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Change in Tea Polyphenol and Purine Alkaloid Composition during Solid-State Fungal Fermentation of Postfermented Tea

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ABSTRACT: The aim of this study was to evaluate tea polyphenol and purine alkaloid contents of pu-erh tea (*Camellia assamica*) in a fermentation solid system with *Aspergillus niger* and *Aspergillus fumigatu*. In addition, the objective was to find the major intermediate product during fermentation by HPLC−MS^n analysis. The results showed the change of catechin, ester-catechins and gallic acid by quantitative analysis. In the early stages, the contents of ester-catechins were lightly increased. Then, ester-catechins were gradually degraded to produce catechins and gallic acid. Furthermore, a major metabolic intermediate compound of catechins was observed and elucidated by HPLC−DAD−MS^n analysis. This study provided a reliable dynamic data description and metabolic pathway of tea polyphenols for postfermented pu-erh tea.

KEYWORDS: *Camellia assamica (Mast.) Chang, Aspergillus niger, Aspergillus fumigatu, fermentation, polyphenols*

INTRODUCTION

Camellia assamica is an important plant source for Chinese dark tea and black tea. Its leaves contain high content of ester-catechins, which were considered to be critical compounds of tea for antitumor and antioxidant properties.[1] Recently, many studies have concerned postfermented tea made of *Camellia assamica* such as pu-erh tea, which was the most reported Chinese dark tea with respect to chemical constituents, biological activities and fermentation technology.[2−4]

Ripened pu-erh tea is a traditional beverage in southwestern China. It is manufactured by piling crude pu-erh tea under high temperature and humidity, the process of which is called postfermentation. During this process, many regionally dominant fungi are involved in the enzymatic reactions of major compounds of tea such as catechins, gallic acid and caffeine. As the famous full-fermented tea, black tea is always believed to be fermented by the polyphenol oxidase.

Aspergillus species have been found to be the prevailing strains in the postfermentation process.[5] Furthermore, the metabolic profiling study also revealed that the process of postfermentation changed the chemical contents of tea when aged for 1−10 years.[3] As a result, postfermentation markedly decreased the level of catechins such as EGCG, ECG and EC compared with unfermented tea.[6] In the previous study, the comparison analysis of ripened pu-erh tea and aged pu-erh also indicated that postfermentation significantly decreased tea polyphenols of ripened pu-erh tea, while the aged pu-erh stored for 5 years showed nearly the same polyphenol contents as green tea.

Solid-state fermentation (SSF) has become the principal technology for rapidly producing ripened pu-erh tea. Under high humidity and temperature condition, artificial strains are introduced into piled raw tea for accelerating the post-fermentation process. After SSF, ripened pu-erh tea showed a similar flavor as aged pu-erh tea stored for a long time. Researchers found some new compounds formed in the postfermentation process of pu-erh tea,[7] but a comprehensive analysis on the metabolic pathway of major tea polyphenols was less addressed and explained simultaneously.

The object of this study was to select fungal strains to ripen pu-erh tea leaves under SSF conditions, aiming to find how catechins, ester-catechins and other major compounds change using HPLC−DAD−MS^n.

MATERIALS AND METHODS

Raw Materials. Fresh pu-erh tea leaves (*Camellia assamica*) from Yun-Nan province were used in this experiment. Fresh leaves were prepared with momentary high temperature to deactivate the enzyme. Then tea leaves (100 g) were dried and smashed with a hand-held pulverizer to make tea powder, subsequently sterilized at 121 °C for 20 min.

Fermentation material was provided by Yun-Nan agricultural university. It was used for isolating the microorganisms involved in postfermentation of ripened pu-erh tea.

Fungi and Identification. One gram of fermentation material was washed with sterile saline (10 mL), and then diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of levels. These dilutions were respectively incubated on potato dextrose agar (PDA) to grow and isolate microorganisms. The extracted DNA of the isolated fungus was subjected to the amplification of ITS region fragment for identification.

Two major fungi isolated from the fermentation bulk of ripened Pu-erh tea were identified to be *Aspergillus niger* (Accession No. EU314996) and *Aspergillus fumigatu* (Accession No. FJ844610). They were identified according to their morphological characteristics and ITS by Beijing Sunbiotech Co., Ltd., and were preserved in our laboratory at −80 °C. The producing strain was prepared on Martin medium and stored at 4 °C.

