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# The nutritional evidence and research on tea

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# The nutritional evidence and research on tea

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# Green Tea Polyphenols Upregulate the Nrf2 Signaling Pathway and Suppress Oxidative Stress and Inflammation Markers in D-Galactose-Induced Liver Aging in Mice

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The beneficial effects of green tea polyphenols (GTPs) on D-galactose (D-Gal)-induced liver aging in male Kunming mice were investigated. For this purpose, 40 adult male Kunming mice were divided into four groups. All animals, except for the normal control and GTPs control, were intraperitoneally injected with D-galactose (D-Gal; 300 mg/kg/day for 5 days a week) for 12 consecutive weeks, and the D-Gal-treated mice were allowed free access to 0.05% GTPs (w/w) diet or normal diet for 12 consecutive weeks. Results showed that GTP administration improved the liver index and decreased transaminases and total bilirubin levels. Furthermore, GTPs significantly increased hepatic glutathione and total antioxidant levels, and the activities of superoxide dismutase, catalase, and glutathione *S*-transferase (GST). Furthermore, GTPs downregulated 8-hydroxy-2-deoxyguanosine, advanced glycation end products, and hepatic oxidative stress markers, such as malondialdehyde and nitric oxide. Additionally, GTPs abrogated dysregulation in hepatic Kelch-like ECH-associated protein 1 and nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream target gene expression [heme oxygenase 1, NAD(P)H:quinone oxidoreductase 1, and GST] and inhibited tumor necrosis factor-α, transforming growth factor-β, and interleukin (IL)-1β and IL-6 in the liver of treated mice. Finally, GTPs effectively attenuated D-Gal-induced edema, vacuole formation, and inflammatory cell infiltration. In conclusion, GTPs showed antioxidant and anti-inflammatory properties in D-Gal-induced aging mice, and may be considered a natural alternative to the effects of hepatic aging.

**Keywords: green tea polyphenols (GTPs), aging, antioxidants, D-galactose, inflammatory cytokines, Nrf2 signaling pathway**

## INTRODUCTION

Aging is one kind of irreversible and perennial natural biological process that accounts for genetic, internal, and external environmental factors. This process is characterized by a progressive loss of physiological integrity, which invariably leads to impairments in the organizational structure and function of organs (1). The aging of tissues/organs makes the human body susceptible to adverse circumstances and is the primary risk factor for major human pathologies (2). Like other organs, after the growth and development, the liver undergoes a series of degeneration processes, such as aging, that encompasses changes in its morphological structure to metabolic functions (3). Aging-related liver diseases mainly include alterations of hepatic structure and function, where the increase of liver volume and decrease of hepatic blood flow and perfusion occur. These changes increase the liver fibrosis, hepatocarcinoma, and mortality rate of susceptible elderly people and can thus be considered adverse prognostic factors (3–5). At the cellular level, the disturbances of proteostasis by protein oxidation aggregates trigger reactive oxygen species (ROS) and inflammatory cytokines production (6, 7). Liver aging is usually manifested as a decrease in albumin (Alb) levels and an increase in total bilirubin (TBiL), alkaline phosphatase (ALP), and aminotransferase levels in the blood (8). In addition, liver aging may be related to cytoplasmic polyploidy and decreases in the surface area of the endoplasmic reticulum and the number of mitochondria, thus imposing a negative impact on the functions of hepatocytes (4). Similarly, the damage of hepatocyte mitochondrial function increases the incidence of autoimmune and other age-related diseases (9). Thus, strategies to counteract/alleviate the harmful effects of aging on liver function are desired from the public health perspective.

Green tea has been one of the most consumed non-alcoholic beverages in more than 160 countries (10). The beneficial health effects of green tea are generally associated with its polyphenols, which may account for up to 30% of its dry weight (10). Green tea polyphenols (GTPs) are mainly composed of monomeric flavan-3-ols, such as catechins (10). GTPs exhibit numerous biological effects; for example, they have anti-obesity, anti-inflammatory, antioxidant, neuroprotective, and antitumor properties (10–12). Most biological effects of GTPs are attributed to their ability to transcriptionally upregulate the nuclear factor erythroid 2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) signaling pathway in response to the regulation of antioxidant and Phase II detoxification enzymes and nuclear factor-kappa β (NF-κβ) in different organs, particularly in liver tissue (13, 14).

To our knowledge, the protective effects of GTPs on liver aging have not been extensively evaluated and findings obtained with different *in vivo* protocols are inconclusive and not convergent. For instance, a previous study has shown that in an adult male Sprague–Dawley rat model of metabolic syndrome induced by a high-fat diet, GTPs were able to decrease liver transaminases, oxidative markers, and inflammatory cytokines in the liver (15). Conversely, in a C57BL/6 mice model of cholesterol-induced steatohepatitis, Hirsch et al. observed that GTPs exacerbated hepatic steatosis, oxidative stress, bile acids, and liver damage (16). Thus, it is of pivotal importance to understand the mechanisms of how GTPs can affect inflammation and oxidative stress using different protocols. Considering the global trend for natural products that can be used as adjuvant agents to decrease the risk of diseases and the scientific gap on the beneficial effects of GTPs on liver aging, this work focused on the effects of GTPs on D-galactose (D-Gal)-induced aging in male Kunming mouse liver. The underpinning mechanisms of action were unveiled by quantifying the main oxidative, inflammation, and senescence markers.

