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Effects of *Eurotium cristatum* Fermentation on Tartary Buckwheat Leaf Tea: Sensory Analysis, Volatile Compounds, Non-Volatile Profile and Antioxidant Activity

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Abstract: Background: *Eurotium cristatum* (*E. cristatum*) is the probiotic fungus in Fu-brick tea, with which fermentation brings a unique flavor and taste and health-promoting effects. Tartary buckwheat leaves are rich in functional active substances such as flavonoids and phenolic compounds, yet are not effectively utilized. Methods: Tartary buckwheat leaves were processed into raw green tea first and subsequently fermented with *E. cristatum* to develop a novel fermented leaf tea. The tea quality was evaluated by the aspects of the sensory scores by E-tongue, the volatile compounds by HS-SPME-GC-MS, the non-volatile profile by biochemical and UPLC-MS/MS methods and the antioxidant activity by the colorimetric assay. Results: Fermented leaf tea displayed a golden yellow color, a unique "flower" aroma and a dark-tea taste, with an improved sensory acceptability. Fermentation raised the content of volatile heterocyclic and aromatic compounds, alkenes and other aromatic components, which produced a unique floral flavor. The proportion of sour, bitter and astringency accounting non-volatile compounds such as phenolic acids and amino acids decreased, while the proportion of umami and sweet accounting substances such as responsible amino acids increased. Fermented leaf tea displayed a relative stronger total antioxidant activity against ABTS. Conclusion: *E. cristatum* fermentation exerted positive effects on Tartary buckwheat leaf tea quality.

Keywords: *Eurotium cristatum*; Tartary buckwheat; leaf tea; solid-state fermentation

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1. Introduction

Tartary buckwheat is a dicotyledonous annual grain crop of buckwheat in Polygonaceous, which originated in China and is widely distributed around the world [1]. As an edible and medicinal crop, it recently attracted considerable interest because of its high-quality proteins [2] and starches [3] and especially for the richness of bioactive ingredients, such as flavonoids and polyphenols [4,5]. Polyphenols and flavonoids are bioactive natural products, which display versatile health benefits, such as antioxidation, reducing weight, hypoglycemia, blood lipid regulation and the prevention and treatment of cardiovascular diseases [6,7]. At present, the processing of Tartary buckwheat-derived materials is limited to grains, which are commonly processed into flour to manufacture food products such as noodles and bread [5]. In fact, many organs of the plant contain high levels of bioactive components, including the seeds, stem, cotyledon, flowers and leaves [8]. The leaves display even higher bioactivities than the grains, which are also rich in flavonoids and polyphenols [8,9]. Nevertheless, the leaves are usually discarded as a by-product during processing [8,9], which is a waste of bioactive components and may cause pollution

problems. The application of Tartary buckwheat leaves as raw materials is extremely rare. *Aspergillus niger* was previously applied to conduct fermentation on Tartary buckwheat leaves [10]. The contents and activity of antioxidants, such as phenolic compounds, increased notably during the early stage of fermentation, indicating fermentation as a powerful method to process Tartary buckwheat leaves into functional food products [10].

E. cristatum is a beneficial fungus naturally formed in the "flowering process" of Fu-brick tea and is commonly referred to as "golden flower" fungus. It secretes polyphenol oxidase, pectinase, cellulase, protease and other extracellular enzymes, which catalyze the chemical transformation of phenols, proteins, sugars and other components in tea leaves [11]. The fermentation by *E. cristatum* endows the Fu-brick tea soup with a special red-brown color, fruit aroma and less astringency and bitterness taste [12]. Previous research has shown that the fermentation of autumn green tea [12], loose dark tea [13] and Pu'er tea [14] with *E. cristatum* greatly improved the tea quality. Moreover, water extracts of the fermented tea displayed significant anti-obesity and hypolipidemic functions [15] that are closely related to the effects of *E. cristatum* fermentation [16]. These results suggest *E. cristatum* fermentation as a promising processing measure to enhance the quality of leaf tea.

Tartary buckwheat leaves were processed into raw green tea by the traditional green tea processing procedure in our lab, yet the flavor was with high astringency, bitterness and low acceptability. Thus, exploring an appropriate leaf tea processing method is helpful to utilize the nutrition of Tartary buckwheat leaves effectively. This study systematically explored the processing procedure of fermented Tartary buckwheat leaf tea with *E. cristatum* and analyzed the impact of fermentation on the tea quality.

2. Materials and Methods

2.1. Sample Processing and Collection

Tartary buckwheat variety Chuanqiao No.1 was planted in the Jintang experimental site of Chengdu University. The 5th to 8th fresh and healthy leaves counting down from the top of the Tartary buckwheat plants were harvested between 8:00 a.m. and 9:30 a.m. from the blooming stage to the filling stage. The *E. cristatum* strain in this study was previously separated from Jingyang Fu-brick tea, identified by ITS sequencing and stored in the Key Laboratory of Coarse Cereal Processing in Chengdu University.

2.2. Processing of Tartary Buckwheat Raw Leaf Tea

Tartary buckwheat raw leaf tea was made referring to traditional green tea processing methods. Briefly, the collected buckwheat leaves were washed gently with water twice and drained. Then, the leaves were evenly spread in a clean and soft bamboo tray at the room temperature of 22–25 °C for 9 h, with the thickness of about 3–4 cm and no overlapping. The leaves were flipped every 1 h. Next, the leaves were cut into rectangles with the areas being about 1.2 mm × 1.2 mm. A frying pan was preheated for 3 min and used for the green fixing. Then, the leaves were put in and fixed at/for different temperatures/times and with different amounts by shaking, tossing, stewing and gently kneading. After cooling down, the fixed leaves were kneaded and twisted in a bamboo soft plaque, squeezing out the tea juice. The specific method was drawing circles and kneading the leaves by the palm and lightly pressing every two circles. This operation was repeated for 5 min, so that the leaves could shape and roll into strips. The kneading was completed when the rolled leaves reached 80%. The twisted leaves were then spread in a flat pan and baking firstly at 120 °C for 10 min to dissipate the moisture, fix the shape and enhance the aroma. Then, the leaves were re-baked at 90 °C for 1.5–2 h, until the tea stalk fold was fragile and easy to be broken off by the fingers. The resulting tea was Tartary buckwheat raw leaf tea.

2.3. Processing of Fermented Tartary Buckwheat Leaf Tea

The fermented Tartary buckwheat leaf tea was processed referring to the manufacturing method of Fu-brick tea. For rehydration, water was added to a certain amount of

Tartary buckwheat raw leaf tea in flasks. After thorough mixing, the flasks were sterilized at 120 °C for 20 min. *Eurotium cirstatum* was recovered on the PDA plate at 28 °C for 5–6 days. The spores were rinsed by sterilized water and adjusted to a concentration of ~1 × 10^6 spores/mL. A different volume of *E. cristatum* spores per 100 g of tea was inoculated into the Tartary buckwheat raw leaf tea mixture and fermented at 28 °C for at least 6 days. After fermentation, the tea was dried at 30 °C for 2 days in a tray until the water content was lower than 6%.

2.4. Sensory Evaluation

First, 3.0 g of tea was soaked in 150 mL of freshly boiled water for 4 min in a tea glass. The tea infusions were sensory-evaluated and scored by 10 well-trained panelists. According to the Chinese National Standard GB/T 23776-2018 and the physiological characteristics of Tartary buckwheat leaves, the sensory evaluation rules for the leaf tea were formulated. For the sensory analysis, color, aroma, taste and leaf shape, respectively, accounted for 25% of the tea overall quality [17].

