethoxybenzene and 1,2,4-trimethoxybenzene were the main contributors to the aroma attributes of stale aroma, and 1,2-dimethoxy-benzene contributed insignificantly to the aged aroma of Pu-erh ripe tea (Table S5). Linalool was the main contributors to the aroma attributes of flavor and sweet aroma, and benzeneacetaldehyde contributed insignificantly to the aged aroma of Pu-erh ripe tea (Table S5).

3.3. Quantitative changes in selected key aroma compounds during processing of Pu-erh tea

The manufacturing process is essential in influencing the aroma quality of tea (Flaig, Qi, Wei, Yang, & Schieberle, 2020). During the PF process, the increase in temperature provides a beneficial environment for the growth of microorganisms, which promotes the production of some enzymes and flavor components (Li et al., 2023). Based on the results of the above analysis, we screened a total of 38 metabolites upregulated by microbial and moist-heat effects for quantification (Table 2 and Fig. S3). These substances will be used as important aroma indicators in the PF process of Pu-erh tea.

The quantitative results of the PF process of Pu-erh tea are shown in Table 2 and Fig. S3, and all the compounds were detected in the tea samples. The key odorant active ingredients mainly included methoxy-phenolic compounds, ketones, alcohols, phenols, esters, and others (Figs. S3 and 3b), which are in agreement with the review by Wang et al. Methoxy-phenolic compounds, primarily produced during the PF of Pu-erh tea, are the main contributor to its unique "stale" flavor (Lv, Zhong, & Lin, 2009;Wang, Li, et al., 2022 ; Wang, Su, et al., 2022). Methoxy-phenolic and similar structure compounds could be generated from gallic acid (GA) by the methylation of microorganism activities in the moist-heat environment (Lv et al., 2012). Previous research has indicated that during the methylation reaction, hydrogen atoms of the hydroxyl groups in GA can be substituted with methyl groups, leading to the formation of methoxy-phenolic compounds (Wang, Li, et al., 2022; Wang, Su, et al., 2022). However, it's still uncertain whether microorganisms or environmental factors such as moisture and heat have a greater impact on these compound formations. In the present study, we further identified microorganisms as the key cause of the production of methoxybenzenes. Alcohol-based compounds, the second most abundant after methoxy-phenolic compounds, are responsible for creating "floral," "sweet," or "woody" scents in Pu-erh tea (Lv et al., 2012; Wang, Li, et al., 2022; Wang, Su, et al., 2022). Linalool and geraniol are released from geranyl pyrophosphate precursors through the actions of geraniol synthase and linalool synthase, respectively (Wang, Li, et al., 2022; Wang, Su, et al., 2022) (Fig. 3b). During the PF process of Pu-erh tea, the levels of these compounds gradually decreasing (Chen et al., 2017). In the present study, these substances showed an increase followed by a decrease. These compounds then transform into their respective oxides (linalool oxides [I, II, III, and IV]) (Fig. 3b). Linalool oxides (I, II, III and IV) generate from the glycoside forms of linalool oxides. Linalool oxide I and II are cleaved from glycation bonds with the sugar moieties of *β-D-glucoside* and *β-primeveroside*, while linalool oxides III and IV are cleaved from glycation bonds with the sugar moieties of *β-acuminoside* (Wang, Li, et al., 2022; Wang, Su, et al., 2022). The concentration of these compounds increased during the fermentation of Pu-erh tea (Wang, Li, et al., 2022; Wang, Su, et al., 2022). This result is in general consistent with our results. In addition, the concentrations of α-pinitol and nerolidol increased during pile fermentation of Pu-erh tea (Wang et al., 2022). It was shown that the key enzymes for the formation of major terpene alcohol aroma components were primeverosidases and glucosidase, and their relative abundance both increased significantly during Pu-erh tea fermentation. Ketone compounds are the main contributors to the "woody" aroma in Pu-erh tea (Fig. 3b) (Ho, Zheng, & Li, 2015; Wang, Li, et al., 2022; Wang, Su, et al., 2022). And the primary precursor of α-ionone is α-carotene, while the main precursor of β-ionone is β-carotene. α-ionone and β-ionone can be produced through enzymatic or non-enzymatic degradation processes. Dihydro-β-ionone is primarily formed when glycosides and β-ionone undergo biotransformation during microbial PF (Ho et al., 2015). The precursor of geranyl acetone is phytofluene, mainly formed through oxidation. However, during the PF of Pu-erh tea, the levels of α-ionone, β-ionone, and geranyl acetone showed a decreasing trend (Chen et al., 2017). The study was relatively consistent with the results of these studies and further pointed out the contribution of microbial action and moist-heat action to the formation of volatile components.

