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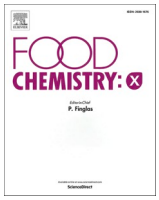

Effects of anaerobic treatment on the non-volatile components and angiotensin-converting enzyme (ACE) inhibitory activity of purple-colored leaf tea

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ABSTRACT

This study investigated the effect of anaerobic treatment on the non-volatile components and angiotensin-converting enzyme (ACE) inhibitory activity in purple-colored leaf tea. Results showed that after 8 h of anaerobic treatment, the γ-aminobutyric acid (GABA) content significantly increased from 0.02 mg/g to 1.72 mg/g ($p < 0.05$), while lactic acid content gradually rose from non-detectable levels to 3.56 mg/g. Notably, certain flavonols like quercetin and myricetin exhibited significant increments, whereas the total anthocyanins (1.01 mg/g) and epigallocatechin-3-(3"-*O*-methyl) gallate (13.47 mg/g) contents remained almost unchanged. Furthermore, the ACE inhibition rate of purple-colored leaf tea increased significantly from 42.16% to 49.20% ($p < 0.05$) at a concentration of 2 mg/mL. Moreover, galloylated catechins showed stronger ACE inhibitory activity than non-galloylated catechins in both *in vitro* ACE inhibitory activity and molecular docking analysis. These findings might contribute to the development of special purple-colored leaf tea products with potential therapy for hypertension.

1. Introduction

Hypertension is a significant modifiable risk factor for cardiovascular disease and premature mortality. The global population of hypertension among individuals aged 30–79 has nearly doubled from 1990 to 2019 (Zhou, Carrillo-Larco, et al., 2021). Angiotensin-converting enzyme (ACE) is a pivotal enzyme in the renin-angiotensin system responsible for regulating vascular tone *via* vasoconstriction, and its dysregulation may lead to hypertension and cardiovascular and cerebrovascular disorders (Li et al., 2017). Inhibiting ACE can effectively lower blood pressure and reduce the risk of thrombosis and cardiovascular disease, and the *in vitro* inhibition activity of potential inhibitors on ACE has long been used as an important factor for screening natural ACE inhibitors (Paiva et al., 2023; Wu et al., 2017).

In recent years, purple-colored leaf teas have garnered global interest for their unique flavor profiles and health benefits (Yang, Meng, et al., 2023). Studies have revealed that purple-colored leaf teas have a significantly higher concentration of bioactive components than conventional green-colored leaf teas (Shi et al., 2021). Many of these components, including epigallocatechin gallate (EGCG) (Dong et al., 2011; Paiva et al., 2023), epigallocatechin-3-(3"-*O*-methyl) gallate (EGCG3"Me) (Kurita et al., 2010), proanthocyanidins (Fan et al., 2022), and anthocyanidins (Ockermann et al., 2021), are the potential natural ACE inhibitors and have shown robust ACE inhibitory activity *in vitro*. Notably, γ-aminobutyric acid (GABA) is considered an important bioactive substance that may play a beneficial role in the prevention of cardiovascular disease, especially in lowering blood pressure (Hinton & Johnston, 2020). GABA-rich green tea exhibits antihypertensive effects in both young and aged Dahl salt-sensitive rats by lowering blood pressure and increasing plasma GABA levels (Abe et al., 1995). Anaerobic treatment, such as CO_2 filling, N_2 filling, vacuum treatment, and alternating anaerobic/aerobic conditions, has been verified to effectively increase GABA content in tea products (Chen et al., 2018; Dai et al., 2020). Additionally, anaerobic treatment can influence the levels of amino acids, TCA cycle metabolites, dimeric flavanols, and lipids in tea leaves, while the levels of methylxanthine alkaloids, flavanols, and flavones/flavonoid glycosides remain relatively unchanged (Dai et al., 2020). Our previous studies have found that different types of teas made

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from the same batch of fresh leaves after anaerobic treatment exhibit varying inhibitory activities on ACE. Among these, green tea shows the highest inhibition rate on ACE, followed by white tea, with black tea having the weakest inhibitory activity (Yang, Shi, et al., 2023).

Nevertheless, the impact of anaerobic treatment on the non-volatile components and ACE inhibitory activity during purple-colored leaf tea processing remains unclear. Therefore, this study investigates the dynamic changes in non-volatile metabolites in purple-colored leaf tea samples during anaerobic treatment using ultrahigh-performance liquid chromatography-quadrupole exactive orbitrap mass spectrometry (UHPLC-Q-Exactive/MS). Additionally, the *in vitro* ACE inhibition activity of tea samples and some key chemical compounds was evaluated using a chromatographic method (Wei et al., 2021; Yin et al., 2022). Molecular docking was employed to elucidate the interaction mechanism between potential inhibitors (mainly, catechins and GABA) and ACE. Overall, this study aims to provide a comprehensive understanding of the effects of anaerobic treatment on the non-volatile components and ACE inhibitory activity of purple-colored leaf tea and contribute to the development of special purple-colored leaf tea products with potential therapy for hypertension.

2. Materials and methods

2.1. Chemicals

Fifty-two authentic standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), Yuanye Biotechnology (Shanghai, China), Macklin (Shanghai, China), Merck (Darmstadt, Germany), Sykam (Munich, Germany) and MedChemExpress (NJ, USA). Mobile phase reagents were purchased from TCI (Tokyo, Japan), and Merck (Darmstadt, Germany). Details of all standards and chemicals are listed in Table S1.

2.2. Sample preparation

The processing method of purple-colored leaf tea was based on the previous work with some modifications (Dai et al., 2020). Specifically, fresh tea leaves (one bud and three leaves) of Zijuan (*Camellia sinensis* var. *assamica*) were plucked from an experimental base in Sanjie town (Shengzhou City, Zhejiang Province, China). Briefly, five kilograms of fresh tea leaves were divided into small portions of 200 g, packed into plastic bags and vacuumed. After vacuum treatment times of 0, 1, 2, 4, 6, and 8 h, tea samples were collected in triplicate (samples were labeled as A-0, A-1, A-2, A-4, A-6, and A-8, respectively) and immediately frozen in liquid nitrogen, then freeze-dried using vacuum freeze-dryer (FD5-10B, GOLD-SIM, California, USA). All samples were ground into powder using a grinder (IKA; Staufen, Germany) and stored at −20 °C for further analysis.

2.3. Analysis of non-volatile metabolites

2.3.1. Determination of amino acids

Amino acids, including GABA and theanine, were quantified using an amino acids analyzer (S—433D, Sykam, Munich, Germany) equipped with a Sykam cation LCAK07/Li column (4.6 mm × 150 mm) based on the previous method (Yang et al., 2018). One hundred milligrams of tea powder were infused with 10 mL of boiling water and extracted at 100 °C for 15 min, vortexed every 5 min. After cooling, the samples were centrifuged in a centrifuge (Centrifuge 5810 R, Eppendorf, Hamburg, Germany) at 8000 rpm for 10 min. The supernatants were filtered through a 0.45 μm membrane and stored at 4 °C for amino acids analysis. Amino acids were separated on a Na^+ model sulfonic acid-base strong acid cation exchange resin. The wavelengths were set at 570 nm and 440 nm, the flow rate was 0.45 mL/min, the injection volume was 10 μL, and the column temperature was set at 40 °C. The peak area of the tea samples was compared with the external amino acid standard solutions to determine the concentration.