2.5. Determination of Water Extracts and Content

The content of the water extracts in the tea was measured according to the Chinese National Standard GB 5009.3-2016 [18]. Each experiment was repeated 3 times.

2.6. Determination of Total Polyphenol Content

The tea polyphenol content was determined according to the Chinese National Standard GB/T 8313-2018 [19]. Briefly, 0.2 g of ground tea sample in a 10 mL centrifuge tube was extracted with 5 mL 70% methanol at 65 °C for 30 min by ultrasonication twice. After cooling down, the tube was centrifuged at a speed of 3500 rpm/min for 10 min. The supernatant was transferred to a new volumetric flask. The tea phenol extraction solution was combined to a constant volume of 10 mL and filtered by a 0.45 mm membrane. The extraction solution could be stored at 4 °C for up to 24 h and was diluted 100 times for the concentration measurement. The total phenolic content of the tea samples was determined by the Folin–Ciocalteu method with some modification [20]. Then, 0.5 mL of extract was mixed with 2.5 mL of 0.2 M Folin–Ciocalteu solution. Within 3–8 min, 2 mL of Na_2CO_3 solution (7.5%) was subsequently added and fixed to a constant 5 mL volume by water. The mixture reacted in 50 °C water for 5 min. After cooling down to room temperature (23 °C ± 2 °C), the absorbance was measured at 760 nm. The assay using the methanol instead of the tea extract was taken as the blank control. A standard curve was plotted using gallic acid as the standard. The polyphenol concentration was described as g gallic acid equivalent (GAE)/100 g of dry matter. Each experiment was repeated 3 times.

2.7. Determination of Total Flavonoid Content

The content of total flavonoid in the tea was analyzed according to the Chinese National Standard NY/T 1295 2007 [21] by the aluminum trichloride colorimetric method [10]. The extraction procedures were similar with the phenol extraction method. First, 1 mL of the properly diluted extract was reacted with 2 mL of aluminum trichloride solution (0.1 M) and 3 mL of potassium acetate solution (1 M) at room temperature for 30 min. The mixture was then fixed by methanol to 10 mL. After centrifugation at 4000 r/min for 10 min, the absorbance of the solution at 420 nm was measured, and a standard curve was plotted using rutin as the standard [21]. Each experiment was repeated 3 times.

2.8. Determination of Soluble Sugar Content

The content of the soluble sugar was determined by the reported phenol–sulfuric acid method [22]. The ground tea sample was extracted with boiling water for 45 min, and the absorbance of the solution was then measured at 490 nm. The anhydrous glucose was used as the standard to draw a standard curve and quantify the concentration of the soluble sugar. Each experiment was repeated 3 times.

2.9. Determination of Free Amino Acids Content

The content of the total amino acid was quantified according to the reported method [23] with some modifications. Briefly, the ground tea sample was extracted with hot boiling water for 45 min. Then, 1 mL of tea infusion was mixed with 0.5 mL of phosphate buffer solution (0.067 M, pH 8.0) and 0.5 mL of 2% ninhydrin solution containing 0.8 mg/mL of tin chloride and incubated for 15 min in a boiling water bath. After cooling down, the mixture was diluted with distilled water to 25 mL and laid aside for 10–15 min. The total free amino acids were determined by measuring the optical absorbance of the solution at 570 nm. Taking theanine as the standard, a standard curve was plotted to quantify the content of free amino acid in the tea. Each experiment was repeated 3 times.

2.10. Determination of the Content of Tea Infusions

The method used for the determination of the water extract content in the tea solution was based on Chinese National Standard GB/T 8305-2013 [24] with modifications [25]. Briefly, 2 g of ground tea sample was extracted in 300 mL of boiling water for 45 min. The tea infusion was then filtered by filter paper under reduced pressure. The tea residue was washed several times by the boiling water, then dried in a drying oven (120 °C ± 2 °C) with a cover for 1 h, followed by another round of drying after cooling down without a cover. Each experiment was repeated 3 times.

2.11. Determination of Tea Pigment Content

First, 3.0 g of ground tea sample were extracted by 125 mL of boiling water for 10 min. The hot solution was filtered and cooled down quickly to obtain the sample extract. Quantitative analysis of the tea pigments, namely theaflavins (TFs), thearubigins (TRs) and theabrownins (TBs), was carried out according to the previous method [26,27]. The absorbance of the solution was measured at 765 nm. The concentration of the tea pigments was described as mg gallic acid equivalents per one gram of tea (mg GAE/g). Each experiment was repeated 3 times.

2.12. Electronic Tongue Intelligent Sensory Analysis

First, 3.0 g of tea leaves were soaked in 150 mL of 80 °C water in a flask for 10 min and then filtered to obtain the tea sample. E-tongue (α-Astree; Alpha MOS Company, Toulouse, France) analysis was employed to characterize the taste of tea infusions. The procedure was repeated three times.

2.13. Analysis of Volatile Aroma Components

The volatile components of tea were detected by the HS-SPME-GC/MS method [28] with some modifications. HS-SPME extraction: The volatile compounds of the ground tea sample were extracted by HS-SPME using a 50/30-PDMS/DVB fiber (Supelco, Bellefonte, PA, USA). The SPME fiber was aged in the GC-MS injection inlet at 250 °C for 30 min prior to use. Then, 2 g of ground tea sample were extracted with 5 mL of boiling water, stirred and sealed rapidly in a 20 mL headspace vial by silicone septa (Sigma, Milwaukee, WI, USA). The PDMS/DVB extraction fiber was aged in the GC inlet at 250 °C for 30 min. After the sample bottle was preheated in the extraction device for 20 min, the adsorptive sample was extracted at 80 °C for 60 min. The extraction fiber head was then immediately inserted into the GC inlet for analysis.

2.14. Determination of Antioxidant Activity

Sample extraction: Accurately weigh 1.0 g of raw green tea and fermented tea sample. The boiling water was taken as the extraction solution. The extraction was carried out with a ratio of material to extraction solution at 1:20 (mg/mL) for 30 min, then the supernatant was collected. After repeating twice, the supernatant was combined, and the volume was fixed at 50 mL. The tea extraction solution with a concentration of 20 mg/mL was obtained

and stored at 4 °C. VC with the determined antioxidant activities was used as the positive control. Each of the following experiments was repeated 3 times.

2.15. Determination of DPPH Free Radical Clearance

The DPPH free radical scavenging activity was determined as previously reported with some modifications [29]. The DPPH solution (0.04 g/L) in ethanol was prepared daily before use. First, 0.2 mL of each test sample was mixed with 3.8 mL of DPPH (0.04 g/L) in ethanol and incubated for 20 min in darkness at room temperature. Then, the absorbance at 517 nm was measured.

2.16. Determination of ABTS Free Radical Clearance

The ABTS free radical scavenging capacity was evaluated as previously reported [30] with slight modifications. First, 7 mM of ABTS store solution was obtained by dissolving ABTS in deionized water. To produce the ABTS radical cation ($ABTS^+$), a 1:1 (v/v) ratio of ABTS solution (7 mM) and potassium persulfate solution (with a 2.45 mM final concentration) was mixed and incubated in darkness at room temperature for 12–16 h. To obtain the ABTS working solution, the $ABTS^+$ solution was diluted to an absorbance of 0.7 ($\pm$0.02) at 734 nm with deionized water before use. Then, 0.1 mL of test sample was reacted with 0.1 mL of 70% ethanol and 3.9 mL of ABTS working solution and incubated in the dark for 6 min. The absorbance at 734 nm was determined.