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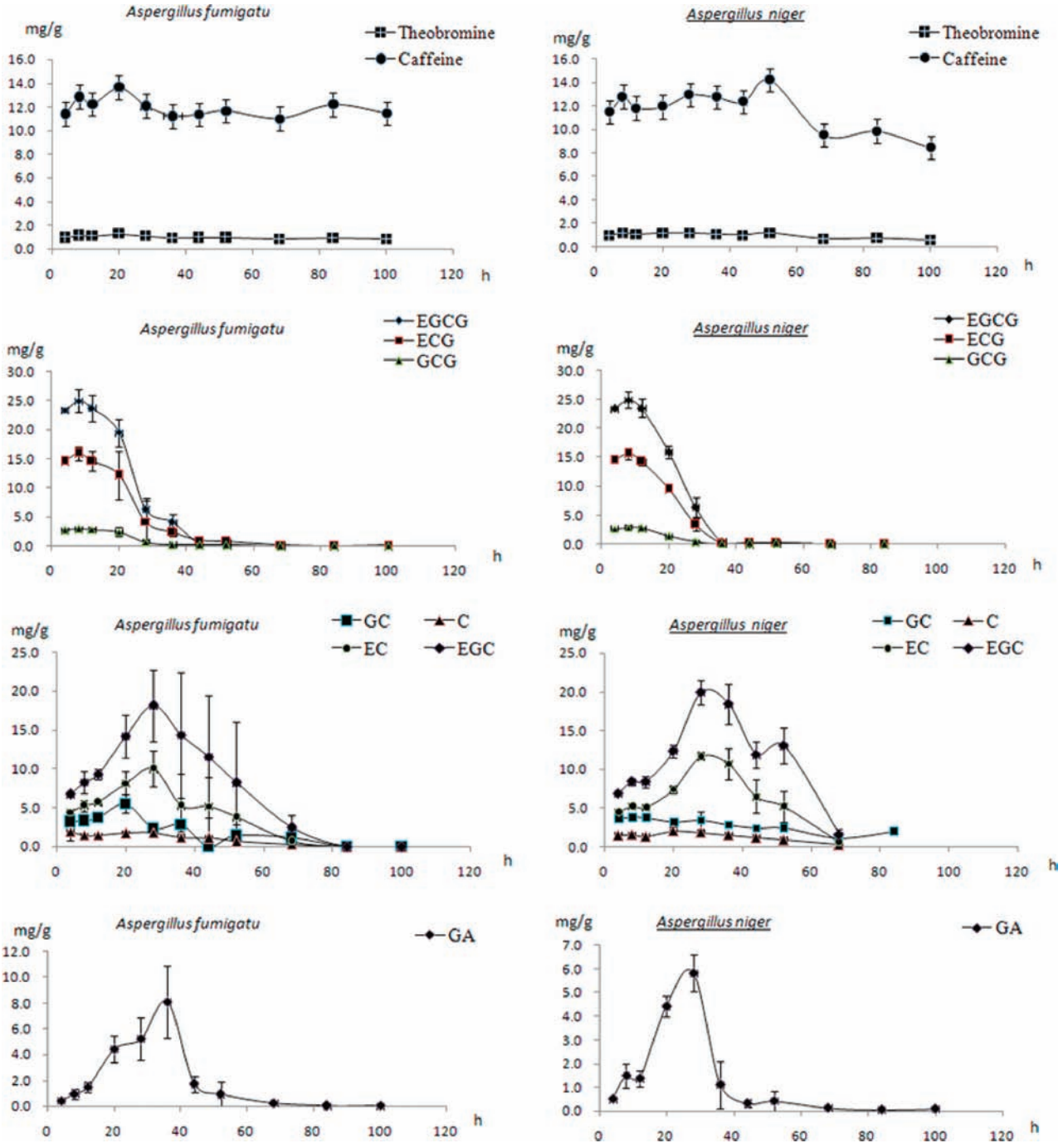

Figure 1. Variation of catechins, gallic acid, caffeine and theobromine in fresh tea leaves of *Camellia assamica* during fermentation with different microorganisms, *A. niger* or *A. fumigatu*.