2.3.2. Determination of organic acids

The organic acid content in tea samples was determined based on the methodology outlined by Li et al. (2022), with slight modifications. Analysis was conducted using an Agilent 1100 high-performance liquid chromatography (HPLC) system equipped with a ZORBAX® SB-C18 column (150 mm × 4.6 mm id, particle size 3.5 μm) (Agilent Technologies, Santa Clara, CA, USA). Briefly, 100 mg of tea powder was immersed in 10 mL of boiling water and extracted at 100 °C for 45 min, with agitation every 10 min. Upon cooling, the mixture was centrifuged at 8000 rpm for 10 min. The supernatant was filtered through a 0.45 μm aqueous membrane filter and stored at 4 °C until analysis. The mobile phase comprised a mixture of 6 mmol/L phosphoric acid (pH 2.1) and methanol (97:3), and elution was isocratic. The flow rate and column temperature were maintained at 1 mL/min and 28 °C, respectively. An injection volume of 10 μL was used, and detection was performed at a wavelength of 220 nm.

2.3.3. Determination of flavonoids

Flavonoid content of the tea samples was evaluated using UHPLC-Q-Exactive/MS (Thermo Fisher, San Jose, CA, USA) according to the previous method (Peng et al., 2022). Briefly, 0.2 g of tea powder was weighed and transferred to a centrifuge tube. Add 10 mL of 70% methanol/water solution (*v*/v) and extract in 70 °C water for 30 min, swirling every 5 min. After cooling, centrifuge the mixture at 4000 rpm for 10 min. Filter the supernatant through a 0.45 μm membrane, dilute it 10-fold with pure water and store at 4 °C for further analysis. The HSS T3 column (100 mm × 2.1 mm, 1.8 μm, Waters, Manchester, UK) was used as the chromatographic column, and it was set at 40 °C. The flow rate was 0.4 mL/min, and the injection volume was 3 μL. The mobile phase was composed of phase A, which was a methanolic solution containing 0.1% formic acid (v/v), and phase B, which was an acetonitrile solution containing 0.1% formic acid (v/v). The elution program was set as follows: 0 min, 2% phase B; 0.5 min, 2% phase B; 10 min, 15% phase B; 18 min, 40% phase B; 20 min, 90% phase B; 20.9 min, 90% phase B; 21 min, 2% phase B; 25 min, 2% phase B. The mass spectrometer operated in positive ESI mode with an ionization voltage of 3.5 kV, a capillary temperature of 300 °C, an S-lens RF level of 50, a sheath gas flow rate of 45 L/min, and a temperature and flow rate of the auxiliary gas set to 350 °C and 10 L/min, respectively. The MS scanning range was set to *m/z* 80–1200.

2.3.4. Determination of total anthocyanins

The total amount of anthocyanins in tea samples was determined using the pH differential method (da Silva et al., 2021). Briefly, the pH 1.0 buffer was comprised of 0.20 mol/L hydrochloric acid-potassium chloride buffer, while the pH 4.5 buffer consisted of 0.4 mol/L sodium acetate. To ensure accuracy, the sample dilution was limited by maintaining the absorbance at pH 1.0 at 520 nm between 0.2 and 0.8. Subsequently, all samples were diluted 4 times with pH 1.0 and pH 4.5 buffers, respectively. After 50 min, the absorbance was measured at 520 nm and 700 nm with a spectrophotometer (UV-3600, Shimadzu, Japan). The total anthocyanin content (milligram cyanidin 3-*O*-glucoside equivalent per gram of extract) was calculated using the following formula:

$$\text{Total anthocyanin content (mg/g)} = \frac{A \times MW \times DF \times V}{\varepsilon \times L \times W.}$$

A: absorbance value; DF: dilution factor; V: final volume (mL); W: weight of tea samples (g); MW: molecular weight of cyanidin 3-*O*-glucoside (449.2 g/mol). L: light path length (cm); ε: molar extinction coefficient of cyanidin 3-*O*-glucoside at 520 nm (2.69×10^4 L × mol^{-1} × cm^{-1}). The specific absorbance A is defined as:

$$A = (A_{520\ nm} - A_{700\ nm})_{pH\ 1.0} - (A_{520\ nm} - A_{700\ nm})_{pH\ 4.5}$$

2.4. Determination of in vitro ACE inhibition activity

The water extract of tea samples was prepared as described in section 2.3.1. In addition, nine catechin and GABA chemical standards were dissolved in pure water, diluted to 1 mg/mL, and stored at 4 °C for testing. The intensity of ACE inhibitory activity of samples and chemical standards was determined with reference to previous studies (Wei et al., 2021; Yin et al., 2022). Hippuryl-histidyl-leucine (HHL) was used as the reaction substrate. In brief, ACE and HHL were dissolved in 0.20 mol/L boric acid-borax buffer solution (0.20 mol/L NaCl, pH 8.3). To initiate the reaction, 20 μL of ACE solution (0.1 UN/mL) and an excess of 100 μL of HHL were combined with 50 μL of water extracts of tea samples at varying concentrations. The enzymatic reaction was carried out at 37 °C for 60 min, and the reaction was stopped by adding 300 μL of 1 mol/L hydrochloric acid. The hippuric acid produced during the reaction was analyzed by high-performance liquid chromatography system (LC-20 A, Shimadzu Corp., Kyoto, Japan) equipped with WondaSil C18 column (250 mm × 4.6 mm, 5 μm). Detection wavelength: 228 nm; column temperature: 30 °C; injection volume: 20 μL; flow rate: 1 mL/min; mobile phase: ultrapure water (containing 0.1% trifluoroacetic acid) and 100% acetonitrile; elution conditions: isocratic elution with 75%/25% (*v*/v) water/acetonitrile. The ACE inhibitory activity was calculated using the following formula:

$$\text{ACE inhibitory activity } (\%) = \frac{\text{Ac} - \text{As}}{\text{Ac} - \text{Ab}} \text{x } 100\%$$

Ac: the peak area of hippuric acid formed when the sample was replaced with boric acid-borax buffer; As: the peak area of hippuric acid formed in the sample; Ab: the peak area of hippuric acid formed when both the sample and ACE were replaced with acid-borax buffer.