2.17. Reducing Power Assay

Determination of the reducing power of the tea sample was performed as described with some modifications [31]. The mixture contained 0.25 mL of test sample, 2.5 mL of potassium ferricyanide (1%, w/v) and 2.5 mL of phosphate buffer (0.2 M PBS, pH 6.6) and was incubated at 50 °C for 20 min in darkness for the reaction. After cooling down, 2.5 mL of trichloroacetic acid (10%, w/v) was added. The mixture was centrifuged at 3000 rpm for 10 min. Then, 1.5 mL supernatant solution was added with 1 mL of ferric chloride (0.1%, w/v) and 5 mL of distilled water for further reaction at 50 °C in darkness for 10 min. The absorbance at 700 nm was then measured.

2.18. UPLC Conditions

The extracts of the tea samples were analyzed by UPLC-ESI-MS/MS (UPLC, Exion LC™ AD, PerkinElmer, Shanghai, China). The Agilent SB-C18 column (1.8 µm, 2.1 mm × 100 mm) was used for UPLC analysis, with the mobile phase being composed of solvent A and B. Solvent A was pure water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. A gradient program was employed to measure the sample, which started with a component of 95% A and 5% B, changed linearly to 5% A and 95% B within 9 min and stayed that way for another 1 min. The condition was adjusted to 95% A and 5.0% B within 1.1 min and stayed the same for a further 2.9 min. The flow velocity and the column oven were set at 0.35 mL/min and 40 °C, respectively. A volume of 2 µL was taken for the injection. The effluent was further analyzed by the ESI-triple quadrupole-linear ion trap (QTRAP)-MS, alternatively.

2.19. ESI-Q TRAP-MS/MS Conditions

The operation parameters of the ESI source were as follows. The source temperature was 500 °C. The ion spray voltage was −4500 V for the negative ion mode and 5500 V for the positive ion mode. The ion source gas I (GSI), gas II (GSII) and curtain gas (CUR) were set at 50, 60 and 25 psi, respectively. The collision-activated dissociation (CAD) was high. QQ scanning was obtained as MRM experiments using collision gas (nitrogen) as the medium. Further optimization of the DP (declustering potential) and CE (collision energy) was carried out for MRM transitions. A specific set of MRM transitions for each period based on the metabolites eluted within this period were monitored.

2.20. HS-SPME-GC/MS Conditions

The volatile aroma components of the tea samples were analyzed using the HS-SPME-GC-MS method. Sample pretreatment: 2 g of crushed tea sample placed in a 20 mL headspace injection bottle mixed thoroughly with 5 mL of boiling water and then sealed quickly. Solid-phase microextraction: The PDMS/DVB fibers were aged at the GC injection port at 250 °C for 30 min. The sample bottle in the extraction device was preheated for 20 min. The adsorption extraction was carried out for 60 min at 80 °C. Then, the extraction fiber head was inserted immediately into the injection port for testing. GC conditions: The carrier gas was helium. The flow rate was set at 1 mL/min. The chromatographic column was HP-5MS (30.0 m × 250 μm, 0.25 μm). The temperature of the injection port was 50 °C initially and raised to 230 °C at a rate of 4 °C/min with no diversion. The temperature of the GC and MS transmission line was 280 °C. MS condition: The ion source temperature was 230 °C. The ionization method was EI. The electron energy was 70 eV.

2.21. Statistical Analysis

Experimental data were analyzed statistically through Excel and SPSS Statistics 25 software, and Origin 2023 software was used for plotting.

3. Results and Discussion

3.1. Processing of Fermented Tartary Buckwheat Leaf Tea

3.1.1. Tartary Buckwheat Leaves at Different Growth Stages

Tartary buckwheat leaves picked at different growth stages (the cotyledon stage, the seedling stage, the flowering stage, the filling stage and the maturation stage) were determined for their quality. As displayed in Table 1, the number, morphology and state of buckwheat leaves varied along with the plant growth. The number of leaves grew quickly from 2 in the cotyledon stage to about 50 in the filling stage, which accompanied the multiplying of the leaf area to about 12 cm × 10 cm. The leaves were soft in the seedling stage, strong and firm in the flowering and filling stages and became aged and dotted in the maturation stage. Flavonoids account for one of the key components in Tartary buckwheat leaves. As in Table 1, the content of the total flavonoids in the leaves gradually rose with the plant growth. The highest content was 47.45 ± 0.82 mg/g in the filling stage, yet the content deceased in the maturation stage to about 36.66 ± 1.11 mg/g. With the aim of making high-valued buckwheat tea, the leaves at the flowering stage were chosen for the following raw tea processing.

Table 1. Morphological changes and the flavonoid content of Tartary buckwheat leaves at different growth stages.

Growth Stages	Leaf Number	Leaf Area	Leaf State	Content of Total Flavonoids
cotyledon	2	quite small	-	40.78 ± 0.67 mg/g
seedling	~10	6 cm × 4 cm	soft	42.21 ± 0.72 mg/g
flowering	~50	10 cm × 10 cm	strong and firm	46.41 ± 0.56 mg/g
filling	~50	12 cm × 10 cm	strong and firm	47.45 ± 0.82 mg/g
maturation	<50	12 cm × 10 cm	aged and dotted	36.66 ± 1.11 mg/g

Note: Each experiment was repeated 3 times.

3.1.2. Optimization of Tartary Buckwheat Raw Leaf Tea Process

Spreading and drying of the fresh leaves are key steps in traditional raw green tea production [32], which have several advantages. First, by dissipating some of the moisture, the fresh leaves become softer and tougher and thus easier to be rolled into strips. Second, spreading and drying can promote substance transformation, such as water, tea polyphenols, protein and starch in fresh leaves, which, in turn, form specific tea characteristics. Third, it can decrease the grassy smell of leaves. Here, Tartary buckwheat leaf samples spread for 3, 6, 9, 12, and 15 h, respectively, were collected and measured for the content

of water, polyphenol, total flavonoid, free amino acid, soluble sugar and water extract. The results are displayed in Figure S1A. Along with the spreading and drying, a continuous decrease was seen in the content of water from 79.37 ± 0.44% to 66.36 ± 0.44% and polyphenol from 4.18 ± 0.16% to 2.69 ± 0.05%. A minor decline was seen for the content of total flavonoid from 47.80 ± 2.44 mg/g to 42.96 ± 3.07 mg/g. Meanwhile, a significant increase was seen for the soluble saccharide content from 4.71 ± 0.09% to 7.52 ± 0.31%. A mild increase was seen for the content of free amino acid and water extract during spreading and cooling. After further processing, the superior tea quality was achieved at 9 h of spreading and drying by sensory evaluation (Figure S1A).

Green fixation utilizes high temperatures to destroy the inner leaf enzyme activity, such as polyphenol oxidase and peroxidase (POD). In that way, the tea forms the inherent quality during processing through the non-enzymatic effects, such as through the Maillard reaction or caramelization reaction. High temperatures can soften the tea tissue and evaporate water, which makes it easier to knead and twist them into thin strands. Moreover, high temperatures enable some low-boiling grassy odors to be volatilized, leaving the pure aroma of tea leaves. Here, the effects of leaf amount, temperature and time of fixation on the peroxidase activity and the tea sensory evaluation were examined. The results are shown in Figure S1B. The leaf POD activity was totally destroyed when the temperature was higher than 180 °C and the time was longer than 4 min. Based on the orthogonal test, the highest sensory score was achieved by fixing at 180 °C for 4 min with 1.5 g/cm^2 of leaf amount (Figure S1B).