In this study, as shown in Table 2 and Fig. S4, the magnitude of changes in key active ingredients and differences between samples were analyzed. Some of the stale aromatics (1,2,3,4-tetramethoxybenzene, 1,2,3-trimethoxybenzene, 1,2,4-trimethoxybenzene, 1,2-dimethoxybenzene, 1-ethenyl-4-methoxybenzene, 1-methoxy-2-(methoxymethyl)-benzene, 1-methoxy-4-methyl-benzene) started to be synthesized in the initial stage of PF (PF1–3), reached the maximum value in the fourth stage of PF (PF4), and decreased in the excessive stage of PF (PF5). Additionally, a number of floral and fruity aroma-like substances were abundantly produced, including mainly citrus-like geranial, fruity, and woody dihydroactinidolide, bitter almond-like and fruity benzyl alcohol, floral and honey-like 2-phenylethanol, floral and woody (*Z*)-linalool oxide (furanoid), flowery alpha-terpineol, and flowery linalool-like 2-phenylethanol, floral and woody (*Z*)-linalool oxide (furanoid), flowery α-terpineol, and flowery linalool. Secondly, from the Fig. S4, we can visualize the trend of the key ingredients more clearly, and many active substances are either undetectable or present in very low levels in the raw material. Aromatics increased gradually with increasing PF time, and most of the aromatic substances reaching their highest values at the fourth stage of PF (i.e., the optimal level of PF) and then declining (the over-PF stage). Therefore, the increase of these substances plays a major role in the flavor characteristics of Pu-erh tea's stale.

3.4. Simulation of the formation mechanism of methoxybenzenes

Previous studies have demonstrated that methoxybenzenes are the main stale substances in Pu-erh ripened tea; it is hypothesized that the synthesis of these substances may occur under microbial action (Wang, Li, et al., 2022; Wang, Su, et al., 2022). The review hypothesized that catechins such as epigallocatechin gallate, epigallocatechin undergo oxidation by tannase and esterase enzymes to form GA, which in the action of microorganisms produces methoxybenzenes (1,2,4-trimethoxybenzene, 1,2,3-trimethoxybenzene, and 1,2-dimethoxybenzene). Preliminary results suggest that microbial action is the main source of synthesizing stale aroma-like substances in Pu-erh tea (Tian et al., 2024; Wang, Li, et al., 2022; Wang, Su, et al., 2022). In our current experiment, methoxybenzenes were quantitatively analyzed in SPPF and STPF samples, and the methoxybenzenes produced in the SPPF samples were approximately seven times more than those made under the action of moist-heat (Fig. S1). In order to further investigate the contribution of GA to the aroma of Pu-erh tea, we designed a related simulation experiment (in material and method 2.9). The results showed that under SPPF, the total amount of methoxybenzenes increased by 25 μg/L relative to that of the unadded samples through the addition of GA. We can see that the content of 1-ethyl-4-methoxybenzene, 1,2,4-trimethoxybenzene, 1,2,3-trimethoxybenzene, and 1,2-dimethoxybenzene increased significantly (Fig. 4b and c). Under sterile action, there was no differential change in methoxybenzene content by the addition of GA. Based on the above analysis, we proved for the first time that methoxybenzenes can be synthesized in large quantities in the effect of microbes. Secondly, in order to further verify the reliability of the conclusions; we examined and analyzed the main fungi in the second simulation samples at different stages of fermentation. As shown in the Fig. S5, we can see that the microbial community is very fast during the

Table 2
Changes in odorant concentrations (relative quantification based on internal standards) during the manufacturing process of Pu-erh tea.