2.5. Molecular docking

To investigate the molecular-level interaction between the compound and ACE, a molecular docking simulation was carried out using Autodock Vina 1.2.3 (Scripps Research Institute, La Jolla, CA, USA) (Eberhardt et al., 2021). The three-dimensional crystal structure of the human ACE-lisinopril complex (Protein ID:1O86) was obtained from the Protein Data Bank (http://www.rcsb.org/) and used as the receptor in the docking process. The three-dimensional structure of the compound was obtained from the National Library of Medicine (https://pubchem.ncbi.nlm.nih.gov/) and energy minimized with ChemBio3DUltra 14.0 software (Cambridge Soft, Cambridge, MA, USA). Before docking, all hetero molecules, such as water, were removed from 1O86 while preserving the cofactors zinc ion and glycine. Polar hydrogens were added to the ACE model. The molecular docking in this study employs a semi-flexible docking approach, where the conformation of the small molecule undergoes limited changes within a reasonable range, while the conformation of the large molecule remains largely unchanged. This method balances the computational volume and the predictive ability of the model and is the most used one (Eberhardt et al., 2021). Grid maps of 22.50 Å × 22.50 Å × 22.50 Å with a grid spacing of 0.375 Å centered on the zinc coordinate (x: 43.82, y: 38.24, and z: 46.71) were used as the binding site. Molecular docking was conducted using Autodock Vina 1.2.3 software, employing the Lamarckian genetic algorithm with 64 runs.

The optimal conformation was determined by selecting the pose with the lowest binding energy in the active pocket. All docking procedures were repeated three times to ensure accuracy. The binding interactions between the ligands and the ACE model were analyzed using the PLIP tool (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index). Protein-ligand interaction diagrams were visualized and analyzed using PyMOL 2.5.4 software (DeLano Scientific LLC, South San Francisco, CA, USA).

2.6. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 8 software (GraphPad Software, San Diego, CA). For the analysis of non-volatile metabolite heatmaps, and the performance of partial least squares discriminant analysis (PLS-DA), online tool MetaboAnalyst 6.0 (https://www.metaboanalyst.ca) was utilized. The IC_{50} value was defined as the concentration of peptide required to inhibit half of the ACE activity and was calculated by non-linear curve fitting of the ACE inhibition rate *versus* inhibitor concentration. Some icons in the graphical abstract were obtained from BioRender (https://app.biorender.com/).

3. Results and discussion

3.1. Dynamic changes of non-volatile compounds in purple-colored leaf tea samples during anaerobic treatment

3.1.1. Dynamic changes of amino acids

The dynamic changes of amino acids contents in fresh leaves of purple-colored leaf tea during anaerobic treatment are shown in Fig. 1 & Table S2. The anaerobic treatment significantly altered the composition of amino acids. Specifically, the GABA levels increased significantly (from 0.02 to 1.72 mg/g, $p < 0.05$) after 8 h of anaerobic treatment, meeting the requirements for GABA tea (GABA content ≥1.50 mg/g). The GABA content in fresh tea leaves is typically low, ranging from 0.18 to 0.45 mg/g in traditional tea products (Zhao et al., 2011). In tea leaves, GABA synthesis occurs mainly through two pathways: the GABA shunt and polyamine degradation (Wu et al., 2018). The former is the primary synthetic pathway, triggered by decreased intracellular pH caused by anaerobic treatment, which activates glutamate decarboxylase to produce GABA by decarboxylating glutamate. The latter pathway involves the conversion of putrescine and spermidine to GABA *via* diamine oxidase and polyamine oxidase, respectively. Previous studies showed that green-colored leaf tea samples treated with filled-N_2 anaerobic method (Huang et al., 2023) and alternating anaerobic-aerobic method (Wang et al., 2006) contained GABA levels of 1.89 mg/g and 1.81 mg/g, respectively, which were close to the result of 1.72 mg/g found in purple-colored leaf tea. Therefore, employing a direct vacuum anaerobic treatment may be a more accessible and cost-effective approach to enhance the GABA content in fresh tea leaves compared to the aforementioned methods.

L-Theanine was the most abundant non-protein amino acid in purple-colored leaf tea, and its content showed a trend of firstly increasing and then decreasing during the anaerobic treatment, which is consistent with the previous study (Dai et al., 2020). Its content reached the highest value (from 9.39 mg/g to 11.18 mg/g) after 1 h of anaerobic treatment, but gradually decreased to 9.37 mg/g over the following 7 h. Other amino acids such as alanine (from 0.09 to 0.44 mg/g), tyrosine (from 0.02 to 0.16 mg/g), valine (from 0.01 to 0.17 mg/g), and glycine (from 0.00 to 0.12 mg/g) also showed significant increases over time. Previous research has reported that the GABA shunt contributes to the accumulation of alanine and tyrosine (Yu & Yang, 2020). In addition, proline, an osmoregulatory substance in plants, also increased continuously up to 0.06 mg/g after anaerobic treatment, and similar results were observed in a recent report on pickled teas treated with prolonged anaerobic treatment (Hou et al., 2023). In contrast, the levels of glutamate and glutamine, which serve as precursors of GABA, exhibited a gradual decline during the anaerobic treatment (from 0.73 to 0.06 mg/g and from 0.45 to 0.22 mg/g, respectively), with a particularly large decrease within the first hour of anaerobic treatment. Additionally, aspartic acid content displayed a significant decreasing trend, whereas asparagine showed the opposite pattern, possibly due to the conversion of aspartic acid to asparagine through partial acylation under anaerobic conditions (Wang et al., 2006).