## MATERIALS AND METHODS

### Chemicals and Reagents

D-Gal (CAS: 59-23-4) of 99% purity was purchased from Sigma–Aldrich Chemical Co. (MO, United States). GTPs [gallic acid (4.9%), catechin (0.42%), epicatechin (4.17%), gallocatechin gallate (1.28%), epigallocatechin (10.76%), epicatechin gallate (7.46%), epigallocatechin-3-gallate (60.97%), anthocyanins (3.42%), leukoanthocyanins (1.34%), and other phenolic acids (5.28%)] were purchased from Hefei Jishi Mingxiang Biotechnology Co. Ltd. (Anhui, China). Commercial kits for measuring the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP, Alb, TBiL, total superoxide dismutase (T-SOD), catalase (CAT), glutathione *S*-transferase (GST), total antioxidant capacity (T-AOC), glutathione (GSH), malondialdehyde (MDA), nitric oxide (NO), and ELISA kits for measuring the levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) and advanced glycation end products (AGEs) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and heme oxygenase 1 (HO-1) ELISA kits were purchased from Sigma–Aldrich (MO, United States), Invitrogen (CA, United States), BD Biosciences (CA, United States), Cell Sciences Inc. (MA, United States), and Abcam (CA, United States), respectively. Mouse Nrf2, Kelch-like ECH-associated protein 1 (Keap1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) ELISA kits were purchased from CUSABIO (Wuhan, China).

### Animals

Healthy male Kunming mice (age: 7–8 weeks, body weight: ∼18–22 g) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. Mice were given a standard laboratory diet and water *ad libitum*.

### Experimental Design for the *in vivo* Protocol

After a week of acclimation, 40 mice (10 in each group) were randomly divided into the following groups: (i) normal control, (ii) D-Gal model, (iii) GTPs intervention, and (iv) GTPs control. The normal control group had free access to the standard diet and received intraperitoneal injections of normal saline for 12 weeks. The D-Gal model group mice that were fed the standard diet had intraperitoneal injections of 300 mg/kg D-Gal (5 days a week) for 12 weeks, the GTPs intervention group mice received a

0.05% GTPs (w/w) diet with the intraperitoneal injection of 300 mg/kg D-Gal for 12 weeks, and the GTPs control group received a 0.05% GTPs (w/w) diet with an intraperitoneal injection of normal saline (5 days a week) for 12 weeks. The mice were anesthetized and sacrificed as per ethical guidelines 24 h after the last administration. The liver of each animal was removed, weighed carefully, and quickly placed into ice-cold phosphate-buffered saline (PBS; 150 mM, pH 7.2). The right liver lobes were fixed with 10% neutral-buffered formalin for >72 h before the preparation of tissue sections for histological examination; the left liver lobes were removed and then stored at −80°C until biochemical analysis. Blood samples were obtained to determine cytokines levels by using ELISA and to evaluate plasma enzyme activities.

## Assessment of Hepatic Function

The plasma levels of hematological biomarkers, including Alb, TBiL, ALP, ALT, and AST, were estimated using an enzyme-labeled instrument and the commercially available colorimetric assay kits according to the manufacturer's protocols. The liver index was calculated according to the following equation: liver weight (g)/body weight (g) × 100%.

## Assessment of Hepatic Antioxidant Markers

The levels or activities of GSH, T-AOC, T-SOD, CAT, and GST in the liver homogenates were measured using an enzyme-labeled instrument by the commercially available colorimetric assay kits according to the manufacturer's protocols.

## Assessment of Hepatic Oxidative Stress and Senescence Markers

The levels of MDA, NO, 8-OhdG, and AGEs in liver homogenates were detected using an enzyme-labeled instrument by the commercially available colorimetric assay kits following the manufacturer's protocol.

## Assessment of Hepatic Inflammatory Mediator Concentrations

The hepatic inflammatory cytokines concentrations, including IL-1β, IL-6, TNF-α, and TGF-β, were assessed using an enzyme-labeled instrument by the commercially available ELISA kits according to the manufacturer's instructions.

## Histopathological Examinations

Fixed liver tissues were embedded in paraffin and cut into coronal sections (4 μm thick), which were then stained with hematoxylin and eosin (H&E) according to Wang et al. (17).

## RNA Isolation and qPCR

The total RNA isolation, reverse transcription, and quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) were performed according to the standard protocol described by Wang et al. (17). The sequences of HO-1, NQO1, GST m1, and GST a1 were designed and synthesized as described by Wang et al. (17).