Aroma compound (μg/Kg, Mean ± SD)	SDGT-B-PF	SPPF-1	SPPF-2	SPPF-3	**SPPF-4**	SPPF-5	Aroma
Geranial	0.05 ± 0.01 b	0.07 ± 0.04 b	0.11 ± 0.02 b	0.17 ± 0.07 b	**0.57 ± 0.13 a**	0.09 ± 0.00 b	Citrus-like
2-Methoxybenzyl alcohol	0.00 ± 0.00 a	0.00 ± 0.00 a	18.49 ± 1.34 a	**25.02 ± 0.95 a**	**43.77 ± 8.65 a**	147.4 ± 213.21 a	–
2-Methylbutan-1-ol	0.00 ± 0.00 f	11.10 ± 1.34 d	23.07 ± 1.68 b	**35.66 ± 2.6 a**	18.42 ± 2.34 c	1.37 ± 0.5 e	Malty
3-Methyl-1-butanol	0.00 ± 0.00 c	0.98 ± 0.67 bc	1.89 ± 0.32 b	**4.24 ± 1.38 a**	**3.16 ± 0.49 a**	0.33 ± 0.11 c	Malty
Benzyl alcohol	12.24 ± 1.08 e	104.3 ± 11.47 c	216.43 ± 19.03 b	**313.13 ± 37.01 a**	241.22 ± 31.4 b	55.09 ± 20.10 d	Fruity
(*Z*)-Linalool oxide (furanoid)	3.35 ± 0.10 d	13.69 ± 1.01 d	35.26 ± 4.42 c	**68.28 ± 7.08 a**	**60.51 ± 4.91 ab**	48.56 ± 15.9 bc	Sweet, woody
Linalool	5.17 ± 0.63 e	36.64 ± 6.08 c	59.35 ± 6.35 b	**69.23 ± 1.52 a**	42.87 ± 3.97 c	15.46 ± 5.02 d	Flowery
(*E*)-Linalool oxide (pyranoid)	5.59 ± 1.13 c	23.89 ± 2.21 c	56.93 ± 6.85 b	**98.45 ± 11.41 a**	**110.68 ± 17.08 a**	70.78 ± 17.27 b	–
(*E*)-Linalool oxide (furanoid)	5.84 ± 0.81 d	30.36 ± 3.58 c	63.54 ± 8.61 b	**107.41 ± 10.38 a**	**101.14 ± 8.64 a**	60.62 ± 18.02 b	Floral
Alpha-terpineol	3.50 ± 0.06 d	6.12 ± 0.90 d	19.82 ± 3.06 c	31.57 ± 2.77 b	25.61 ± 2.96 bc	49.53 ± 14.21 a	Flowery
(+)-2-Bornanone	0.17 ± 0.02 b	0.44 ± 0.3 b	0.74 ± 0.12 b	0.61 ± 0.24 b	**3.84 ± 3.38 a**	0.14 ± 0.00 b	Camphor-like
4-Methyleneisophorone	0.00 ± 0.00 d	0.03 ± 0.02 cd	0.07 ± 0.01 b	**0.12 ± 0.04 a**	**0.13 ± 0.03 a**	0.06 ± 0.00 b	–
Acetophenone	0.00 ± 0.00 e	4.92 ± 0.70 c	6.9 ± 1.21 b	**11.33 ± 0.27 a**	6.95 ± 0.43 b	2.9 ± 1.33 d	Bitter almond
1-ethyl-2,5-Pyrrolidinedione	0.00 ± 0.00 d	20.54 ± 4.16 d	99.2 ± 10.21 c	187.64 ± 26.43 b	**392.67 ± 57.89 a**	0.00 ± 0.00 d	–
dihydro-5-methyl-2(3H)-Furanone	0.00 ± 0.00 f	1.80 ± 0.51 e	5.68 ± 0.48 c	**10.43 ± 0.91 a**	8.61 ± 0.99 b	3.33 ± 1.11 d	–
Dimethyl sulfone	0.00 ± 0.00 c	10.65 ± 3.36 c	89.16 ± 5.52 b	114.54 ± 3.19 b	**234.91 ± 34.27 a**	0.00 ± 0.00 c	Fatty, sulfuric
2(5H)-Furanone	4.50 ± 1.60 c	9.76 ± 3.24 c	35.56 ± 5.56 b	**49.20 ± 2.67 a**	**49.83 ± 5.93 a**	11.64 ± 4.74 c	–
Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate	0.23 ± 0.03 c	1.93 ± 1.29 bc	3.57 ± 0.52 b	**7.88 ± 2.88 a**	**7.76 ± 1.62 a**	2.22 ± 0.13 bc	–
2-Ethylhexyl trans-4-methoxycinnamate	2.76 ± 0.89 a	5.53 ± 3.89 a	3.14 ± 1.56 a	**3.15 ± 0.21 a**	**5.37 ± 1.79 a**	7.53 ± 6.23 a	–
1,4-Dibutyl benzene-1,4-dicarboxylate	71.17 ± 12.98 ab	20.99 ± 9.78 b	130.27 ± 42.61 a	**83.58 ± 15.74 ab**	**98.71 ± 36.34 ab**	122.08 ± 97.93 a	–
1,2,3,4-Tetramethoxybenzene	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	1.41 ± 0.49 b	**13.91 ± 3.57 a**	19.48 ± 7.32 a	Stale, musty
1,2,3-Trimethoxybenzene	0.00 ± 0.00 d	8.52 ± 3.81 d	164.85 ± 17.77 d	462.62 ± 45.57 c	**1062.44 ± 154.59 a**	713.81 ± 179.38 b	Stale, musty
1,2,4-Trimethoxybenzene	0.00 ± 0.00 e	4.46 ± 1.22 e	68.75 ± 7.18 d	141.68 ± 19.89 c	**274.02 ± 49.31 a**	214.3 ± 56.82 b	Stale, musty
1,2-Dimethoxy-Benzene	0.00 ± 0.00 d	6.31 ± 1.28 d	70.66 ± 10.61 c	171.53 ± 19.81 b	**201.1 ± 21.65 ab**	240.04 ± 79.37 a	Stale, musty
1-Ethenyl-4-methoxy-Benzene	0.00 ± 0.00 c	0.37 ± 0.26 bc	1.23 ± 0.96 ab	**1.74 ± 0.68 a**	**1.68 ± 0.43 a**	0.56 ± 0.03 bc	Stale, musty
1-Methoxy-2-(methoxymethyl)-Benzene,	0.00 ± 0.00 c	0.00 ± 0.00 c	0.10 ± 0.01 b	**0.22 ± 0.08 a**	**0.2 ± 0.04 a**	0.07 ± 0.01 bc	Stale, musty
1-Methoxy-4-methyl-Benzene	0.00 ± 0.00 d	0.10 ± 0.07 d	0.46 ± 0.08 bc	**0.71 ± 0.29 ab**	**0.85 ± 0.23 a**	0.22 ± 0.03 cd	Stale, musty
Ethylbenzene	43.54 ± 6.08 b	35.65 ± 22.68 b	46.77 ± 5.69 b	73.16 ± 28.21 b	**128.93 ± 27.52 a**	43.98 ± 7.57 b	–
2,4-bis-[1,1-dimethylethyl]-phenol	7.31 ± 1.02 d	9.24 ± 2.91 cd	12.64 ± 1.39 bc	**19.8 ± 2.83 a**	**16.56 ± 2.22 ab**	9.99 ± 4.11 cd	Leather-like
2-Phenylethanol	22.27 ± 2.63 d	337.51 ± 65.11 c	683.27 ± 86.91 b	**1173.93 ± 160.41 a**	**1119.69 ± 175.12 a**	810.76 ± 220.97 b	Floral, honey
4-Ethylphenol	0.00 ± 0.00 d	20.68 ± 6.03 c	36.33 ± 7.41 b	**46.07 ± 3.63 a**	25.16 ± 2.97 c	6.99 ± 2.76 d	Phenolic
2,3-Dimethoxyphenol	0.00 ± 0.00 d	0.63 ± 0.18 d	9.46 ± 0.67 d	34.4 ± 5.46 c	**97.20 ± 16.64 a**	55.45 ± 13 b	–
2-Propenoic acid, 3-(4-methoxyphenyl)- 2-ethyl-hexyl ester	0.00 ± 0.00 c	22.67 ± 15.5 cb	35.76 ± 11.87 b	22.31 ± 1.83 cb	**86.51 ± 30.38 a**	44.45 ± 17.72 b	–
Anisole	0.00 ± 0.00 c	0.11 ± 0.07 c	0.49 ± 0.08 b	0.67 ± 0.27 b	**1.17 ± 0.29 a**	0.00 ± 0.00 c	Gasoline
Octamethyl-cyclotetrasiloxane	0.00 ± 0.00 c	40.04 ± 15.83 b	59.8 ± 15.04 b	**101.62 ± 34.92 a**	**105.93 ± 9.47 a**	0.00 ± 0.00 c	–
Dihydroactinidolide	0.00 ± 0.00 e	1.05 ± 0.25 d	1.91 ± 0.16 c	2.68 ± 0.30 b	**3.88 ± 0.73 a**	0.00 ± 0.00 e	Fruit, woody
1-Ethyl-2-formyl-1H-pyrrole	4.86 ± 0.2 c	1.99 ± 0.19 c	7.78 ± 1.02 c	23.78 ± 0.43 b	**44.94 ± 2.40 a**	22.47 ± 8.95 b	Burnt roasted