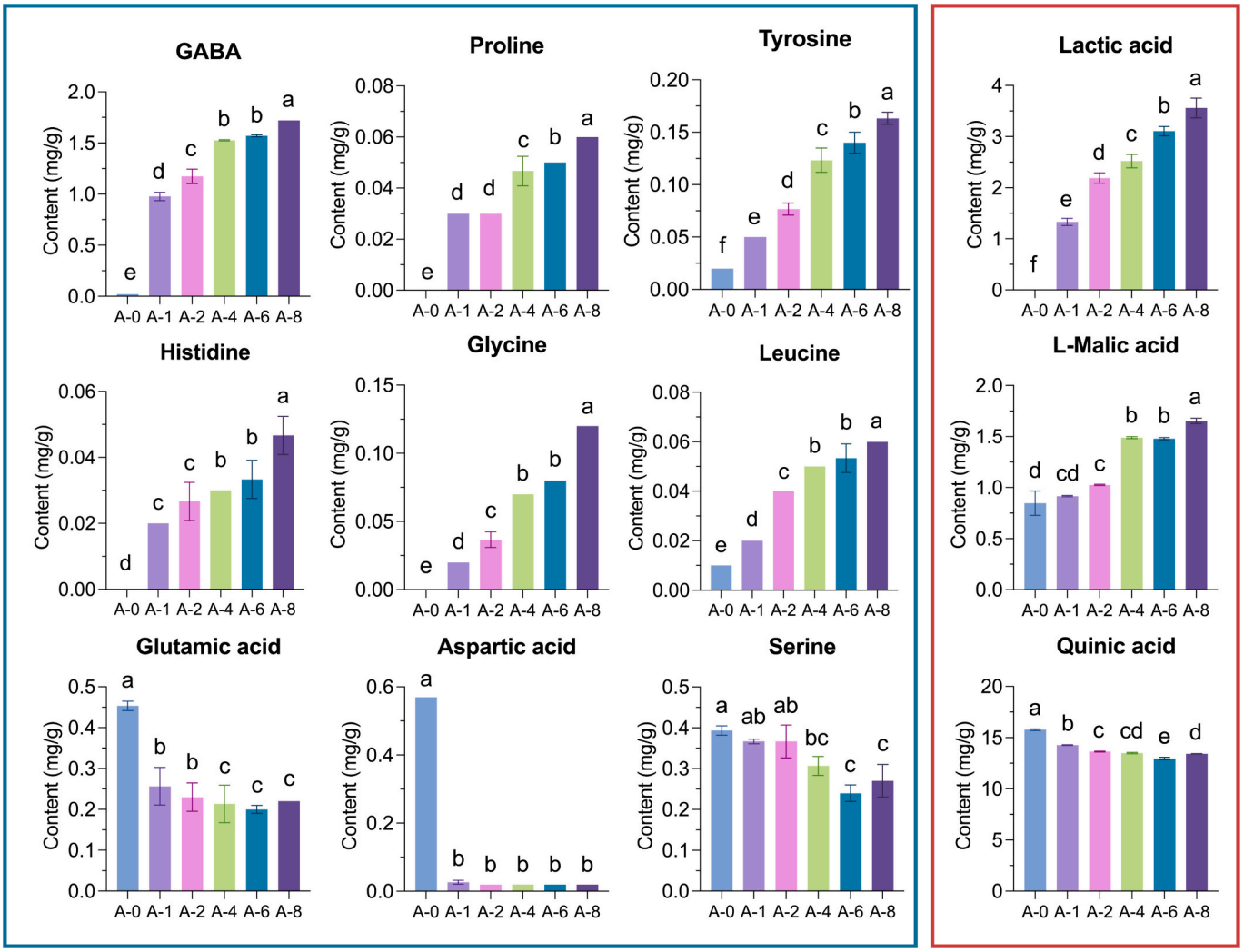

Fig. 1. Amino acids (A) and organic acids (B) with significant changes in the content of tea samples during anaerobic treatment. The different letters represent statistically significant differences by Tukey's multiple comparisons test ($p < 0.05$)

3.1.2. *Dynamic changes of organic acids*

According to recent reports, organic acids such as succinic acid, lactic acid, and citric acid directly contribute to the acidity profile (Zhang et al., 2022). Previous studies have indicated that anaerobic treatment of fresh tea leaves often results in the development of a distinct sharp acidic taste (Chen et al., 2018), suggesting a possible correlation between this taste and changes in organic acid content of tea leaves. In this study, nine common organic acids including oxalic acid, D-malic acid, and citric acid, were accurately quantified using a high-performance liquid chromatography system, as shown in Table S2. After 8 h of anaerobic treatment, the total content of the nine organic acids significantly increased (from 30.20 mg/g to 31.65 mg/g, $p < 0.05$). Among them, quinic acid was the most abundant, accounting for approximately 50% of the total organic acid content, and showed a significant decrease during anaerobic treatment (from 15.76 mg/g to 13.43 mg/g, $p < 0.05$). Additionally, succinic acid, chlorogenic acid, and citric acid exhibited similar decreasing trends. Conversely, with prolonged anaerobic treatment, the content of lactic acid and L-malic acid gradually increased. Lactic acid was not detected in fresh tea leaves, but after 8 h of anaerobic treatment, its content increased to 3.56 mg/g. The increase in content may be due to anaerobic respiration by cells in tea leaves under anoxic conditions. Furthermore, unlike the sharp decrease in malic acid content during the withering process of black tea (Yu et al., 2022), the content of D-malic acid (the main form of malic acid) remained relatively stable during anaerobic treatment (approximately 8 mg/g), while the content of L-malic acid significantly increased (from 0.85 mg/g to 1.65 mg/g, $p < 0.05$). It has been reported that lactic acid and malic acid may be important contributors to the acidity of green tea (Zhang et al., 2022), suggesting that the increase in lactic acid and L-malic acid content may be the primary reason for the development of acidity in tea samples after anaerobic treatment.

3.1.3. *Dynamic changes of flavonoids*

Tea flavonoids, as the most representative secondary metabolites, mainly include flavanones, flavanols, flavonols, flavones, and anthocyanins, and play crucial roles in the flavor qualities and health benefits of tea (Yang, Meng, et al., 2023). Previous investigations have demonstrated that purple-colored leaf tea exhibits higher levels of anthocyanins, catechins and proanthocyanidins in comparison to green-colored leaf tea (Shi et al., 2021). The dynamic changes of flavonoid components in tea samples during anaerobic treatment are shown in Table 1. Anthocyanins are the characteristic components of the purple-colored leaf tea, and the levels of total anthocyanins (1.01 mg/g) remained relatively stable throughout the anaerobic treatment ($p > 0.05$). Interestingly, the content of flavonols such as quercetin and myricetin increased significantly after 8 h of anaerobic treatment ($p < 0.05$). A recent study indicated that the increase in free-form flavonols in tea samples during anaerobic treatment primarily resulted from the hydrolyzation of

Table 1
Dynamic changes of flavonoids in tea samples during anaerobic treatment.