Chemicals. Gallic acid (GA), caffeine, theobromine, (+)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-gallocatechin gallate (GCG), (−)-epigallocatechin gallate (EGCG) and (−)-epicatechin gallate (ECG) standards (>98%) were purchased from Shanghai Tongtian biotechnology company and identified by our laboratory for the quantitative analysis. Flavone glycoside standards (>95%) including apegenin-6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-glucopyranoside,[1] myricetin-3-*O*-β-D-galactopyranoside,[2] apigenin-8-*C*-glucose-rhamnose,[3] quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside],[4] kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside,[5] quercetin-3-*O*-β-D-glucopyranoside,[6] kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside,[7] quercetin-3-*O*-α-L-rhamnoside,[8] kaempferol-3-*O*-β-D-glucopyranoside[9] isolated from ripened pu-erh tea were used to study the chemical changes of pu-erh tea during SSF. All of these standards were dissolved and diluted using 1:1 CH_3OH/H_2O.

Culture Medium and Fermentation Conditions. The fermentation medium used for substrate moistening was potato dextrose agar. Sterilization of the medium was performed at 121 °C for 20 min. *Aspergillus fumigatu* (*A. fumigatu*) and *Aspergillus niger* (*A. niger*) were used to ferment sterile dry pu-erh tea samples.

In the present study, fermentation was carried out in a thermostat incubator. One milliliter of fungus inoculums was inoculated on PDA with 3×10^5 CFU/mL for pure cultures at 37 °C and humidity at 75%, and then fungus was maintained at this condition on a PDA Petri dish for 1 day. Fifteen grams of sterile tea powder was placed with either *A. fumigatu* or *A. niger* to start fermentation. During SSF, 1 g of sample

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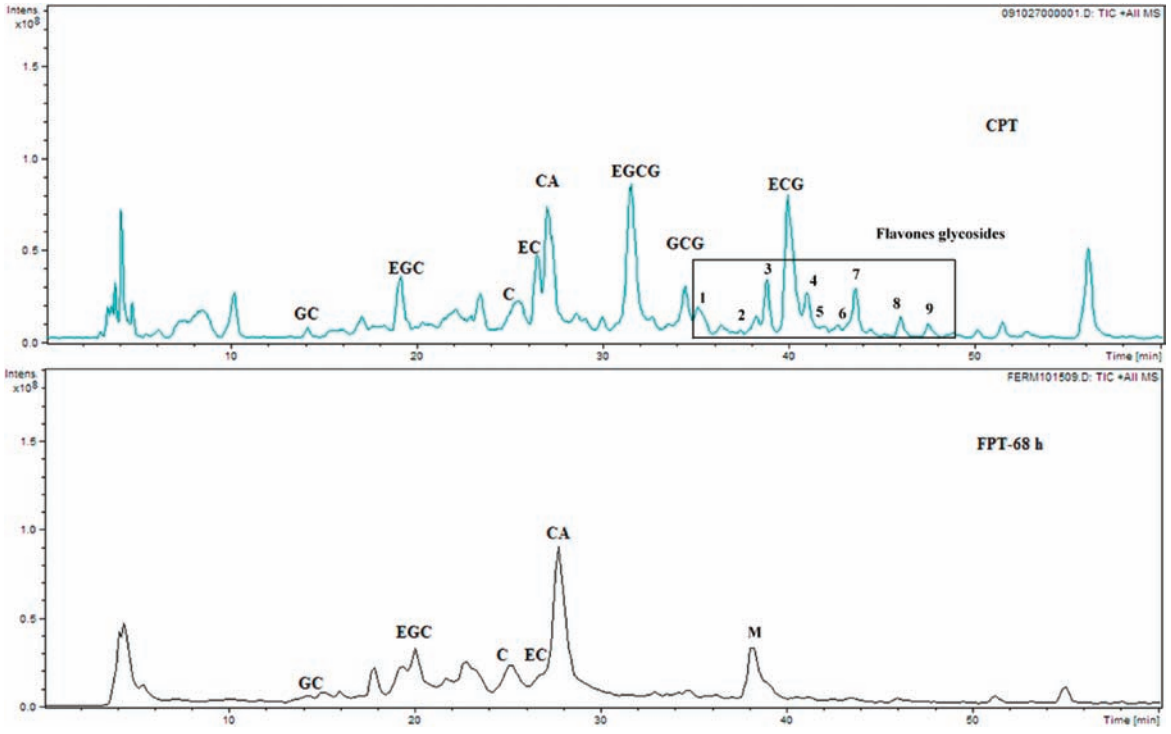

Figure 2. HPLC−MS profiles (detected wavelength at 280 nm) of pu-erh tea before and after SSF (the 68th hour). After SSF, the catechin contents were significantly decreased, while the flavone glycosides (compounds 1−9) were not detected at the 68th hour. CPT, crude pu-erh tea; FPT, fermented pu-erh tea.