3.1.3. Processing of Fermented Tartary Buckwheat Leaf Tea by *E. cristatum*

The "flowering" process is the crucial step in the manufacturing process of Fu-brick tea [11]. By controlling the temperature and humidity, a large number of *E. cristatum* proliferate vigorously and form spots on the tea leaves, namely "golden flowers". The metabolic activities of *E. cristatum*, including absorbing and utilizing nutrients such as polyphenols and carbohydrates, and the secreted extracellular enzymes will further change the substances in the tea, forming a unique flavor of dark tea different from other types of tea [11]. To explore the effects of *E. cristatum* on Tartary buckwheat leaves, *E. cristatum* was artificially inoculated to the raw Tartary Buckwheat leaf tea, and the flowering conditions were strictly investigated. The effects of the water content, inoculation amount, temperature and time of fermentation on the quality of fermented tea were examined. The content of water infusion and total flavonoid and the sensory score of the tea are measured.

An appropriate temperature is conducive to the moist heat effect, the enzymatic activity and microbial metabolism, which can prevent the growth of harmful bacteria and, finally, improve the overall quality of tea. As shown in Figure 1A, with the increase in temperature from 20 °C to 36 °C, the content of water infusion in fermented tea water increased mildly, while the total flavonoid content decreased significantly. The results indicated that high temperatures promote the enzyme activity of *E. cristatum* and the substance conversion rate. The highest sensory evaluation score was achieved at 28 °C, which is the most suitable temperature for *E. cristatum* growth reported previously [12]. Therefore, the fermentation temperature was set at 28 °C for the subsequent experiments.

A larger amount of inoculation starter can shorten the fermentation time and advance the fermentation cycle effectively. However, with excessive microbes, the nutrients in tea cannot meet the needs of microbial growth. The free substances obtained in the early stage of fermentation will be utilized by the microbes, thus affecting the nutrition and quality of tea [11]. The effects of different amounts of inoculation starter ranging from 1×10^6 to 11×10^6 spores per 100 g tea were explored in the Tartary buckwheat tea fermentation process. As displayed in Figure 1B, with the increase of the inoculation amount, both the content of the water infusion and total flavonoid showed a descending tendency, indicating the consuming of the tea nutrients. Meanwhile, with a larger amount of inoculation starter and limited culture medium, *E. cristatum* enters the aging period much earlier, which leads to the faster brown color of the tea surface and a decrease in sensory scores (Figure 1B).

The superior sensory scores were achieved at about 5×10^6, 7×10^6 and 9×10^6 spores of inoculation starter. Thus, 5×10^6 spores of inoculation starter were adopted for the following optimization.

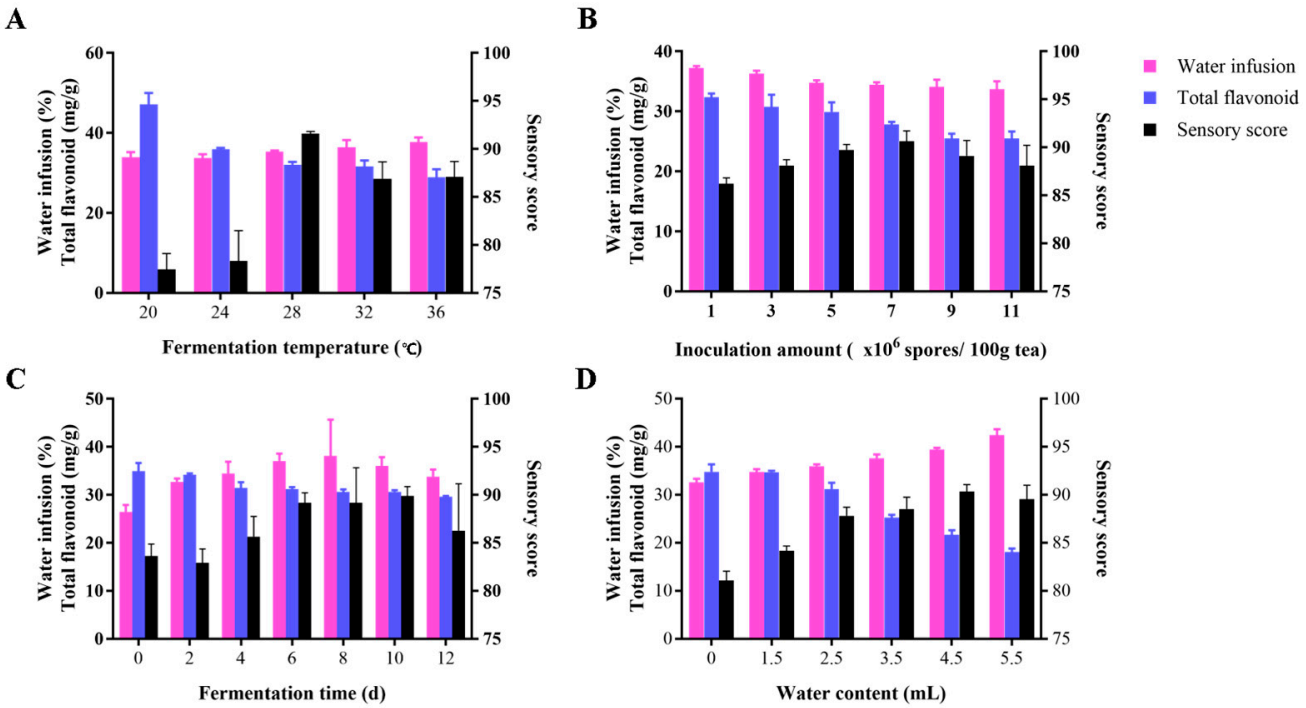

Figure 1. Effects of fermentation conditions on the quality of fermented buckwheat leaf tea. (**A**) Fermentation temperature. (**B**) Inoculation amount. (**C**) Fermentation time. (**D**) Water content. Each experiment was repeated 3 times.

Short-time incomplete fermentation could not achieve high-quality tea, while long-time fermentation can lead to the reuse of tea nutrients and the production of adverse products. As shown in Figure 1C, with the extension of the fermentation time, the flavonoid content decreased gradually, while the water infusion content increased continuously before 8 days and decreased after. The superior sensory score was obtained from the tea fermented for 8 or 10 days (Figure 1C).

Fu-brick tea stores an extremely low water content. The water and humidity of tea leaves are particularly important for the "flowering" process. As in Figure 1D, with the water amount being added from 0 mL/100 g to 5.5 mL/100 g to the fermentation system, the content of the water infusion increased gradually from 32.59% to 42.48%, while the total flavonoid content decreased significantly from 34.82% to 18.08%. An appropriate water content promotes vigorous microbial growth and metabolism and the secretion of extracellular enzymes, which subsequently increases the content of water-soluble carbohydrates and the oxidative decomposition of bitter and astringent substances in the tea and, finally, improves the tea quality. Yet, excessive water would alter the growth characteristics of *E. cristatum*, affecting the appearance and taste of tea. As shown in Figure 1D, the sensory scores of fermented tea are superior when the added water amounts are 2.5 mL/100 g, 3.5 mL/100 g and 4.5 mL/100 g. Meanwhile, the "golden flowers" are more stable with 2.5 mL/100 g of water. Therefore, the water content was set at 2.5 mL/100 g for the following fermentation system.