Compounds	Content (mg/g of the dry weight)					
	A-0	A-1	A-2	A-4	A-6	A-8
Total anthocyanins	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.00 ± 0.01^{a}	1.01 ± 0.01^{a}
C	16.53 ± 0.06^{e}	17.02 ± 0.06^{c}	16.92 ± 0.13^{cd}	16.79 ± 0.08^{d}	17.51 ± 0.09^{b}	18.06 ± 0.06^{a}
EC	24.66 ± 0.16^{b}	24.55 ± 0.28^{b}	24.58 ± 0.11^{b}	24.27 ± 0.07^{b}	26.40 ± 0.74^{a}	26.62 ± 0.47^{a}
GC	11.51 ± 0.10^{ab}	11.68 ± 0.10^{a}	11.45 ± 0.07^{ab}	11.33 ± 0.01^{bc}	11.51 ± 0.13^{ab}	11.12 ± 0.08^{c}
EGC	48.15 ± 0.01^{a}	47.89 ± 0.49^{a}	46.53 ± 0.13^{b}	46.71 ± 0.10^{b}	46.15 ± 0.03^{b}	45.85 ± 0.21b
CG	29.82 ± 0.68^{b}	29.50 ± 0.15^{b}	30.80 ± 1.01^{ab}	31.13 ± 0.05^{a}	29.34 ± 0.03^{c}	29.48 ± 0.22^{bc}
ECG	36.36 ± 0.76^{b}	36.00 ± 0.23^{b}	37.64 ± 1.24^{ab}	38.05 ± 0.09^{a}	34.59 ± 0.04^{c}	35.95 ± 0.34^{bc}
GCG	1.43 ± 0.03^{c}	1.42 ± 0.04^{c}	1.40 ± 0.01^{c}	1.42 ± 0.04^{c}	1.84 ± 0.09^{b}	2.14 ± 0.16^{a}
EGCG	74.33 ± 1.06^{a}	73.86 ± 0.89^{a}	73.66 ± 0.59^{a}	74.61 ± 0.06^{a}	74.50 ± 0.10^{a}	74.65 ± 0.59^{a}
EGCG3"Me	13.25 ± 0.22^{bc}	13.42 ± 0.16^{b}	14.03 ± 0.09^{a}	13.97 ± 0.02^{a}	12.94 ± 0.24^{c}	13.47 ± 0.11^{b}
Dihydromyricetin	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Astragalin	0.33 ± 0.02^{b}	0.33 ± 0.01^{b}	0.36 ± 0.00^{a}	0.36 ± 0.00^{a}	0.33 ± 0.01^{b}	0.34 ± 0.01^{ab}
Rutin	1.35 ± 0.01^{abc}	1.31 ± 0.02^{c}	1.37 ± 0.03^{ab}	1.37 ± 0.02^{ab}	1.33 ± 0.02^{bc}	1.38 ± 0.00^{a}
Luteolin	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
Quercetin	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}	0.01 ± 0.00^{d}	0.01 ± 0.00^{c}	0.01 ± 0.00^{b}	0.02 ± 0.00^{a}
Quercetin-3-galactoside	1.86 ± 0.07^{ab}	1.87 ± 0.00^{ab}	1.93 ± 0.03^{a}	1.93 ± 0.03^{a}	1.85 ± 0.02^{ab}	1.81 ± 0.01^{b}
Quercetin-3-glucoside	0.36 ± 0.01^{ab}	0.36 ± 0.02^{b}	0.39 ± 0.02^{a}	0.36 ± 0.01^{ab}	0.35 ± 0.06^{b}	0.35 ± 0.01^{b}
Kaempferol	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.01 ± 0.00^{a}
Myricetin	0.08 ± 0.00^{c}	0.08 ± 0.00^{c}	0.10 ± 0.005^{b}	0.12 ± 0.00^{a}	0.12 ± 0.01^{a}	0.12 ± 0.01^{a}
Myricetin-3-galactoside	4.17 ± 0.04^{abc}	4.17 ± 0.06^{abc}	4.25 ± 0.02^{ab}	4.28 ± 0.04^{a}	4.10 ± 0.01^{c}	4.14 ± 0.07^{bc}
Myricetin-3-glucoside	1.10 ± 0.02^{bc}	1.08 ± 0.01^{bc}	1.16 ± 0.03^{a}	1.12 ± 0.00^{b}	1.07 ± 0.00^{c}	1.11 ± 0.01^{bc}
Procyanidin B1	0.57 ± 0.01^{ab}	0.58 ± 0.00^{a}	0.53 ± 0.00^{c}	0.55 ± 0.01^{b}	0.58 ± 0.00^{a}	0.56 ± 0.00^{b}
Procyanidin B2	3.20 ± 0.05^{a}	3.20 ± 0.05^{a}	3.19 ± 0.05^{a}	3.15 ± 0.07^{a}	3.24 ± 0.00^{a}	3.20 ± 0.00^{a}
Procyanidin B3	1.69 ± 0.02^{a}	1.58 ± 0.04^{bc}	1.45 ± 0.04^{d}	1.51 ± 0.04^{cd}	1.65 ± 0.01^{ab}	1.56 ± 0.03^{c}
Procyanidin A1	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.00 ± 0.00^{b}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
Procyanidin A2	0.02 ± 0.00^{b}	0.03 ± 0.00^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}
Procyanidin C1	2.95 ± 0.06^{a}	2.99 ± 0.02^{a}	2.74 ± 0.11^{c}	2.91 ± 0.06^{ab}	2.97 ± 0.05^{a}	2.96 ± 0.01^{a}
Neochlorogenic acid	1.84 ± 0.01^{a}	1.82 ± 0.02^{ab}	1.76 ± 0.05^{b}	1.80 ± 0.02^{a}	1.68 ± 0.04^{c}	1.82 ± 0.01^{ab}
Chlorogenic acid	0.23 ± 0.00^{a}	0.22 ± 0.00^{bc}	0.22 ± 0.00^{bc}	0.22 ± 0.00^{ab}	0.22 ± 0.00^{c}	0.21 ± 0.00^{d}

Note: The different letters in the same row indicate the significant differences between the tea samples by Tukey's multiple comparisons test ($p < 0.05$). GC, gallocatechin; EGC, epigallocatechin; C, catechin; EGCG, epigallocatechin gallate; EC, epicatechin; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; EGCG3"Me, epigallocatechin-3-(3"-*O*-methyl) gallate. Tea samples were labeled according to their anaerobic treatment times of 0, 1, 2, 4, 6, and 8 h as A-0, A-1, A-2, A-4, A-6, and A-8.

flavonoid glycosides, such as isoquercitrin and the *O*-methylation of relevant flavonoids (Hou et al., 2023).

Flavanols mainly include catechins and proanthocyanidins; the former are the most abundant subclass of tea flavonoids, while the latter are mainly oligomers of catechins and are generally found in smaller amounts in tea leaves. The levels of most catechins did not exhibit significant changes during the anaerobic treatment. For instance, EGCG, one of the most abundant catechins, its content remained stable ($p > 0.05$). However, there was a significant decreasing trend in the content of epigallocatechin (EGC) (from 48.15 to 45.85 mg/g, $p < 0.05$), while the content of catechin (C) showed a significant increasing trend (from 16.53 to 18.06 mg/g, $p < 0.05$) after 8 h anaerobic treatment. Additionally, there were significant increases in the contents of gallocatechin gallate (GCG) and epicatechin (EC) after anaerobic treatment (from 24.66 to 26.62 mg/g and 1.43 to 2.14 mg/g, respectively). Notably, high levels of EGCG3"Me (about 13 mg/g) were detected in all the tea samples. The results showed that EGCG3"Me content was relatively stable during anaerobic treatment, suggesting that it is feasible to produce GABA tea products with a high EGCG3"Me content (≥ 10 mg/g). Previous studies have reported that alternating anaerobic and aerobic treatment of green tea did not result in significant changes in the content of most of the catechins, which is consistent with our findings (Wang et al., 2006). Furthermore, proanthocyanidins B1, B2, A2, A1 and C1 exhibited minimal changes and no significant differences were observed after 8 h of anaerobic treatment ($p > 0.05$).

In summary, the majority of flavonoids in purple-colored leaf tea exhibited relative stability during anaerobic treatment. This might be attributed to the reduced oxygen supply, which slows down the oxidation rate of flavonoids, and the restricted metabolic activities of enzymes in the absence of oxygen, contributing to the maintenance of tea flavonoid stability (Zhou, Iqbal, et al., 2021).