Note: “SDGT-B", sun-dried green tea before piling fermentation; “SPPF”, spontaneous piling fermentation after; SPPF-processed samples [SPPF-1 (7 days), SPPF-2 (14 days), SPPF-3 (22 days), SPPF-4 (32 days), SPPF-5 (38 days)]. ‘a, b, c, d, e, f’ values in the same row are labelled with different superscript letters (a-f) differ significantly ($p < 0.05$); and statistical analysis was One-Way ANOVA with Post Hoc Multiple comparisons by the method of Duncan.

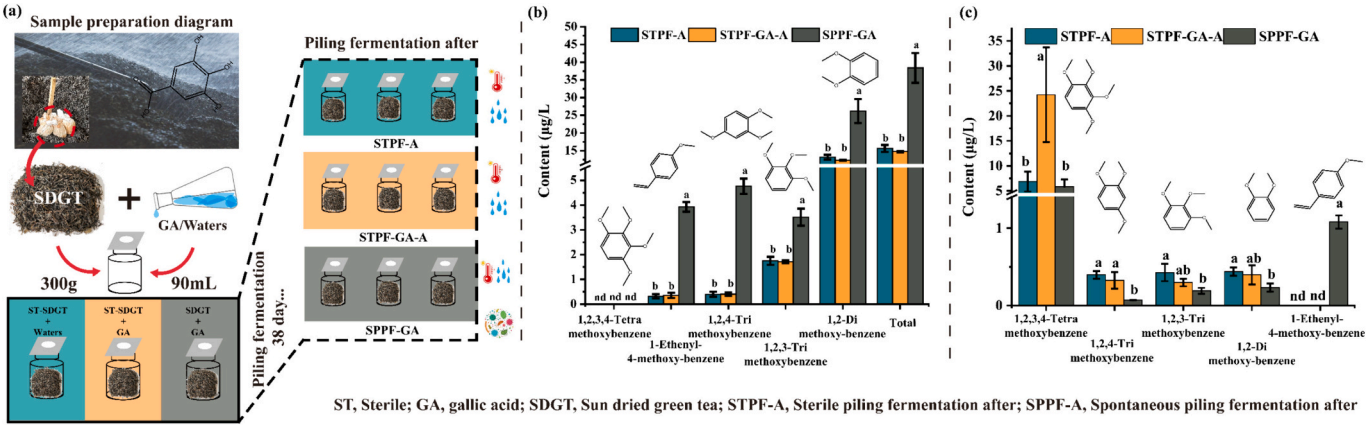


Fig. 4. Schematic diagram of simulated experiments of gallic acid degradation (a) and histogram of changes in synthesis of methoxybenzenes (b and c).