After orthogonal optimization of the fermentation conditions based on the sensory quality and the nutrients, the superior fermented Tartary buckwheat leaf tea was obtained on Day 8, with 4.5 mL of water addition and 2.5 mL of inoculation amount per 10 g raw green tea. The final fermented leaf tea displayed a golden yellow color (Figure 2) and a unique "flower" aroma.

3.2. Quality Evaluation of Fermented Tartary Buckwheat Leaf Tea

3.2.1. Sensory Characteristics

The taste, aroma and color of tea are fundamental factors influencing the tea quality. To evaluate the fermented leaf tea quality, 10 random volunteers were recruited to taste and score the tea by five aspects, namely blossom, appearance, color, aroma and taste. As shown in Figure 2, the unfermented leaf tea represented a darker appearance, while the fermented leaf tea displayed a golden yellow color (Figure 2A,B). The color of the tea infusions changed from yellow-green to reddish-brown after fermentation (Figure 2C,D). The color shift indicated the tea pigment formation during fermentation. The fermented leaf tea displayed a unique "flower" aroma and a thick, heavy and mellow taste, which is similar to other types of dark tea. These results suggested that *E. cristatum* fermentation significantly changed the characteristics of Tartary buckwheat leaf tea, including the color, aroma and taste. The average sensory score of the fermented leaf tea was 90.5, significantly higher than that of unfermented leaf tea (79.3). In summary, the overall tea quality was significantly improved by *E. cristatum* fermentation.

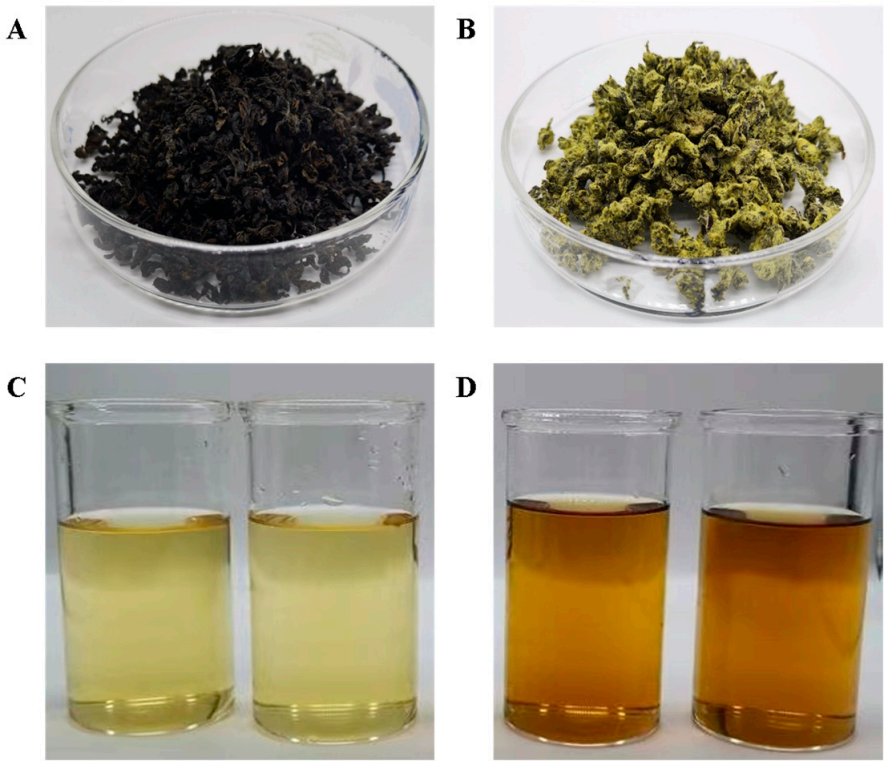

Figure 2. The Tartary buckwheat leaf tea and soup. (**A**) Raw leaf tea (T). (**B**) Fermented leaf tea (FT). (**C**) Raw leaf tea soup. (**D**) Fermented leaf tea soup.

Electronic tongue can detect tastes that humans cannot percept [33], thus could better distinguish the sweet, sour, fresh and salty flavors in tea soup samples. As shown in Figure 3, after fermentation, the bitter and sour taste of the tea soup decreased, while the umami and aftertaste of bitterness increased. Fermentation enhanced the tea sweet taste mildly (Figure 3). Taste perception and the substances in tea have a complex connection, which is often the results of chemical components acting in concert. In dark tea, the decrease in bitter taste is related to the decrease in tea polyphenols, flavonoids and other substances, while the increase in umami and sweet taste is mainly related to the increase in soluble substances in the tea soup, including amino acids and soluble saccharides [34]. Tea pigments also contribute to the umami taste of tea soup [34].

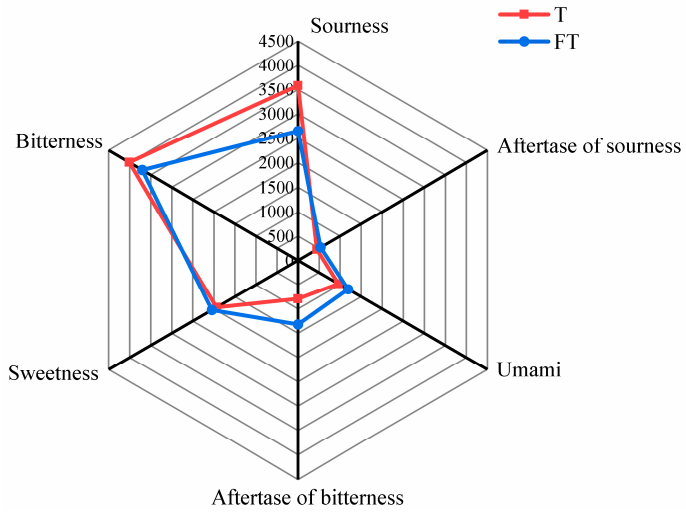

Figure 3. E-tongue analysis of the taste characteristics of Tartary buckwheat leaf tea fermented with *E. cristatum* or not. T: raw Tartary buckwheat leaf tea. FT: fermented Tartary buckwheat leaf tea.

3.2.2. Analysis of Volatile Components

The volatile compounds in the tea samples were quantitatively analyzed by HS-SPME-GC-MS. A total of 58 and 42 volatile compounds were identified, respectively, in Tartary buckwheat leaf tea before and after fermentation, which were constituted with heterocyclic, alkane, alcohol, alkene, aldehydes, esters, ketones, acids, etc. Methylbenzene compounds were reported to be the most abundant compounds in black tea and impart fungal flavor and aging aroma to tea leaves [35,36]. As shown in Figure 4, the heterocyclics were the dominant compounds in the fermented tea, accounting for 71% of the total volatile compounds, which was 171.7% higher than the content in the unfermented tea. Yet, the content of alkane (50.95%) was the highest in the volatile components of unfermented tea. Alkenes are important contributors to "flowers" and fruit aromas in tea [37]. In the fermented tea, five alkenes accounting for 4.17% of the identified volatile compounds were detected, which was significantly higher than the content in the unfermented tea (0.7%). As in Figure 4, apart from heterocyclics and alkenes, a significant increase was also seen in ketones (from 0.78% to 1.38%) and acids (from 0.19% to 0.64%). Meanwhile, after fermentation, the content of alkanes (from 50.95% to 13.6%), alcohols (from 10.3% to 7.98%), aldehydes (from 3.68% to 0.79%) and esters (from 4.28% to 0.78%) decreased significantly, respectively.