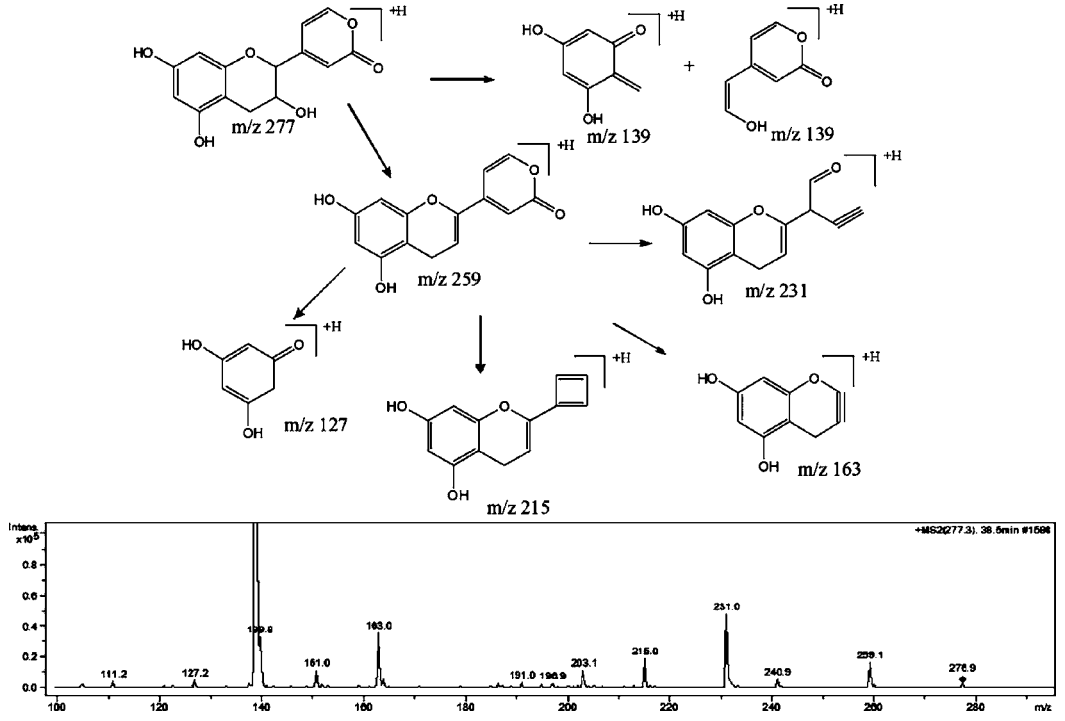

Figure 3. The HPLC−MS^n fragment analysis for metabolic intermediate compound M at positive ionization.

was removed after 4, 8, 12, 20, 28, 36, 44, 52, 68, 84, and 100 h, subsequently placed at 70 °C for 4 h to stop the fermentation and evaporate the water. 0.3 g of dry sample was extracted with 3 mL of methanol at 37 °C by sonic extract for 30 min. Extract was filtered through a 0.22 μm micropore filter for HPLC−MS analysis.

Analytical Methods. The HPLC−MS analytical method used to determine catechins, gallic acid and purine alkaloids was the same as previously reported.[8] Separations were carried out using an Agilent SB-Aq C_{18} reverse phase column (250 × 4.6 mm i.d., 5 μm, Agilent), protected with a security guard cartridge (Gemini C_{18}, 4 × 2.0 mm i.d., Phenomenex). Under these conditions, all analytes had good resolution in HPLC.