3.1.4. Principal component analysis and correlation analysis

To obtain an overview of the dynamic changes in amino acids and flavonoids levels in tea samples during anaerobic treatment, PLS-DA was performed using the contents of 61 non-volatile compounds. The score plot is shown in Fig. 2A, the groups with different anaerobic treatment times exhibited clear separation, indicating a significant impact of anaerobic treatment on the non-volatile components of purple-colored leaf tea. Cross-validation using leave-one-out cross-validation method showed that the fit index (R^2X) of the independent variables was 97.2%

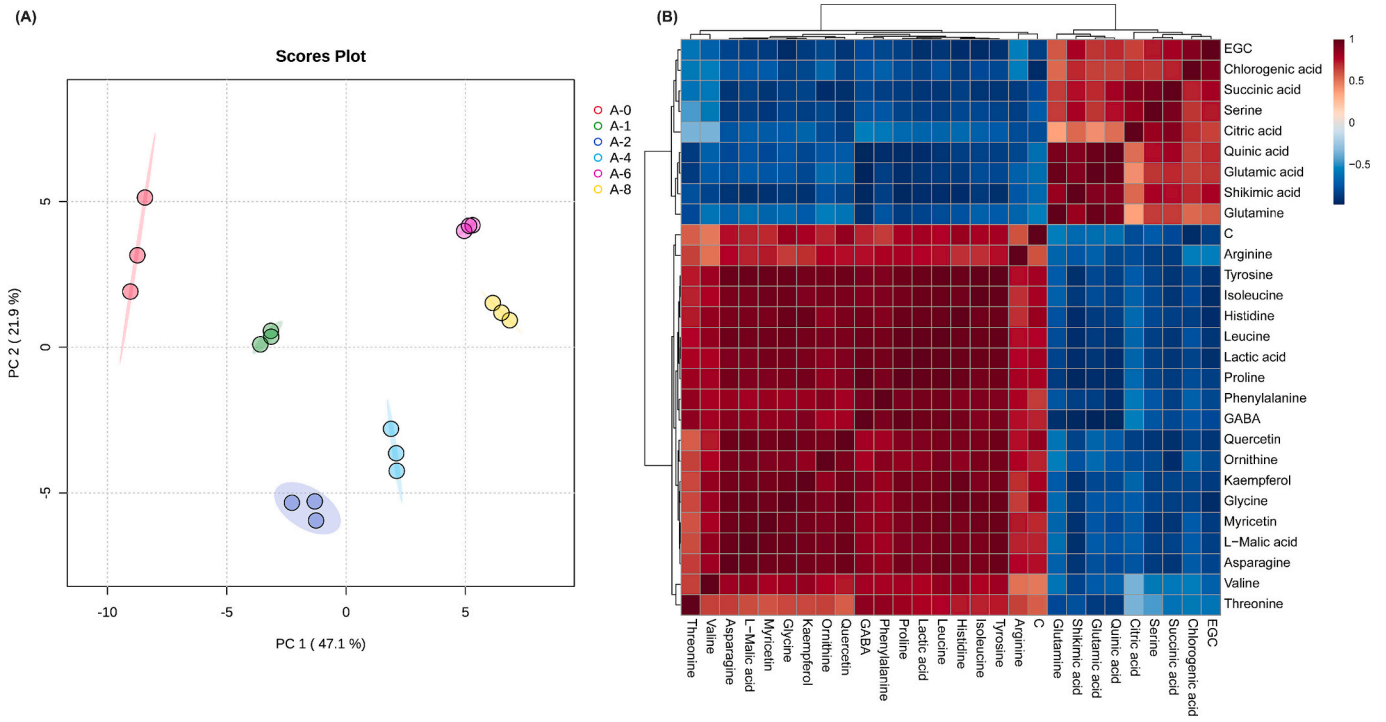

Fig. 2. The partial least squares discriminant analysis (PLS-DA) scores plot (A) and correlation heatmap of VIP $\geq$ 1 (B) for non-volatile components.

and the predictive index (Q^2) of the model was 95.7%, implying that the model has good predictive ability. Based on the PLS-DA model, we identified 28 non-volatile compounds with variable importance in projection (VIP) values $\geq$1 across all tea samples and conducted a correlation analysis of these components (Fig. 2B). Strong correlations were observed among certain amino acids (arginine, tyrosine, isoleucine, histidine, leucine, proline, phenylalanine, and GABA) and organic acids (lactic acid, D-malic acid), whose levels gradually increased during anaerobic treatment of tea leaves. These constituents play an important role in contributing to the flavor quality of tea. Their accumulation may be due to cellular respiration in fresh leaves and protein degradation during prolonged anaerobic treatment (Dai et al., 2020).

3.2. *In vitro* ACE inhibition activity analysis

The *in vitro* inhibition activity of ACE inhibitors is widely used as an important factor for screening natural ACE inhibitors. As shown in Fig. 3A, the A-8 tea samples exhibited slightly higher ACE inhibition activity compared to the A-0 tea samples at different concentrations. The IC_{50} values of ACE inhibitory activity for A-8 and A-0 tea samples were 1.96 mg/mL and 2.39 mg/mL, respectively. The IC_{50} values of A-8 tea samples were lower than those of A-0 tea samples, indicating that anaerobic treatment could enhance the ACE inhibitory activity of purple-colored leaf tea samples. Specifically, at a concentration of 2 mg/mL, A-8 tea samples demonstrated a significantly higher ACE inhibition of 49.20%, while A-0 tea samples showed 42.16% inhibition ($p < 0.05$). Combined with the analysis of the dynamics of the chemical composition in the previous section, we can infer that the enhanced ACE inhibitory activity of the tea samples may be related to the increase of GCG, EC, C, and GABA components in the samples after anaerobic treatment. Our study has preliminarily evaluated tea's potential blood pressure-lowering effects under *in vitro* conditions. However, future studies should include a broader range of experiments, including animal studies and clinical trials.

Moreover, we conducted an assay to evaluate the *in vitro* ACE inhibitory activity of nine catechins and GABA chemical standard (Fig. 3B). Interestingly, the galloylated catechins (including GCG, EGCG3"Me, CG, EGCG, ECG) exhibited significantly stronger inhibitory activity compared to the non-galloylated catechins (including GC, EGC, EC, C) ($p < 0.05$). This difference in activity may be attributed to the different chemical structures of these catechins (Fig. 3C). Additionally, gallic acyl groups have been shown to enhance various biological activities of catechins. For example, galloylated catechins have significantly stronger inhibitory effects on histamine release and amylase compared to non-galloylated catechins (Pan et al., 2023). Previous reports have indicated that the methylated EGCG3"Me not only exhibits greater bioavailability *in vivo* compared to EGCG, but also demonstrates stronger *in vitro* ACE inhibitory activity (Kurita et al., 2010; Zhang et al., 2018).

Furthermore, the ACE inhibitory activity of all nine catechins was significantly stronger than that of GABA at the same concentration ($p < 0.05$), indicating that catechins are possibly the major bioactive components that exert *in vitro* ACE inhibitory activity in tea samples. However, recent research suggests that anaerobic treatment can increase the GABA content and enhance the *in vitro* ACE inhibitory activity of mulberry leaves (Tu et al., 2022), supporting the potential of GABA as an ACE inhibitor. It is important to note that the finding from *in vitro* studies may not directly translate to *in vivo* effects, and GABA may exert hypotensive activity *in vivo* through other pathways. GABA has been shown to have a higher oral bioavailability and better ability to cross the blood-brain barrier than catechins, which might contribute to its superior antihypertensive activity compared to catechins (Oketch-Rabah et al., 2021). Previous reports have demonstrated potent antihypertensive biological activity of GABA in animal models of hypertension, suggesting that GABA may also act *in vivo* through inhibition of noradrenaline release *via* presynaptic $GABA_B$ receptors or the inhibition of post-ganglionic sympathetic cardiac nerves *via* $GABA_A$ receptors (Hinton & Johnston, 2020). Therefore, further investigation is required to elucidate the mechanisms underlying the *in vivo* antihypertensive effects of catechins and GABA.