process; in the raw material, mainly *Debaryomyces*, *Aspergillus* and *Blastobotrys* dominate. In the first stage of fermentation, *Aspergillus* was as long as the dominant bacterium, followed by *Blastobotrys*. In the second and third stages of fermentation, *Rasamsonia* dominates, followed by *Aspergillus* and *Blastobotrys*. In the last stage of fermentation, we can find *Blastobotrys* as the main fungal community. We speculate that the variation of different microbial communities has a significant influence on the aroma of Pu-erh tea. Combined Pearson correlation analysis showed that *Blastobotrys, Candida, Rasamsonia*, and *Thermomyces* were positively correlated with the key aroma components 1-ethyl-4-methoxybenzene, 1,2,4-trimethoxybenzene, 1,2,3-trimethoxybenzene, and 1,2-dimethoxybenzene. In contrast, *Aspergillus* showed an overall negative correlation with stale aroma substances. Previous studies have shown that *Aspergillus* is capable of generating methoxybenzene aroma substances (Ye et al., 2016) and is the main aroma-producing microorganism in the early stage of PF (Xu et al., 2022). Li et al. pointed out that *Rasamsonia* is the dominant laccase-producing flora and is the core microorganism in the late stage of Pu-erh tea PF (Li et al., 2018; Zhu, Li, Zhou, Ouyang, & Wu, 2019). And the abundance of *aspergillus* decreased substantially in the late PF stage, while the relative abundance of *Rasamsonia emersonii* and *Thermomyces lanuginosus* increased rapidly (Xu et al., 2022). The fungal community structure changed significantly in the late PF stage, with *Blastobotrys adeninivorans* being the dominant fungal population in the ripe tea samples of Pu-erh tea, followed by *Rasamsonia emersonii*, *Thermomyces lanuginosus* and *Aspergillus penicillioides* (Li et al., 2022). *Blastobotrys* is the dominant bacterial group in the PF process and is capable of producing a variety of extracellular enzymes: proteases, convertases, etc., which also play a crucial role in the transformation of the quality of Pu-erh tea (Abe et al., 2008). Additionally, *Rasamsonia* and *Debaryomyces* can produce both catalase and laccase, which are the core functional microorganisms for aroma production in the late stage of Pu-erh ripe tea PF (Zhu et al., 2019). It would be interesting to explore the contribution of a single fungus to the aroma of Pu-erh tea in future studies.

4. Conclusion

This study will, for the first time, elucidate the respective contributions of microbial activity and moist heat to aroma during the fermentation process of Pu-erh tea. This study systematically assessed the aroma profile and aroma formation mechanism of SPPF and STPF samples processed from the same raw materials. Based on the quantitative descriptive analysis results showed that microbial action plays a significant role in producing the aroma attributes of Pu-erh tea's stale (musty) aroma. The moist-heat effect mainly contributed to the flavor characteristics of plum fragrance, and sweet (like honey) of Pu-erh tea. Based on k-mean trend analysis, it was found that 16 markers were upregulated by microorganisms, 28 markers were upregulated by moisture and heat action, and 28 markers were upregulated owing to synergistic effects. According to ROAVs and AEDA analyses revealed that the top five primary aroma-active contributors of SDGT were *β*-phytolone, (*E,E*)-2,4-heptadienal, methyl-2-methylbutanoate, and (*Z*)-4-heptenal. The top five main aroma contributors of SPPF samples were 1,2,3-trimethoxybenzene, 1,2-dimethoxybenzene, 1,2,4-trimethoxybenzene, 1,2,3,4-tetramethoxybenzene, and linalool The top five main aroma contributors in the STPF samples were 1,2,3-trimethoxybenzene, linalool, (*Z*)-4-heptenal, 1,2-dimethoxybenzene, and 1-octen-3-one. And the GA addition experiment simulated the synthesis experiment of methoxybenzenes and successfully verified the research conclusion that microorganisms are primarily responsible for promoting aromatic compound synthesis during PF. Finally, *Blastobotrys, Candida, Rasamsonia*, and *Thermomyces* were positively correlated with the key aroma components (1-ethyl-4-methoxybenzene, 1,2,4-trimethoxybenzene, 1,2,3-trimethoxybenzene, and 1,2-dimethoxybenzene). This study provides a comprehensive analysis of the contributions of key influencing factors, namely microbial activity and moist heat, to the aroma of Pu-erh tea during the fermentation process. This analysis offers a theoretical basis for the formation of Pu-erh tea's quality.

Authors' contributions

Tiehan Li participated in the conception and design of the study, acquisition of data, analysis, interpretation of data drafting the article and revising. Yuming Wei and Mingxia Lu participated in acquisition of data and data analysis. Yida Wu, Yanqun Jiang, and Han Ke participated in related experiments and data acquisition. Aiju Shao provided the samples. Jingming Ning participated in the analysis, interpretation of data drafting the article and revising. All authors read and approved the manuscript.