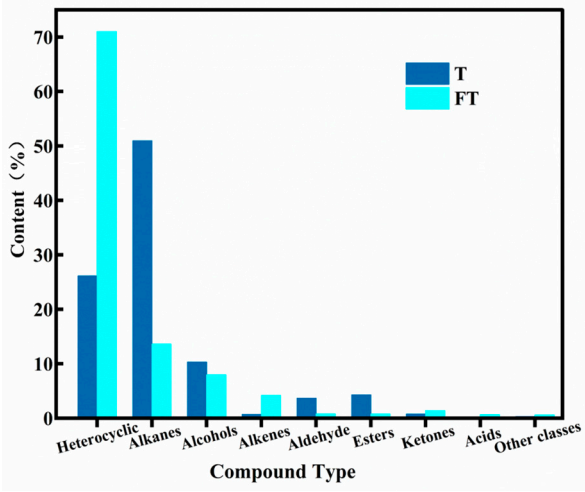

Figure 4. Relative contents of different types of volatile aroma components. T: raw Tartary buckwheat leaf tea. FT: fermented Tartary buckwheat leaf tea.

3.2.3. Analysis of Non-Volatile Components

1. Main Tea Chemical Composition

The non-volatile composition of the main tea components, including moisture, tea polyphenols, total flavonoids, soluble saccharides, water infusion and tea pigments, was analyzed in the leaf tea before or after fermentation. As shown in Figure 5A, no significant variation was seen in the content of the total flavonoids and moisture, while the content of the soluble saccharides and theaflavin displayed a significant decrease after fermentation. The content of the water infusions increased from 26.35% to 35.35%, as the content of the tea polyphenols was downregulated significantly from 2.53% to 1.6% due to *E. cristatum* fermentation. Tea pigments, consisting of theaflavins, thearubigins and theabrownins, are directly related to the color of tea [38]. Thearubigins showed a red-brown color, as theabrownins are dark brown [38]. In fermented Tartary buckwheat leaf tea, the content of both thearubigins (from 1.31% to 4.23%) and theabrownins (from 1.80% to 3.47%) increased markedly. These results were consistent with other black tea [39,40]. Saccharides can be the most important carbon source for microbial growth [32,41], contributing to the tea taste, together with the water infusions and tea pigments. The variation in tea pigment contents may illustrate the color change of the fermented tea. Moreover, theabrownins had a variety of functional activities, such as lowering blood fat [42] and blood sugar [43] and antioxidation [44]. A higher amount of theabrownins indicates potential higher bioactivities of the fermented leaf tea.

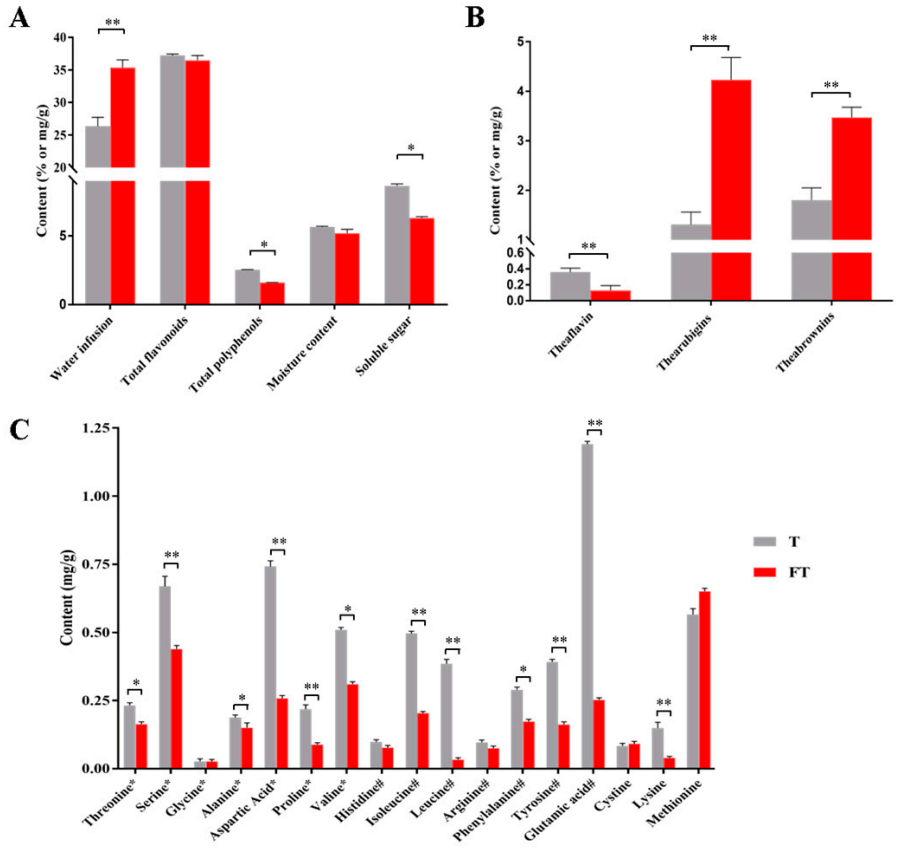

Figure 5. Changes in the contents of major tea substances of Tartary buckwheat leaf tea before (T) and after *E. cristatum* fermentation (FT). (**A**) Water infusion, total flavonoids, total polyphenols, moisture contents and soluble sugars. (**B**) Tea pigments. (**C**) Amino acids. T: raw Tartary buckwheat leaf tea. FT: fermented Tartary buckwheat leaf tea. Star-marked (*) groups represent umami and sweet amino acids. Octothorpe-marked (#) groups represent the sour and bitter amino acids. Each experiment was repeated 3 times. Statistical significance was determined by Students t-test (* p value < 0.05, ** p value < 0.01).

2. Free amino acid

Tea amino acids are important contributors to the tea taste and precursors of the tea aroma. Their composition, content, degradation and transformation products directly affect the quality of tea [45]. A sharp decrease was seen in the content of the total amino acids after fermentation, from 6.33 mg/g to 3.19 mg/g (Figure 5C). Briefly, the amino acids are commonly divided into three groups: fresh sweet amino acids, sour bitter amino acids and tasteless amino acids. He subsequent analysis showed that the total content ratio of sour and bitter amino acids (His, Leu, Ile, Asp, Phe, Tyr and Glu) decreased remarkably from 46.55% to 30.5% after fermentation, while the total content ratio of sweet amino acids (Thr, Ser, Gly, Ala, Asn, Pro and Val) increased from 40.67% to 44.9%. The content ratio of tasteless amino acids increased from 12.62% to 24.49%. The degradation and release of free amino acids occurred simultaneously during the fermentation of black tea [37]. On the one hand, peptides and proteins are hydrolyzed into free amino acids by microorganisms [22,46]. On the other hand, some free amino ends are converted into aromatic compounds through the Maillard reaction. The variation in amino acids effectively improved the umami taste and reduced the sour and bitter tastes of fermented leaf tea.