## Preparation of Cytosolic and Nuclear Fractions and Assessment of the Hepatic Nrf2 Pathway

Hepatic cytosolic and nuclear fractions were prepared using a commercially available Minute Cytosolic and Nuclear Extraction Kit (Invent Biotechnologies, Inc.) according to the manufacturer's protocol. Briefly, liver tissues (25 mg/mouse) were washed once with cold buffer solution A on ice for 5 min, and the samples were twisted and ground with a grinding pestle for 1 min (40–60 times). Homogenates were centrifuged at 14,000 g for 5 min, the supernatants were recovered as cytosolic fractions, and the nuclear pellets were lysed in cold buffer solution B on ice for 5 min. After centrifugation at 500 g for 5 min, the nuclei were resuspended in cold buffer solution N for 5 min. Thereafter, 50 mg of protein extract powder and cold buffer solution A were added, and the solution was mixed for 1 min. Hepatic homogenates were centrifuged at 10,000 g for 5 min, and the supernatants were recovered as nuclear fractions for a subsequent ELISA.

## Statistical Analysis

Data are presented as mean ± standard error of the mean. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post-hoc* test were used to compare the treatments. $P < 0.05$ were considered statistically significant.

# RESULTS

## Effects of GTPs on the Liver Function Markers

The beneficial effects of GTPs on liver function were investigated by assessing organ index and multiple hematological indicators. The results in **Table 1** and **Figure 1** show that the liver index and hematological indicators were significantly increased after the exposure to D-Gal, whereas the liver index and the hematological indicators were significantly lower in the GTP-treated group. In particular, plasmatic ALP, ALT, and AST levels of the D-Gal mice were significantly increased (**Figure 1C**), revealing that the liver function in the D-Gal model group was disrupted. Moreover, plasmatic ALP, ALT, and AST levels were significantly lower in the D-Gal-treated mice supplemented with GTPs. In addition, plasmatic Alb level was 24% lower and plasma TBiL level was

**TABLE 1** | Effects of GTPs on body weight and liver indexes.

| Groups | Body weight | | Liver weight (g) | Liver index (%) |
|---|---|---|---|---|
| | Initial | Final | | |
| Normal control | 24.8 ± 1.8 | 37.2 ± 2.6 | 1.92 ± 0.14 | 5.16 ± 0.28 |
| D-Gal model | 25.2 ± 1.8 | 36.5 ± 2.2 | 2.45 ± 0.16** | 6.71 ± 0.35* |
| GTPs intervention | 25.0 ± 1.6 | 37.8 ± 2.4 | 2.23 ± 0.12 | 5.90 ± 0.31# |
| GTPs control | 25.1 ± 1.7 | 37.9 ± 2.5 | 2.02 ± 0.13 | 5.32 ± 0.26 |

*D-Gal, D-galactose; GTPs, green tea polyphenols.*
**P < 0.05 and **P < 0.01 vs. normal control group; #P < 0.05 vs. D-Gal model group.*

92% higher in the D-Gal model group compared with the normal control group. Furthermore, plasmatic Alb and plasma TBiL levels were significantly higher and lower, respectively, in the D-Gal model mouse were supplemented with GTPs (**Figures 1A,B**). However, the administration of GTPs did not affect the liver index and liver damage indexes.

## Effects of GTPs on Liver Antioxidant, Oxidative Stress, and Senescence Markers

In this study, liver antioxidant and oxidative stress markers were screened to explore the protective effects of GTPs against D-Gal-induced liver aging. In the D-Gal model group, antioxidant and oxidative stress marker values were significantly different from those of the normal control group. Hepatic antioxidant markers, namely T-SOD, CAT, GST, GSH, and T-AOC, significantly decreased in the D-Gal-treated group (**Figures 1D–F**). Furthermore, hepatic oxidative stress and senescence markers, namely MDA, NO, 8-OhdG, and AGE, significantly increased in the D-Gal-treated group (**Figures 2A–C**). Moreover, these antioxidant markers were significantly increased, and the oxidative stress and senescence markers were significantly inhibited in the D-Gal model mice supplemented with GTPs (**Figures 1**, **2**). These results highlight the regulatory role of GTPs in relation to the redox imbalance and oxidative stress in the liver of D-Gal-treated mice. However, the supplementation of GTPs did not affect hepatic markers of antioxidant, oxidative stress, and senescence.

## Effects of GTPs on Liver Inflammatory Mediators

The hepatic level of TNF-α (proinflammatory cytokine) in the D-Gal-treated mice was significantly higher than that in the normal control group (**Figure 2D**). Furthermore, the hepatic TNF-α level of the GTPs intervention group was significantly lower than that in the D-Gal model group (**Figure 2D**). Similarly, the level of hepatic TGF-β was significantly higher in the D-Gal model mouse; furthermore, the hepatic TGF-β level was lower in the D-Gal model mice supplemented with GTPs (**Figure 2D**). Additionally, the administration of GTPs significantly inhibited the production of both IL-1β and IL-6. However, only GTPs did not affect hepatic inflammatory mediators.

## Effects of GTPs on Hepatic Histopathological Alterations

The histopathological analysis found that D-Gal caused edema, vacuoles, cytoplasmic porosity, inflammatory cell infiltration, and degeneration in the liver (**Figure 3**). GTP was able to counteract these harmful effects and reduce the pathological damage of the liver induced by D-Gal (**Figure 3**). However, only GTPs did not affect hepatic histopathological changes.