3.3. *Molecular docking results*

Although *in vitro* ACE inhibition assays demonstrate a dose-response

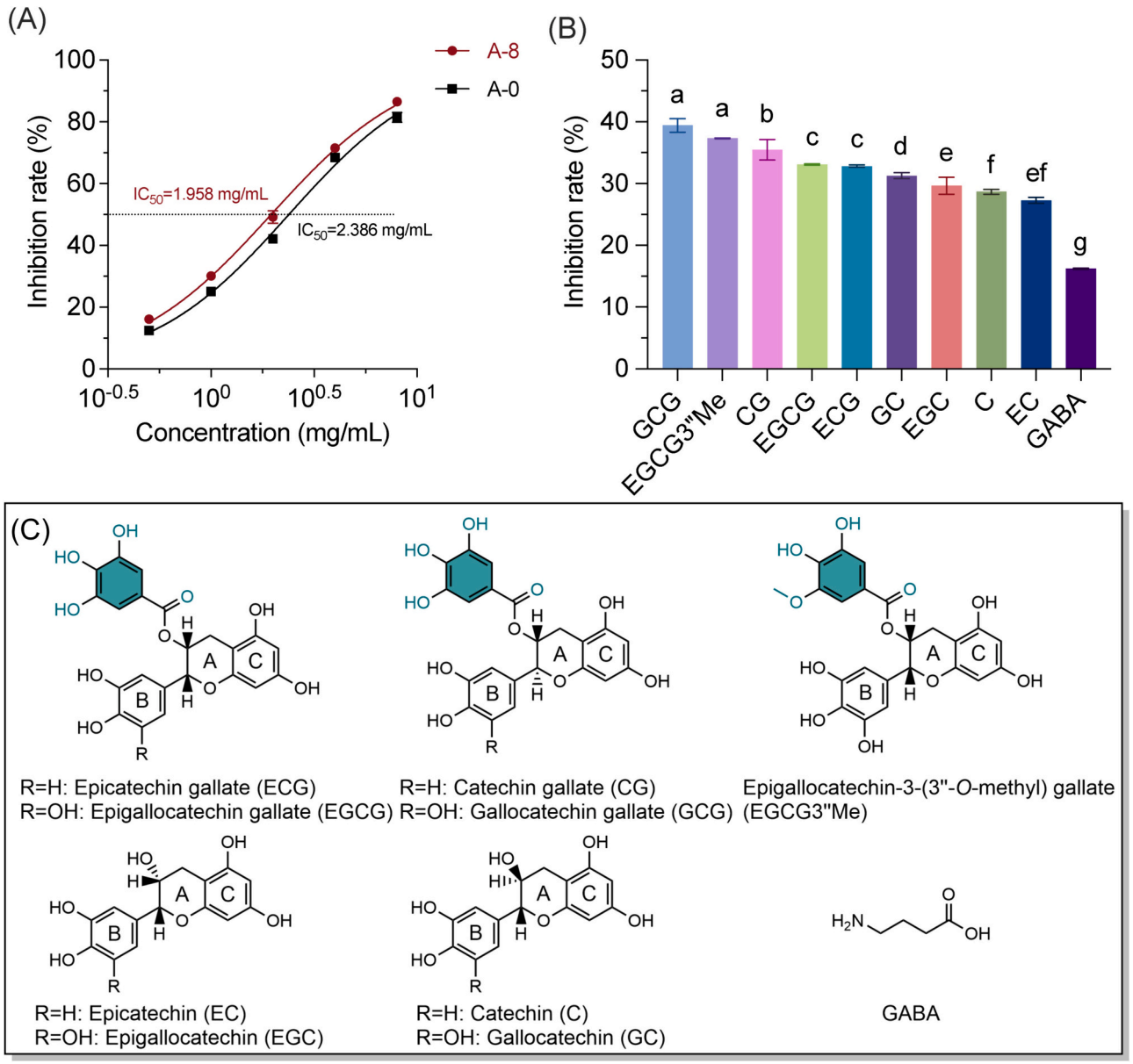

Fig. 3. *In vitro* ACE inhibitory activity was assessed for different tea samples (A-0 and A-8) (A) and chemical standards (nine catechins and GABA) (B). (C) Chemical structures of nine catechins and GABA.

relationship between the inhibitor and ACE activity, they do not directly reveal the structure-activity relationship. The molecular docking results are presented in Table 2. The binding affinity represents the energy released during the binding process, and a higher absolute value indicates a greater likelihood of binding (Wu et al., 2022). Among the nine catechins, GCG exhibited the highest absolute value of binding affinity at 10.23 kcal/mol. In contrast, GABA had a significantly smaller absolute value of binding affinity at 3.88 kcal/mol compared to the nine catechins ($p < 0.05$). Notably, the galloylated catechins (including GCG, CG, EGCG3"Me, and EGCG) displayed significantly higher absolute value of binding affinity compared to the non-galloylated catechins (including EGC, GC, EC, and C). This suggests that under the simulated molecular docking conditions, galloylated catechins have a greater propensity to binding to ACE protein receptors than non-galloylated catechins, consistent with the results in section 3.3. The galloyl group on the C ring may be the primary functional group influencing the ACE inhibitory activity of catechins (Wang et al., 2021). Fig. S1 illustrates a significant linear correlation between the binding energy of molecular docking and the ACE inhibition rates of the nine catechins and GABA standards ($R^2 = 0.91$, $p < 0.01$), which can be utilized for activity prediction and interaction analysis of structurally similar compounds.

Table 2
Binding energy of nine catechins and GABA with ACE.

No.	Compounds	Binding affinity (kcal/mol)
1	GCG	-10.23 ± 0.03^{a}
2	CG	-9.65 ± 0.07^{b}
3	EGCG3"Me	-9.44 ± 0.02^{c}
4	EGCG	-9.35 ± 0.03^{d}
5	ECG	-9.26 ± 0.04^{d}
6	EGC	-8.46 ± 0.04^{e}
7	GC	-8.39 ± 0.01^{ef}
8	C	-8.32 ± 0.01^{f}
9	EC	-8.30 ± 0.03^{f}
10	GABA	-3.88 ± 0.05^{g}

Note: The different letters in the same column indicate the significant differences between the compounds by Tukey's multiple comparisons test ($p < 0.05$).