Compliance with ethical standards

The authors declare that they have complied with ethical standards.

CRediT authorship contribution statement

Tiehan Li: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yuming Wei:** Methodology, Investigation, Formal analysis. **Mingxia Lu:** Formal analysis, Data curation. **Yida Wu:** Software, Methodology. **Yanqun Jiang:** Data curation. **Han Ke:** Methodology, Data curation. **Aiju Shao:** Resources. **Jingming Ning:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge the financial support of the National Key Research and Development Program of China (2021YFD1601102) and supported by the earmarked fund for Agriculture Research System of China (CARS-19).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101553.

References

Abe, M., Takaoka, N., Idemoto, Y., Takagi, C., Imai, T., & Nakasaki, K. (2008). Characteristic fungi observed in the fermentation process for Puer tea. *International Journal of Food Microbiology, 124*(2), 199–203. https://doi.org/10.1016/j.ijfoodmicro.2008.03.008

Akacha, N. B., & Gargouri, M. (2015). Microbial and enzymatic technologies used for the production of natural aroma compounds: Synthesis, recovery modeling, and bioprocesses. *Food and Bioproducts Processing, 94*, 675–706. https://doi.org/10.1016/j.fbp.2014.09.011

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*, 852–857. https://doi.org/10.1038/s41587-019-0190-3

Cao, L. T., Guo, X. M., Liu, G. J., Song, Y. L., Ho, C. T., Hou, R. Y., … Wan, X. C. (2018). A comparative analysis for the volatile compounds of various Chinese dark teas using combinatory metabolomics and fungal solid-state fermentation. *Journal of Food and Drug Analysis, 26*(1), 112–123. https://doi.org/10.1016/j.jfda.2016.11.020

Chen, B., Man, H. P., Jiang, D. H., Luo, Z. G., Gao, X. L., & Li, Z. H. (2017). Changes of aroma components during Pu'er tea fermentation process by headspace–solid–phase microextraction and gas chromatography–mass spectrometry. *Journal of Food Safety and Quality, 8*, 2191–2198. https://doi.org/10.3969/j.issn.2095-0381.2017.06.040

Deng, X. J., Huang, G. H., Tu, Q., Zhou, H. J., Li, Y. L., Shi, H. Y., … Xu, W. T. (2021). Evolution analysis of flavor-active compounds during artificial fermentation of Pu-erh tea. *Food Chemistry, 357*(2), Article 129783. https://doi.org/10.1016/j.foodchem.2021.129783

Fang, S. M., Ning, J. M., Huang, W. J., Zhang, G., Deng, W. W., & Zhang, Z. Z. (2019). Identification of geographical origin of keemun black tea based on its volatile composition coupled with multivariate statistical analyses. *Journal of the Science of Food and Agriculture, 99*(9), 4344–4352. https://doi.org/10.1002/jsfa.9668

Feng, Z. H., Li, Y. F., Li, M., Wang, Y. J., Zhang, L., Wan, X. C., & Yang, X. G. (2019). Tea aroma formation from six model manufacturing processes. *Food Chemistry, 285*, 347–354. https://doi.org/10.1016/j.foodchem.2019.01.174

Flaig, M., Qi, S., Wei, G., Yang, X. G., & Schieberle, P. (2020). Characterization of the key odorants in a high-grade Chinese green tea beverage (*Camellia sinensis*; Jingshan cha) by means of the Sensomics approach and elucidation of odorant changes in tea leaves caused by the tea manufacturing process. *Journal of Agricultural and Food Chemistry, 68*(18), 5168–5179. https://doi.org/10.1021/acs.jafc.0c01300

GB/T. (2017). *GB/T 14487–2017: Tea vocabulary for sensory evaluation*.

Ho, C. T., Zheng, X., & Li, S. (2015). Tea aroma formation. *Food Science and Human Wellness, 4*(9–27), 2213–4530. https://doi.org/10.1016/j.fshw.2015.04.001

Huang, W. J., Fang, S. M., Wang, J., Zhuo, C., Luo, Y. H., Yu, Y. L., … Ning, J. M. (2022). Sensomics analysis of the effect of the withering method on the aroma components of Keemun black tea. *Food Chemistry, 395*, Article 133549. https://doi.org/10.1016/j.foodchem.2022.133549

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

Li, T. H., Wei, Y. M., Feng, W. Z., Lu, M. X., Ke, H., Li, Y. F., … Ning, J. M. (2023). Exploring the mysterious effect of piling fermentation on Pu-erh tea quality formation: Microbial action and moist-heat action. *LWT- Food Science and Technology, 185*, Article 115132. https://doi.org/10.1016/j.lwt.2023.115132

Li, T. H., Zhang, Y. Y., Jia, H. Y., Zhang, J. X., Wei, Y. M., Deng, W. W., & Ning, J. M. (2022). Effects of microbial action and moist-heat action on the nonvolatile components of Pu-Erh tea, as revealed by metabolomics. *Journal of Agricultural and Food Chemistry, 70*(49), 15602–15613. https://doi.org/10.1021/acs.jafc.2c05925