3. The metabolic change after *E. cristatum* fermentation

To see the metabolic changes of buckwheat leaf tea before and after *E. cristatum* fermentation in more detail, raw Tartary buckwheat leaf tea and fermented Tartary buckwheat leaf tea were subjected to UPLC-MS/MS analysis. In total, 13 types of compounds were identified, including flavonoids, phenolic acids, amino acid and derivatives, nucleotides and derivatives, lignans and coumarins, organic acids, terpenoids, alkaloids, steroids, lipids, quinones, tannins and others.

As for the amino acids and derivatives, the metabolic dynamic of free amino acids is consistent with the upper biochemical results in which the sour or bitter amino acids (His, Leu, Asp, Phe, Tyr and Glu) decreased and the sweet-tasting Gly increased after fermentation (Figure 6A). Soluble saccharides are also sweet-responsible substances in tea [47]. Here, after fermentation, the level of some high-sweetness saccharides increased remarkably (Figure 6A), such as D-glucose ($\log_2$FC = 2.49), D-mannitol ($\log_2$FC = 4.70), D-sorbitol ($\log_2$FC = 4.98) and DL-xylose ($\log_2$FC = 1.98). As rutin was merely detected in the buckwheat leaf tea samples before or after fermentation, the increase in rutinose may partially be from the degradation of rutin during tea fixing with high temperatures [48]. Besides amino acids, sour-related ingredients mainly contain organic acids and free phenolic acids (Figure 6A). The level of main sour-related organic acids in the black tea [49], including malic acid ($\log_2$FC = −1.22), succinic acid ($\log_2$FC = −1.3) and citric acid ($\log_2$FC = −1.27), declined mildly in the fermented tea, partially explaining the lowering of the sour taste in the fermented tea.

Flavonoids are critical phenolic components in Tartary buckwheat with strong health benefits and are also important taste and coloring substances in tea infusions [45]. Flavones, flavonols and flavanols contribute largely to the bitterness and astringency of tea infusions [11]. Here, all 18 differential flavanols were significantly downregulated after fermentation (Figure 6C), including catechin ($\log_2$FC = −3.66), epicatechin ($\log_2$FC = −3.5), gallocatechin 3-O-gallate ($\log_2$FC = −2.77), catechin-catechin-catechin (merely detected after fermentation), epicatechin glucoside ($\log_2$FC = −4.58), epigallocatechin-3-O-gallate ($\log_2$FC = −3.57), catechin gallate ($\log_2$FC = −8.68) and epicatechin gallate ($\log_2$FC = −8.72). Additionally, 27 out of 43 flavonols were downregulated remarkably, such as quercetin ($\log_2$FC = −1.37), dihydroquercetin ($\log_2$FC = −3.38), kaempferol-3,7-O-dirhamnoside ($\log_2$FC = −4.70), eriodictyol-8-C-glucoside ($\log_2$FC = −2.54), kaempferol ($\log_2$FC = −2.40), dihydrokaempferol ($\log_2$FC = −3.68), etc. Proanthocyanidin is one type of dimeric or polymeric compound with catechins or epicatechins as precursors, linking through C4–C8 or C4–C6 bonds of flavane-3-ol [50], which also represents bitterness and astringency taste. Here, after fermentation, two proanthocyanidin compounds identified were enormously downregulated (galloylprocyanidin B4, $\log_2$FC = −6.26 and procyanidin B2, $\log_2$FC = −6.86).

The degradation or conversion of flavonols, flavones and proanthocyanidin by *E. cristatum* fermentation [51] could partially explain the bitterness and astringency decline of the fermented tea and the special taste formation of fermented leaf tea.

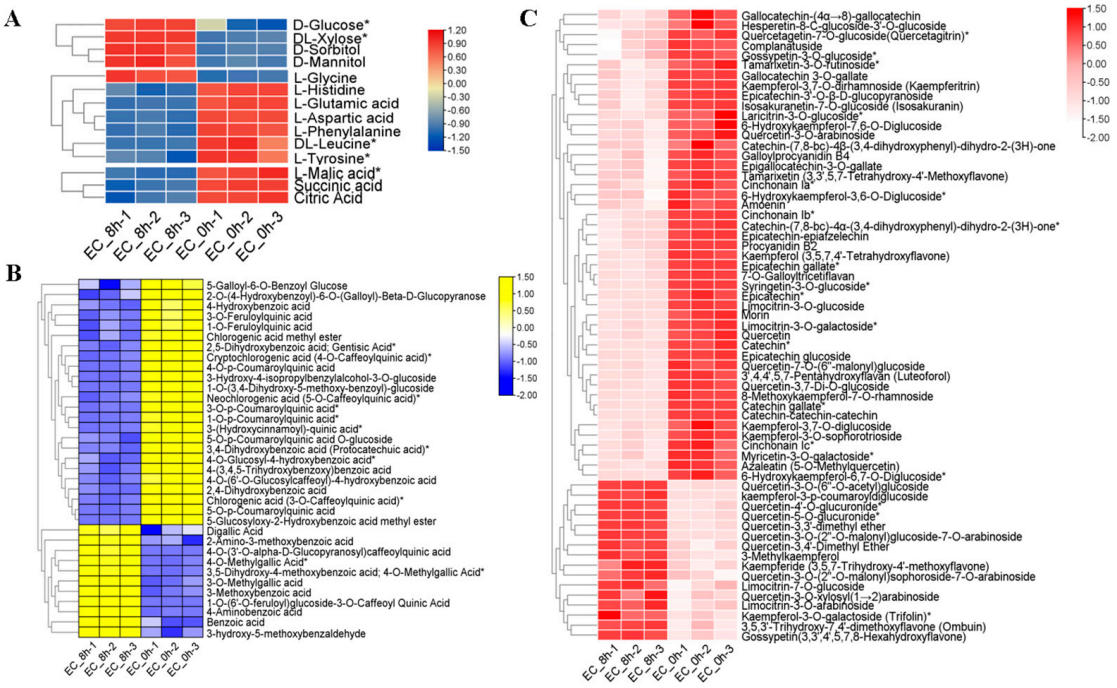

Figure 6. Hierarchical cluster analysis of the differential levels of chemical compounds in the tea samples. (**A**) Part of the taste-related free amino acids, saccharides and organic acids. (**B**) Part of the phenolic acids. (**C**) Part of flavonoids and proanthocyanins. Star-marked (*) groups indicate possible isomers exist.

Phenolic acids are another type of critical health-promoting components in Tartary buckwheat. In addition, as essential substances to synthesize flavonols and catechins, phenolic acids are thus also important taste- and color-responsible compounds in the tea. Previous research has indicated that most phenolic acids greatly declined during fermentation by *E. cristatum* [52]. Here, 71 out of 125 differential phenolic acids decreased after fermentation, which is in accordance with the change in total polyphenols determined by the colorimetric method (Figure 6B). Astringency-related phenolic acids mainly comprise benzoic acid- and hydroxycinnamic acid-type compounds and their derivatives, such as gallic acid, quinic acid, chlorogenic acid, caffeioyl quinic acid substances and coumaroyl quinic acid substances. With the decline in tea bitterness and rise in bitter aftertaste by E-tongue analysis after fermentation, the astringency of fermented tea was also attenuated (tasted by sensory evaluation). Coordinately, quinic acid ($\log_2$FC = −1.7), chlorogenic acid ($\log_2$FC = −6.33), cryptochlorogenic acid ($\log_2$FC = −4.93), neochlorogenic acid ($\log_2$FC = −5.23), chlorogenic acid methyl ester ($\log_2$FC = −2.92), 4-O-p-coumaroylquinic acid ($\log_2$FC = −3.74), 5-O-p-coumaroylquinic acid ($\log_2$FC = −2.76), 1-O-p-coumaroylquinic acid ($\log_2$FC = −3.11), 3-(hydroxycinnamoyl)-quinic acid ($\log_2$FC = −3.11), 3-O-p-coumaroylquinic acid ($\log_2$FC = −3.40), 5-O-p-coumaroylquinic acid O-glucoside ($\log_2$FC = −5.73) and 3-O-feruloylquinic acid ($\log_2$FC = −5.78) were found to be markedly reduced by *E. cristatum* fermentation (Figure 6B). 4-O-p-coumaroylquinic acid was identified as the astringent compound [53]. Whether the similarly downregulated coumaroylquinic acid analogs 5-, 3- and 1-O-p-coumaroylquinic acids are also astringent compounds needs to be further investigated. Yet, some of the gallic acid derivatives: digallic acid, 3-O-methylgallic acid and 4-O-methylgallic acid increased substantially (Figure 6B), indicating active methylase activity during *E. cristatum* fermentation.