## Effects of GTPs on the Hepatic Nrf2 Signaling Pathway

To investigate the underlying mechanism of the anti-aging effect caused by the administration of GTPs, the signal changes of the Nrf2/Keap1 pathway in mice treated with D-Gal were investigated. Mice treated with D-Gal had significant changes in Keap1 and Nrf2 and its downstream target genes in the liver (**Figure 4**). Hepatic Keap1 levels were 40% higher in response to D-Gal treatment (**Figure 4A**). Hepatic Nrf2 (cytoplasmic and nuclear) had a different pattern compared to Keap1 in the GTP-treated group; both cytoplasmic and nuclear Nrf2 levels in the liver were significantly higher in the GTPs-treated group compared within the D-Gal model mice (**Figure 4B**). Furthermore, the HO-1 and NQO1 protein expression levels in the liver of aged mice were significantly lower (by 47 and 36%, respectively) in the D-Gal model group compared within the normal control group (**Figure 4C**). The drastic decreases in HO-1 and NQO1 levels were abrogated in the D-Gal model mice supplemented with GTPs (**Figure 4C**). D-Gal injection significantly downregulated the gene expression of HO-1, NQO1, GST m1, and GST a1 in the liver (**Figure 4D**). Moreover, the Nrf2-targeted gene expression levels were increased in the D-Gal model mice supplemented with GTPs (**Figure 4D**).

# DISCUSSION

Aging is a normal physiological phenomenon and is an independent risk factor for various chronic diseases (2). Therefore, discovering and using natural products that may effectively decrease the risk of aging-related diseases are crucial tasks. Oxidative stress and proinflammatory responses are the detrimental causative factors leading to imbalances between oxidative damage and antioxidant function in the development of age-associated conditions (18, 19). Recent evidence suggests that age-related progressive hepatic capacity dysfunction enhances the senescence of hepatocytes (9).

D-galactose has been widely applied to induce an aging-like condition in various organs to study different biomarkers (20–22). High doses of D-Gal can induce the formation of hydrogen peroxide, which is invariably linked to ROS, thus leading to hepatic metabolic disorders and, ultimately, liver aging (23, 24). Therefore, in our study, the D-Gal-induced liver aging model in mice was used. The hepatic antioxidant system plays an important role in maintaining the normal liver function, and changes in antioxidants in this system impact the endogenous antioxidant capacity (24). Although the antioxidant properties of GTPs were confirmed using this *in vivo* model, this is the first study to investigate their ability to alleviate the hepatic oxidative stress in D-Gal-treated mice. Thus, our results showed that GTPs can improve hepatic antioxidant capacity and maintain hepatic redox balance during simulated aging. D-Gal significantly increased hepatic damage by changing the levels of plasmatic ALP, ALT, and AST, when compared with the control mice. The increase in aminotransferases in D-Gal-induced liver aging has been reported elsewhere (21). Moreover, plasma TBiL level was remarkably increased in these mice. The catabolism process of bilirubin depends on liver function; therefore, a high level of TBiL reflects hepatocellular dysfunction (25). Consistently, we found that GTPs significantly counteracted liver function abnormalities in D-Gal-treated mice (**Figure 1**). The proposed mechanism of action of GTPs is shown in **Figure 5**.