Li, Z., Feng, C., Luo, X., Yao, H., Zhang, D., & Zhang, T. (2018). Revealing the influence of microbiota on the quality of Pu-erh tea during fermentation process by shotgun metagenomic and metabolomic analysis. *Food Microbiology, 76*, 405–415. https://doi.org/10.1016/j.fm.2018.07.001

Liao, X. L., Yan, J. N., Wang, B., Meng, Q., Zhang, L. Y., & Tong, H. R. (2020). Identification of key odorants responsible for cooked corn-like aroma of green teas made by tea cultivar 'Zhonghuang 1'. *Food Research International, 136*, Article 109355. https://doi.org/10.1016/j.foodres.2020.109355

Lv, H. P., Zhang, Y., Shi, J., & Lin, Z. (2016). Phytochemical profiles and antioxidant activities of Chinese dark teas obtained by different processing technologies. *Food Research International, 100*(pt.3), 486–493. https://doi.org/10.1016/j.foodres.2016.10.024

Lv, H. P., Zhong, Q. S., & Lin, Z. (2009). Study on the aroma components in Pu-erh tea with stale flavor. *Journal of Tea Science, 29*, 219–224. https://doi.org/10.3969/j.issn.1000-369X.2009.03.007

Lv, H. P., Zhong, Q. S., Lin, Z., Wang, L., Tan, J. F., & Guo, L. (2012). Aroma characterisation of pu-erh tea using headspace-solid phase microextraction combined with gc/ms and gc–olfactometry. *Food Chemistry, 130*(4), 1074–1081. https://doi.org/10.1016/j.foodchem.2011.07.135

Ma, L. J., Peng, Y. X., Du, L. P., Zhang, L. Q., Tong, W. Z., Weng, Y. R., … Huang, J. H. (2022). Effect of main taste compounds on the release of methoxyphenolic compounds in Pu-erh tea. *LWT- Food Science and Technology, 160*, Article 113293. https://doi.org/10.1016/j.lwt.2022.113293

Mo, H. Z., Zhu, Y., & Chen, Z. M. (2008). Microbial fermented tea – A potential source of natural food preservatives. *Trends in Food Science and Technology, 19*(3), 124–130. https://doi.org/10.1016/j.tifs.2007.10.001

Pang, X. L., Yu, W. S., Cao, C. D., Yuan, X. X., Qiu, J., Kong, F. Y., & Wu, J. H. (2019). Comparison of potent odorants in raw and ripened Pu-Erh tea infusions based on odor activity value calculation and multivariate analysis: Understanding the role of pile fermentation. *Journal of Agricultural and Food Chemistry, 67*(47), 13139–13149. https://doi.org/10.1021/acs.jafc.9b05321

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research, 41*, D590–596. https://doi.org/10.1093/nar/gks1219

Rong, Y. T., Xie, J. L., Yuan, H. B., Wang, L. L., Liu, F. Q., Deng, Y. L., … Yang, Y. Q. (2023). Characterization of volatile metabolites in Pu-erh teas with different storage years by combining GC-E-nose, GC–MS, and GC-IMS. *Food Chemistry: X, 18*, Article 100693, 2590–1575 https://doi.org/10.1016/j.fochx.2023.100693.

Shi, J., Zhu, Y., Zhang, Y., Lin, Z., & Lv, H. P. (2019). Volatile composition of Fu-brick tea and Pu-erh tea analyzed by comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry. *LWT- Food Science and Technology, 103*, 27–33. https://doi.org/10.1016/j.lwt.2018.12.075

Tian, D., Huang, G. H., Ren, L., Li, Y. D., Yu, J., Lu, Q., … Zhou, H. J. (2024). (2024) effects of *Monascus purpureus* on ripe Pu-erh tea in different fermentation methods and identification of characteristic volatile compounds. *Food Chemistry, 440* (138249), 0308–8146. https://doi.org/10.1016/j.foodchem.2023.138249

Wang, C., Li, J., Wu, X. J., Zhang, Y., He, Z. R., Zhang, Y., … Liu, Z. H. (2022). Pu-erh tea unique aroma: Volatile components, evaluation methods and metabolic mechanism of key odor-active compounds. *Trends in Food Science and Technology, 124*, 25–37. https://doi.org/10.1016/j.tifs.2022.03.031

Wang, M. Q., Ma, W. J., Shi, J., Zhu, Y., Lin, Z., & Lv, H. P. (2020). Characterization of the key aroma compounds in Longjing tea using stir bar sorptive extraction (SBSE) combined with gas chromatography-mass spectrometry (GC–MS), gas chromatography-olfactometry (GC-O), odoractivity value (OAV), and aroma recombination. *Food Research International, 130*, Article 108908. https://doi.org/10.1016/j.foodres.2019.108908

Wang, Z. X., Su, D., Ren, H. T., Lv, Q., Ren, L., Li, Y. L., & Zhou, H. J. (2022). Effect of different drying methods after fermentation on the aroma of Pu-erh tea (ripe tea). *LWT- Food Science and Technology, 171*(114129). https://doi.org/10.1016/j.lwt.2022.114129, 0023–6438.