Although taste substances have their own specific taste properties, they also have synergistic, antagonistic and other interactions, which lead to taste multiplication and other situations [54]. The interaction between flavoring substances is complex and influenced by various factors such as substance concentration, flavor intensity and the threshold of flavor substances [54]. *E. cristatum* could secrete extracellular enzymes, such as polyphenol oxidase, pectinase, cellulase and protease [11–14], which catalyze the chemical transformation of phenols, proteins, sugars and other components in tea leaves. The upper differential taste-accounting metabolites and their interactions together form the special taste and color of fermented Tartary buckwheat leaf tea.

3.2.4. Analysis of the Antioxidant Activity

The scavenging assays of DPPH, ABTS radicals and the ferric ion reducing power were used to evaluate the antioxidant activity of tea extract. As shown in Figure 7, tea extract displayed a dose-responsive antioxidant ability consistent with VC. For the DPPH radical-clearing rate and the total antioxidant capability, fermented Tartary buckwheat leaf tea showed rather similar levels of antioxidant activities with unfermented leaf tea (Figure 7A,C). As shown in Figure 7B, the ability of fermented tea to scavenge ABTS free radicals (IC_{50} = 1.082 mg/mL) is significantly stronger than that of unfermented tea, with the concentrations being above 0.6 mg/mL. ABTS can reflect the electron and proton transfer abilities of antioxidants [55]. The antioxidant active substances in tea mainly include tea polyphenols and other phenolic compounds [56]. These substances have different mechanisms of action on various free radicals and will show different trends in scavenging different free radicals. These results indicated that the total antioxidant activity of Tartary buckwheat leaf tea was not significantly downregulated by *E. cristatum* fermentation, while the antioxidant activity against ABTS was enhanced. As flavonoids and phenolic acids were important and nonnegligible antioxidants in Tartary buckwheat, the contents of some of the flavonoids and phenolic acids were upregulated remarkably after fermentation (Figure 6C), such as 3-methylkaempferol (log_2FC = 4.95), 3-O-methylgallic acid (log_2FC = 4.81), digallic acid (log_2FC = 2.18), etc. This result may partially explain the increased antioxidant activity against ABTS.

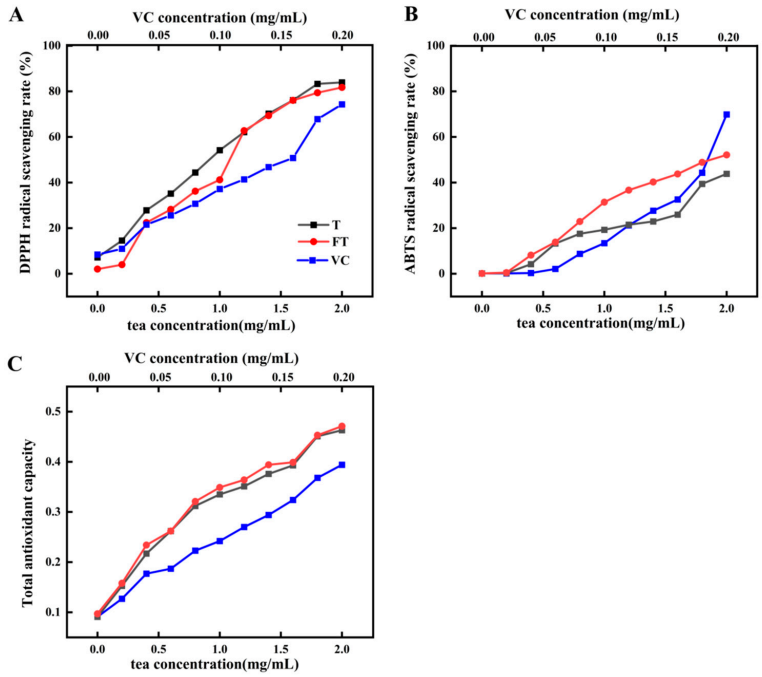

Figure 7. The antioxidant activity of the raw leaf tea and fermented leaf tea. (**A**) DPPH radical scavenging rate. (**B**) ABTS radical scavenging rate. (**C**) Total antioxidant activity. Each experiment was repeated 3 times.

4. Conclusions

In this study, the sensory quality and volatile and non-volatile components of Tartary buckwheat leaf tea fermented by *E. cristatum* were analyzed. After fermentation, fermented tea was covered with golden flowers, with the soup displaying a reddish-brown and bright color and a unique "flowery" and pleasant smell. The sensory quality of fermented tea was effectively improved, with the bitter and astringent taste reduced remarkably and the umami and sweet taste increased. The volatile compounds analysis showed that heterocyclic aromatic compounds and alkenes may be the main contributors to the special "mushroom flower" flavor of fermented leaf tea. The major non-volatile compounds analysis by the colorimetric method indicated that no significant variation was seen in the content of the total flavonoids and the moisture, while the content of the water infusions and tea pigments increased significantly after fermentation. UPLC-MS/MS analysis represented a significant differential regulation of part of the amino acids, flavonoids, phenolic acids and organic acids after fermentation. The antioxidant ability analysis indicated that fermented Tartary buckwheat leaf tea has a relatively high antioxidant activity. In conclusion, Tartary buckwheat leaves can be utilized to produce high-quality dark tea after fermentation. Fermentation with *E. cristatum* exerts a positive effect on improving the leaf tea sensory and nutritional quality. The fermented Tartary buckwheat leaf tea displays similar characteristics to those of dark teas, such as Fu-brick tea.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation10070369/s1: Figure S1: Effects of spreading and drying on the quality of Tartary buckwheat leaves.

Author Contributions: Conceptualization, L.J. and D.X.; methodology, L.J., L.W., X.H., H.Z., X.W., G.M. and D.X.; software, L.J., H.Z. and Y.T.; validation, X.H. and L.W.; formal analysis, L.J., X.H., Y.T., L.W. and C.L.; investigation, L.J. and D.X.; resources, Y.W. and D.X.; data curation, X.Z.; writing—original draft preparation, X.H., L.J., L.W. and X.Z.; writing—review and editing, L.J., X.H., D.X. and Y.T.; visualization, L.J. and C.L.; supervision, D.X.; project administration, L.J. and D.X.; funding acquisition, L.J., Y.T. and D.X. All authors have read and agreed to the published version of the manuscript.

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