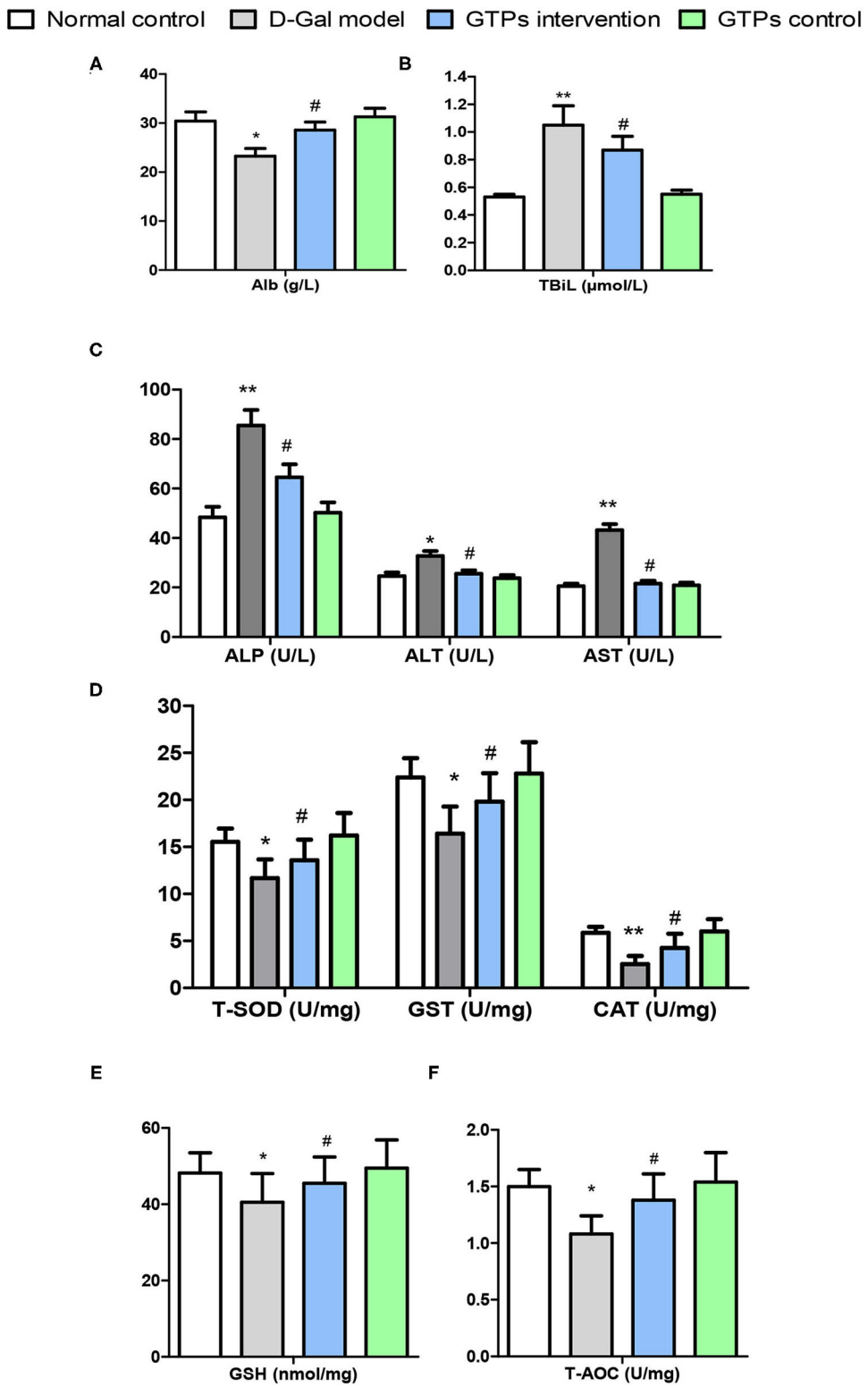


**FIGURE 1 |** Effects of GTPs on liver damage indexes and antioxidant markers of D-gal treated mice. **(A)** Plasma levels of Alb. **(B)** Plasma levels of TBiL. **(C)** Plasma levels of ALP, ALT, and AST. **(D)** Hepatic contents of T-SOD, GST, and CAT. **(E)** Hepatic levels of GSH. **(F)** Hepatic levels of T-AOC. $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. D-Gal model group. Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; D-Gal, D-galactose; GSH, reduced glutathione; GST, glutathione S-transferase; GTPs, green tea polyphenols; T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity; TBil, total bilirubin.