Statistical Analysis. The data generated from this study were subjected to one-way analysis of variance (ANOVA) at 5% level of

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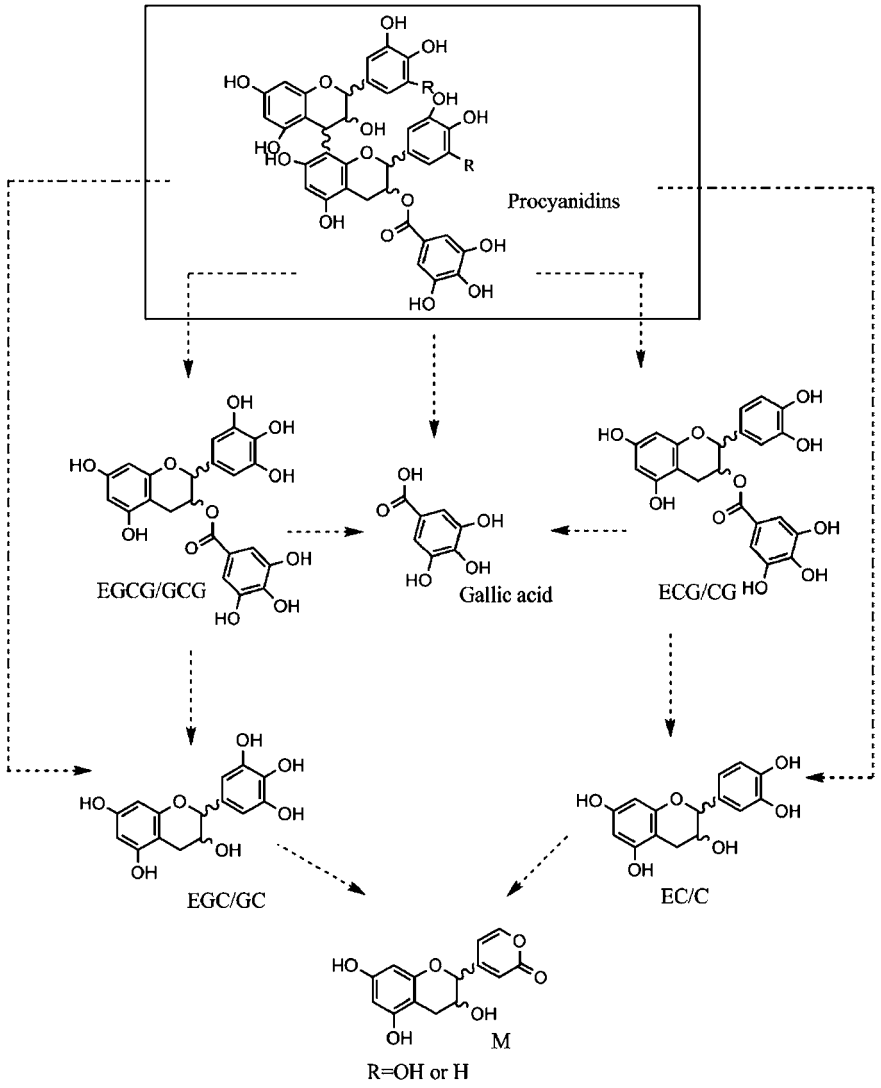

Figure 4. Proposed biotransformation pathway of polyphenols compounds in the fermentation of *Aspergillus* species.

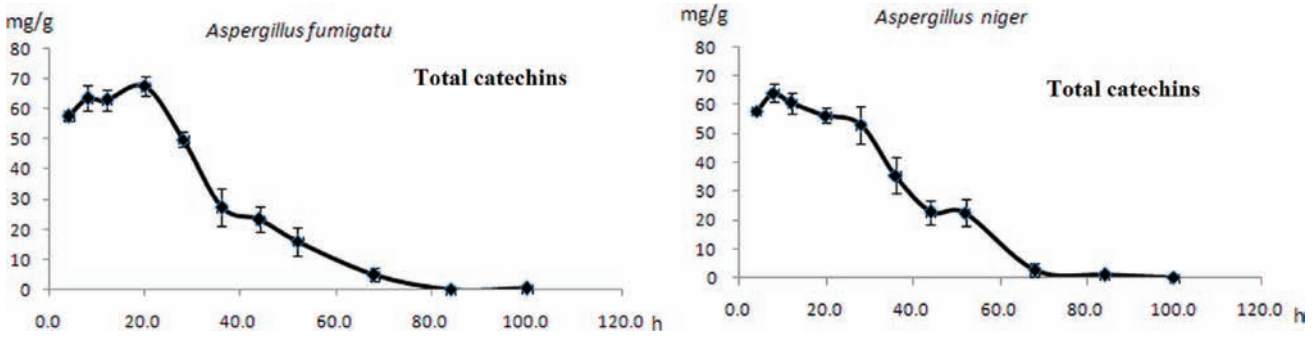

Figure 5. The total catechin contents of pu-erh tea during SSF with different microorganisms, *A. niger* or *A. fumigatu*.

significance. Means were compared by Tukey's test. All determinations were carried out in triplicate.