Understanding the receptor-ligand interactions between ACE and inhibitors is crucial for screening natural bioactive inhibitors (Wu et al., 2017). Fig. 4 illustrates the binding pockets and interaction analysis of four representative compounds (including EGCG3"Me, EGCG, C, and GABA) with ACE protein receptors in molecular docking studies. The main active site of ACE consists of three active pockets (S1, S2, and S1') and the HEXXH binding motif (His383, Glu384, and His387) with zinc ion. The S1 pocket contains Ala354, Glu384 and Tyr523, the S2 pocket contains Gln281, His353, Lys511, His513 and Tyr520, while the S1' pocket contains only Glu162 (Mirzaei et al., 2018; Shih et al., 2019). All four compounds can fit into the hydrophobic pocket of ACE, forming stable hydrophobic bonds. Certain functional groups, such as hydroxyl and carboxyl groups, which can act as hydrogen bond acceptors or donors, have been reported to contribute to ACE inhibitory activity (Al Shukor et al., 2013).

Among these compounds, EGCG3"Me primarily interacts with ACE residues Asn70, Glu143, Glu384, Glu411, Arg522, and Tyr523, forming eight hydrogen bonds with bond distances ranging from 2.70 to 3.10 angstroms (Å). EGCG3"Me, which has a similar chemical structure to EGCG, is produced by methylating a hydroxyl group on the galloyl portion of EGCG, resulting in the formation of an *O*-methylated compound. During the interaction with ACE, EGCG3"Me forms an additional hydrogen bond with the Tyr306 residue, with a bond distance of 2.80 Å, in addition to the previously mentioned binding sites of EGCG. In addition, compared to EGCG3"Me and EGCG, C forms only three hydrogen bonds with the ACE residues Ala356, Glu403, and Gly404 due to the lack of gallic acyl groups, and the C ring has an imidazole ring at His387 forming a π-stack at a distance of 4.00 Å. GABA also forms three hydrogen bonds with the ACE residues Tyr523 and Glu411, and GABA can tunnel deeply to chelate directly with the zinc ion (bond distances: 2.40 Å). The molecular docking results indicated that catechins primarily interact with the S1 pocket of ACE, while GABA can interact with the zinc ion of ACE. It is noteworthy that the binding affinity of galloylated catechins with ACE is stronger than that of non-galloylated catechins, possibly due to the presence of gallic acyl groups in their molecular structure, enabling them to interact with multiple structural domains in ACE. On the other hand, the binding ability of GABA with ACE is weaker compared to catechins, which may be attributed to its relatively smaller molecular weight and simpler structure, resulting in weaker interactions with ACE.

This study primarily focused on flavonoids and amino acids, and potentially overlooked other bioactive compounds in tea leaves that could influence ACE inhibitory activity. Additionally, while molecular docking suggested interactions, the detailed biochemical mechanisms of these compounds on ACE required further investigation.

4. Conclusion

The findings of this study indicate that anaerobic treatment of purple-colored fresh tea leaves is an effective method for enhancing their *in vitro* ACE inhibitory activity. While the amino acid and organic acid compositions undergoe significant changes due to anaerobic treatment, most flavonoid components with potential ACE inhibitory activity are minimally affected. The combined factors of restricted enzyme activity and reduced oxygen availability during anaerobic treatment may contribute to the preservation of these flavonoids. The *in vitro* ACE inhibitory activities of all nine catechin components were significantly stronger than GABA, with galloylated catechins showing greater activity than non-galloylated catechins. The enhanced ACE inhibitory activity of purple-colored leaf tea after anaerobic treatment is likely due not only to the increase in GABA content but also to changes in catechins and other components. Purple-colored leaf tea products enriched with GABA might have considerable market potential to meet the specific demands of tea consumers seeking for healthy food with therapy for hypertension. Further *in vivo* studies are necessary to determine whether the GABA-rich purple-colored leaf tea products offer superior hypotensive efficacy compared to the conventional tea products.

CRediT authorship contribution statement

Gaozhong Yang: Writing – original draft, Software, Methodology, Investigation. **Yin Zhu:** Software, Methodology, Investigation. **Jiang Shi:** Software, Methodology, Investigation. **Qunhua Peng:** Resources, Investigation. **Zhi Lin:** Writing – review & editing, Project administration. **Haipeng Lv:** Writing – review & editing, Project administration, Methodology, Investigation.

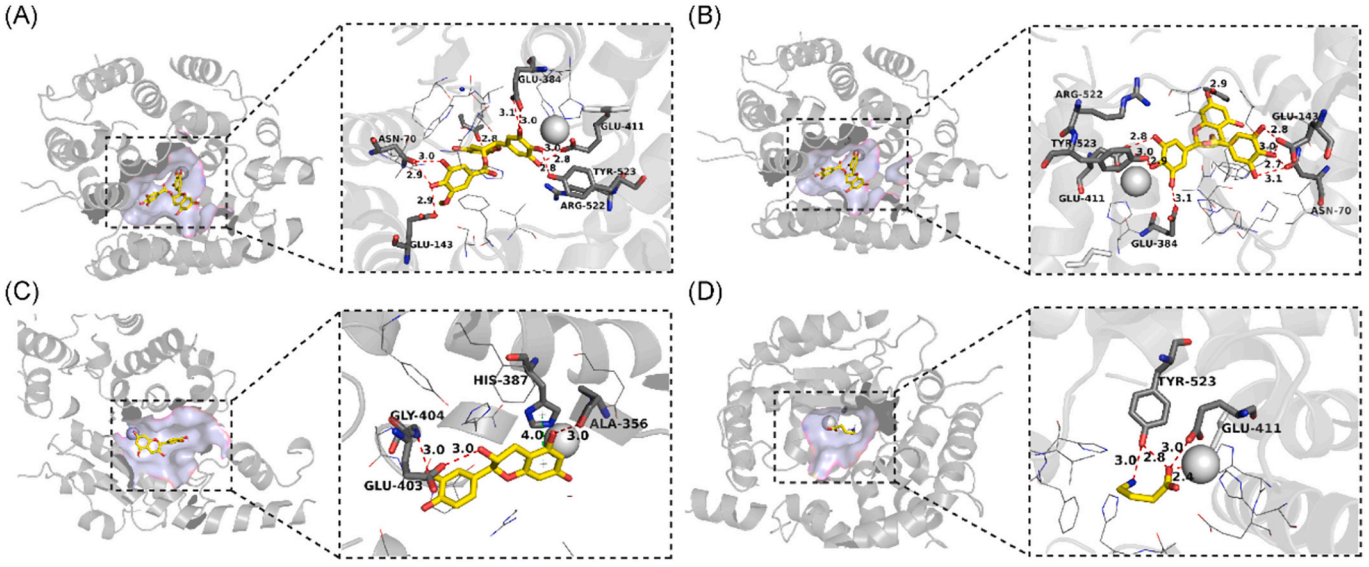

Fig. 4. Molecular docking interaction analysis of four representative compounds with ACE. (A) EGCG3"Me; (B) EGCG, (C) C; (D) GABA. The red dotted line represented the hydrogen bond, and the green dotted line represented the π-stacking. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101649.

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