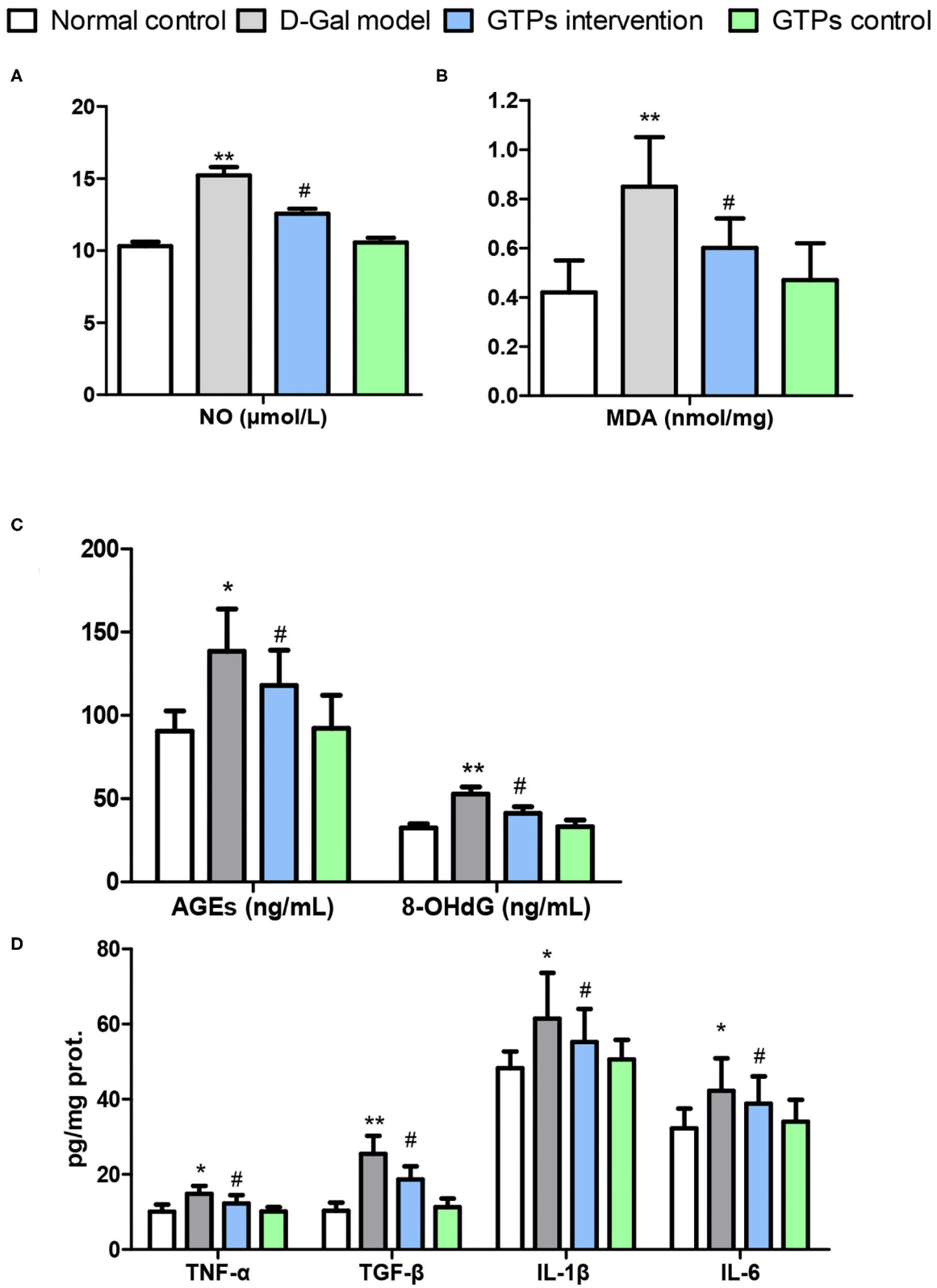


**FIGURE 2 |** Effects of GTPs on liver oxidative stress markers and inflammatory mediators of D-gal-treated mice. **(A)** Plasma level of NO. **(B)** Hepatic contents of MDA. **(C)** Plasma levels of AGEs and 8-OHdG. **(D)** Hepatic contents of TNF-α, TGF-β, IL-1β, and IL-6. $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. D-Gal model group.