Wei, Y. M., Yin, X. C., Wu, H. T., Zhao, M. J., Huang, J. L., Zhang, J. X., … Ning, J. M. (2022). Improving the flavor of summer green tea (*Camellia sinensis L.*) using the yellowing process. *Food Chemistry, 388*, Article 132982. https://doi.org/10.1016/j.foodchem.2022.132982

Wei, Y. M., Zhang, J. X., Li, T. H., Zhao, M. J., Song, Z. S., Wang, Y. J., & Ning, J. M. (2024). GC–MS, GC–O, and sensomics analysis reveals the key odorants underlying the improvement of yellow tea aroma after optimized yellowing. *Food Chemistry, 431*, Article 137139. https://doi.org/10.1016/j.foodchem.2023.137139

Xie, H. F., Kong, Y. S., Li, R. Z., Nothias, L. F., Melnik Alexey, V., Zhang, H., … Ling, T. J. (2020). Feature-based molecular networking analysis of the metabolites produced by in vitro solid-state fermentation reveals pathways for the bioconversion of epigallocatechin Gallate. *Journal of Agricultural and Food Chemistry, 68*, 7995–8007. https://doi.org/10.1021/acs.jafc.0c02983

Xu, J., Wei, Y., Li, F. L., Weng, X. C., & Wei, X. L. (2022). Regulation of fungal community and the quality formation and safety control of Pu-erh tea. *Comprehensive Reviews in Food Science and Food Safety, 21*(6), 4546–4572. https://doi.org/10.1111/1541-4337.13051

Yang, P., Song, H. L., Wang, L. J., & Jing, H. (2019). Characterization of key aroma-active compounds in black garlic by sensory-directed flavor analysis. *Journal of Agricultural and Food Chemistry, 67*(28), 7926–7934. https://doi.org/10.1021/acs.jafc.9b03269

Ye, J., Wang, W., Ho, C., Li, J., Guo, X., Zhao, M., Jiang, Y., & Tu, P. (2016). Differentiation of two types of Pu-erh teas by using an electronic nose and ultrasound-assisted extraction dispersive liquid–liquid microextraction-gas chromatography mass spectrometry. *Analytical Methods, 8*, 593–604. https://doi.org/10.1039/C5AY02652K

Zhai, X. T., Zhang, L., Granvogl, M., Ho, C. T., & Wan, X. C. (2022). Flavor of tea (*Camellia sinensis*): A review on odorants and analytical techniques. *Comprehensive Reviews in Food Science and Food Safety, 21*(5), 3867–3909. https://doi.org/10.1111/1541-4337.12999

Zhang, H., Wang, J. J., Zhang, D. D., Zeng, L., Liu, Y. N., Zhu, W., … Huang, Y. Y. (2021). Aged fragrance formed during the post-fermentation process of dark tea at an industrial scale. *Food Chemistry, 342*, Article 128175. https://doi.org/10.1016/j.foodchem.2020.128175

Zhang, J. X., Xia, D. Z., Li, T. H., Wei, Y. M., Feng, W. Z., Xiong, Z. C., … Ning, J. M. (2023). Effects of different over-fired drying methods on the aroma of Lu'an Guapian tea. *Food Research International, 173*(1), Article 113224. https://doi.org/10.1016/j.foodres.2023.113224

Zhang, L., Zhang, Z. Z., Zhou, Y. B., Ling, T. J., & Wan, X. C. (2013). Chinese dark teas: Post-fermentation, chemistry and biological activities. *Food Research International, 53*(2), 600–607. https://doi.org/10.1016/j.foodres.2013.01.016

Zhang, Y. Z., Li, Y. Q., Ren, X. X., Zhang, X., Wu, Z. F., & Liu, L. L. (2023). The positive correlation of antioxidant activity and prebiotic effect about oat phenolic compounds. *Food Chemistry, 134231*. https://doi.org/10.1016/j.foodchem.2022.134231, 0308–8146.

Zhao, M., Zhang, D. L., Su, X. Q., Duan, S. M., Wan, J. Q., Yuan, W. X., … Pan, Y. H. (2015). An integrated metagenomics/metaproteomics investigation of the microbial communities and enzymes in solid-state fermentation of Pu-erh tea. *Scientific Reports, 5*, 10117. https://doi.org/10.1038/srep10117

Zhu, J. C., Niu, Y. W., & Xiao, Z. B. (2022). Aromatic profiles and enantiomeric distributions of chiral volatile compounds in Pu-Erh tea. *Journal of Agricultural and Food Chemistry, 70*(27), 8395–8408. https://doi.org/10.1021/acs.jafc.2c03264

Zhu, M., Li, N., Zhou, F., Ouyang, J., & Wu, J. (2019). Microbial bioconversion of the chemical components in dark tea. *Food Chemistry, 312*, Article 126043. https://doi.org/10.1016/j.foodchem.2019.126043