RESULTS AND DISCUSSION

Changes of Chemical Constituents during SSF. As shown in Figure 1, the content of theobromine in pu-erh tea fermented with *A. fumigatu* was nearly the same as that of unfermented leaves after the SSF. The effects of *A. niger* on caffeine were similar to the previous result,[9] which reported that *A. niger* lowered the level of caffeine in tea. *A. niger* significantly decreased the levels of caffeine only when the fermentation continued for a long time.

At the beginning stage (0−8th hour), the levels of EGCG were slightly increased from 23.392 ± 0.470 mg/g and 23.431 ± 0.318 mg/g to 24.983 ± 1.905 mg/g and 24.897 ± 1.362 mg/g in tea fermented by *A. fumigatu* or *A. niger* alone ($P < 0.05$). After 8 h, the contents of EGCG, GCG and ECG of tea were gradually decreased by fermentation of *A. fumigatu* and *A.*

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niger. After long-term fermentation, the levels of EGCG were only 0.477 ± 0.278 mg/g and 0.460 ± 0.330 mg/g, which were highly decreased compared with those of fresh tea ($P < 0.001$). Similar results were obtained from the analysis of ECG and GCG on tea leaves during fermentation.

C, EC, GC and EGC are simple catechin, the molecular structure of which is flavanol without the ester bond of gallic acid. During the beginning period of fermentation (0−18 h), these simple catechins were accumulated from the decomposing of EGCG, ECG and GCG by fungus, but the contents of simple catechins were still decreased highly after long-term fermentation.

Gallic acid is found to be free and part of the tannins in tea leaves. An article reported that the gallic acid was elevated in fermented tea such as black tea and pu-erh tea.[6] When fresh tea leaves were fermented with *A. fumigatu* and *A. niger*, gallic acid presented a significant increasing in the range of 0 to 28th hour of fermentation. Then, the levels of gallic acid decreased until undetected at the 120th hour.

HPLC−DAD−MS^n Analysis of Metabolic Product during SSF. The contents of tea polyphenols and purine alkaloids were determined during fermentation; HPLC was also employed to study chemical variations of fresh tea sample before and after fermentation at the wavelength of 280 nm. Obviously, in the early stage of fermentation, esters-catechins were highly decreased as shown in Figure 2.

An apparent peak (M) attributable to the metabolites derived from the catechins was observed from the chromatogram of fermented pu-erh tea from the 44th hour to the 84th hour in tea fermented with both *A. niger* and *A. fumigates*. HPLC−DAD−MS^n analysis showed that peak M (t_R, 38.1 min; λ_{max}, 280 nm) was tentatively to be assigned as molecular weight at 276 because of $[M - H]^-$ at m/z 275, $[M + Na]^+$ at m/z 299 and $[M + H]^+$ at m/z 277, which was fragmented to produce MS^2 ions at m/z 139, 231, 163, 259, 215, 203, 151, 127, 111. Compared with the absorbance spectrum and fragments of catechins, compound M can be preliminarily assigned as a catechin oxidation product with the 3,6-dihydro-6-oxo-2*H*-pyran moiety. The fragmentation pathway of this metabolic compound is shown in Figure 3.

Although a study reported that catechins were converted to polymeric substances during the postfermentation process, the exact polymeric structures have not been clarified yet.[1] On the contrary, the existing compounds formed in postfermentation were derivatives of catechins such as puerins A and B.[7] In this study, only puerin A was detected by HPLC−MS^n analysis. Clearly, chemical reaction pathways, enzymatic processes and metabolic intermediate product are critical for exploring the characteristics of postfermented teas.