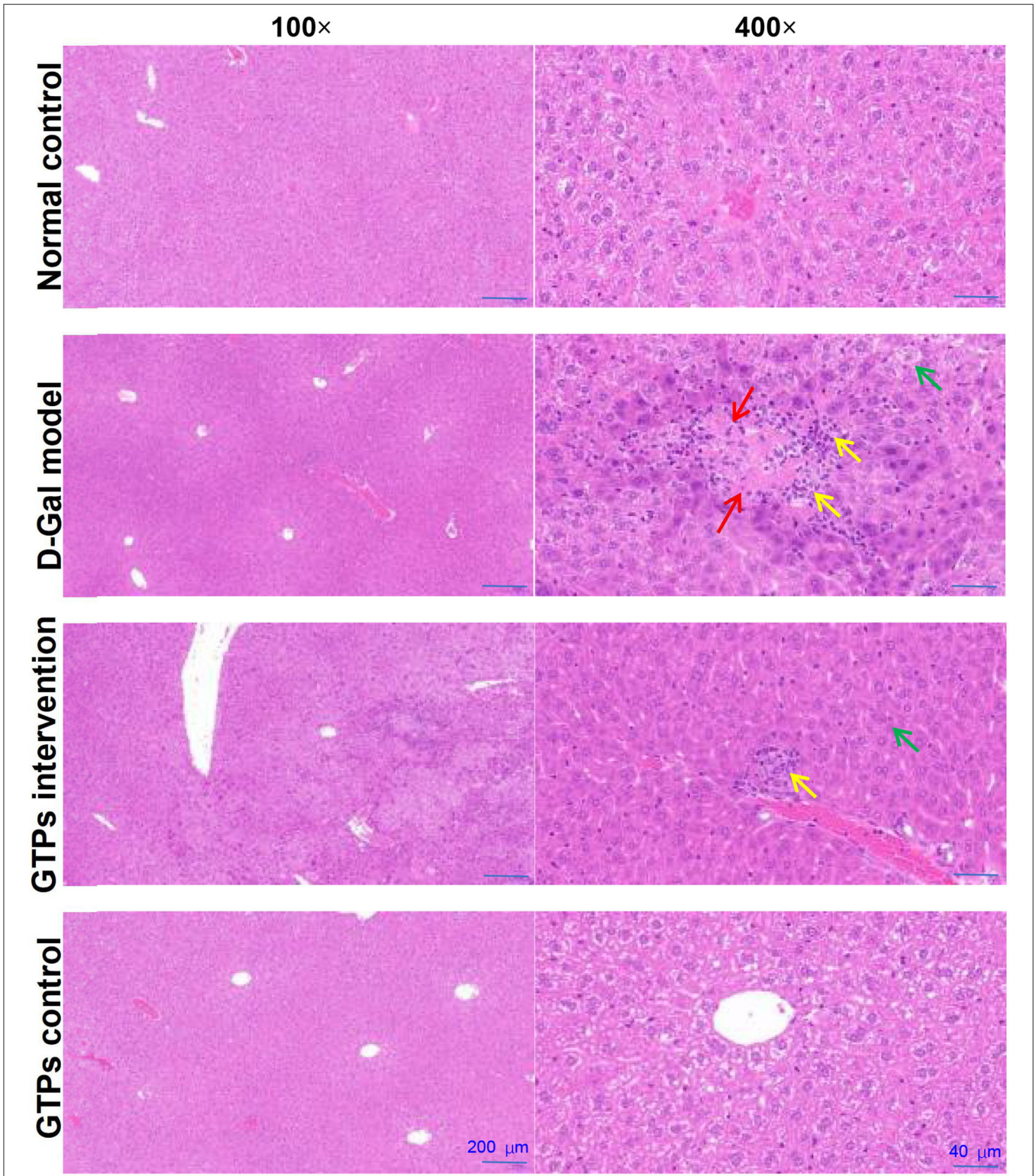


**FIGURE 3 |** Effects of GTPs on hepatic histopathological alterations in D-Gal-treated mice. Representative HE-stained sections of the liver tissues from rats in each group (100× and 400×). Green arrow indicates edema, vacuoles, and cytoplasmic porosity. Yellow arrow indicates inflammatory cell infiltration. Red arrow indicates degeneration. Scale bars (200 and 40 μm).