In the present study, HPLC−MS^n analysis showed a representative intermediate product during SSF of pu-erh tea. Based on the transformation of catechins, ester-catechins and gallic acid, the degradation of the gallate ester bond was supposed to be the main metabolic way in the initial stage. Furthermore, an oxidative product of catechin on the B-ring of flavanols should be responsible for the decreasing of catechins in the late period of SSF.

HPLC−MS analysis also indicated that most of the flavone glycosides were degraded after SSF. Interestingly, the flavonoid aglycons such as kaempferol and quercetin were not detected on HPLC−MS. These results prompted that the degradation of flavone glycosides was more complicated, maybe resulting from multiple enzymatic effects.

In conclusion, the biotransformation profiling of ester-catechins, simple catechins and gallic acid was deduced as shown in Figure 4. The dynamic changes of EGCG, ECG and GCG were parallel. This result indicated that the transformation between different spatial configurations of ester-catechins was hardly proceeded. On the contrary, the gallate ester bond was supposed to be a sensitive chemical group with respect to *Aspergillus* enzymes. Further biochemical study on the related enzymes may contribute to reveal the mechanism of the postfermentation process.

In the whole fermentation, the ratios of catechins−gallic acid/ester-catechins could be determined over time. In view of active polyphenols, an optimum time in the period of fermentation could be decided to get the highest contents of total catechins (Figure 5). The present study also supposed that the *Aspergillus* species could be used to manufacture the tea polyphenols with a specific proportion of compounds.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

A., *Aspergillus*; GA, gallic acid; C, (+)-catechin; EC, (−)-epicatechin; GC, (−)-gallocatechin; EGC, (−)-epigallocatechin; GCG, (−)-gallocatechin gallate; EGCG, (−)-epigallocatechin gallate; ECG, (−)-epicatechin gallate; CG, catechin gallate; PDA, potato dextrose agar; CPT, crude pu-erh tea; FPT, fermented pu-erh tea

REFERENCES

(1) Jiang, H. Y.; Shii, T.; Matsuo, Y.; Tanaka, T.; Jiang, Z. H.; Kouno, I. A new catechin oxidation product and polymeric polyphenols of post-fermented tea. *Food Chem.* **2011**, *129*, 830−836.

(2) Zhang, L.; Ma, Z. Z.; Che, Y. Y.; Li, N.; Tu, P. F. Protective effect of a new amide compound from pu-erh tea on human micro-vascular endothelial cell against cytotoxicity induced by hydrogen peroxide. *Fitoterapia* **2011**, *82*, 267−271.

(3) Jeng, K. C.; Chen, C. S.; Fang, Y. P.; Hou, R. C. W.; Chen, Y. S. Effect of microbial fermentation on content of statin, GABA, and polyphenols in pu-erh tea. *J. Agric. Food Chem.* **2007**, *55*, 8787−8792.

(4) Hou, Y.; Shao, W.; Xiao, R.; Xu, K.; Ma, Z.; Johnstone, B. H.; Du, Y. Pu-erh tea aqueous extracts lower atherosclerotic risk factors in a rat hyperlipidemia model. *Exp. Gerontol.* **2009**, *44*, 434−439.

(5) Zhao, Z. J.; Tong, H. R.; Zhou, L.; Wang, E. X.; Liu, Q. J. Fungal colonization of pu-erh tea in Yunnan. *J. Food Saf.* **2010**, *30*, 769−784.

(6) Zuo, Y. G.; Chen, H.; Deng, Y. W. Simultaneous determination of catechins, caffeine and gallic acids in green, oolong, black and pu-erh teas using HPLC with a photodiode array detector. *Talanta* **2002**, *57*, 307−316.

(7) Zhou, Z. H.; Zhang, Y. J.; Xu, M.; Yang, C. R. Puerins A and B, Two new 8-C substituted flavan-3-ols from pu-er tea. *J. Agric. Food Chem.* **2005**, *53*, 8614−8617.

(8) Zhang, L.; Li, N.; Ma, Z. Z.; Tu, P. F. Comparison of the chemical constituents of aged pu-erh tea, ripened pu-erh tea, and other teas using HPLC-DAD-ESI-MSn. *J. Agric. Food Chem.* **2011**, *59*, 8754−8760.

(9) Wang, X.; Wan, X.; Hu, S.; Pan, C. Study on the increase mechanism of the caffeine content during the fermentation of tea with microorganisms. *Food Chem.* **2008**, *107*, 1086−1091.