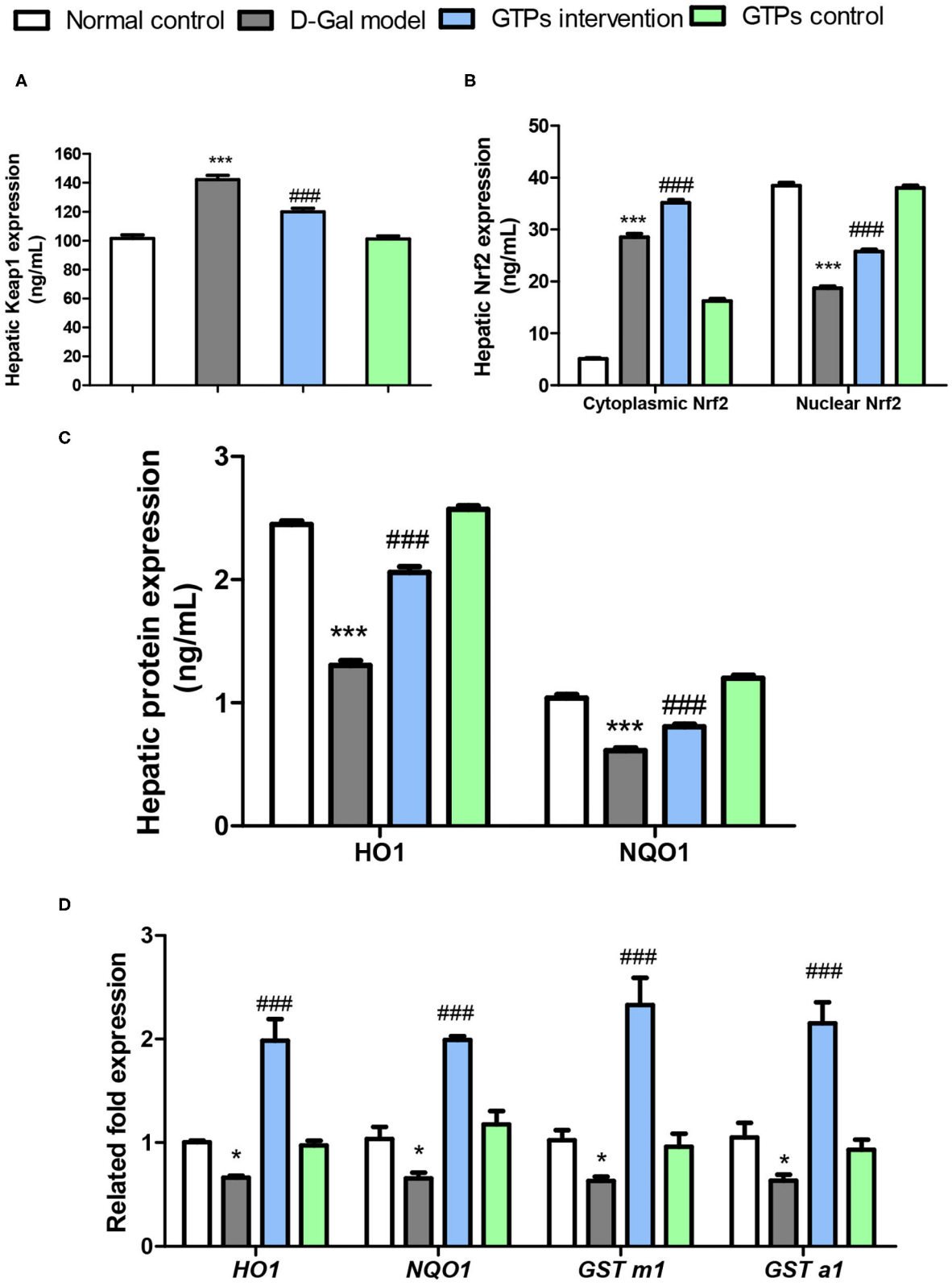


**FIGURE 4 |** Effects of GTPs on the expressions of Nrf2 pathway in the liver of D-Gal-treated mice. **(A)** Hepatic protein levels of Keap1. **(B)** Hepatic protein levels of cytoplasmic and nuclear Nrf2. **(C)** Hepatic protein levels of HO1 and NQO1. **(D)** Hepatic mRNA expressions of Nrf2-targeted genes. $^{***}P < 0.001$ vs. control group; $^{###}P < 0.001$ vs. the D-Gal model group.

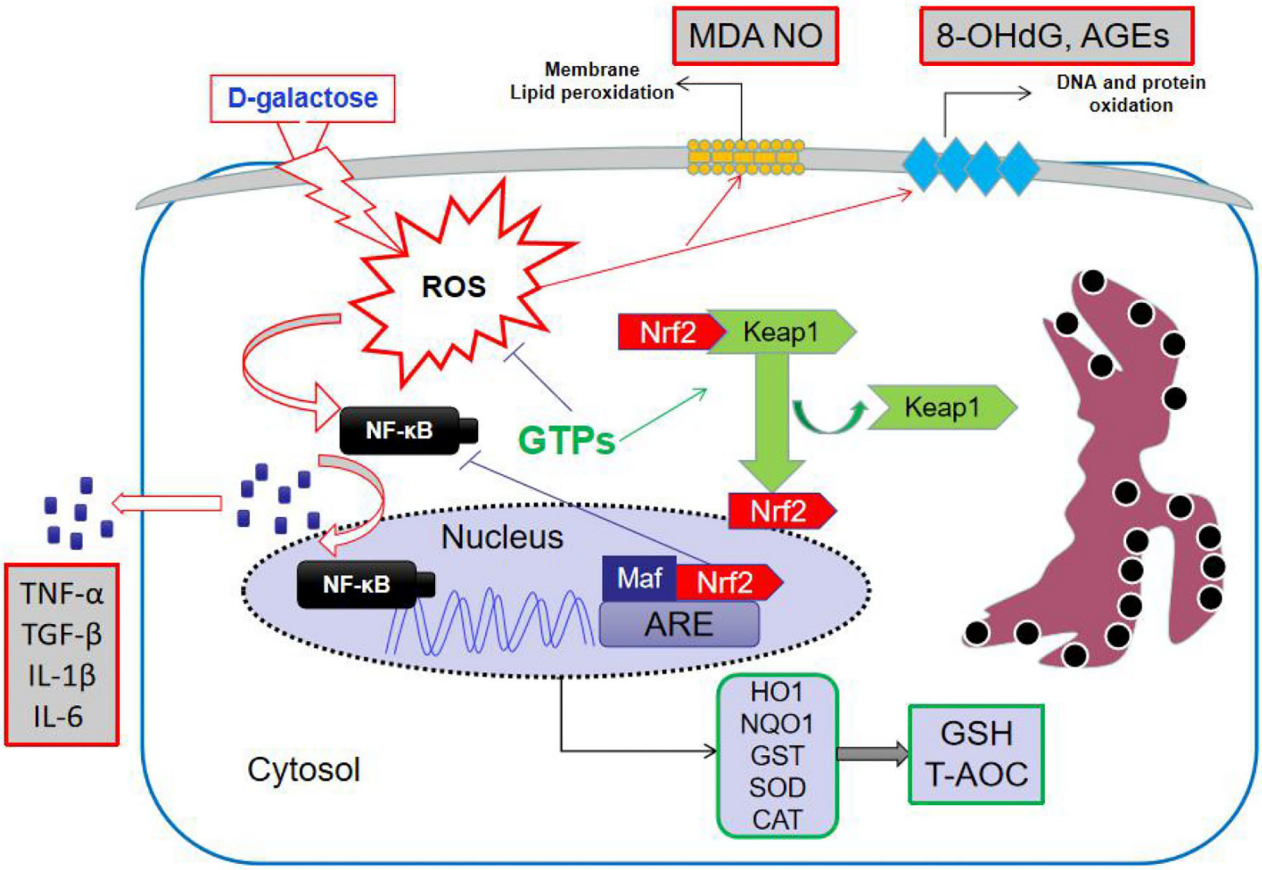


**FIGURE 5 |** Mechanism of action of GTPs in D-galactose-induced aging in mice liver. GTPs, green tea polyphenols; ROS, reactive oxygen species; MDA, malondialdehyde; NO, nitric oxide; 8-OhdG, 8-hydroxydeoxyguanosine; AGEs, advanced glycation end products; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor 2; NF-kB, nuclear factor kappa B; ARE, antioxidant response element; HO-1, hemeoxygenase; NQO1, NAD(P)H:quinone oxidoreductase 1; GST, glutathione S-transferase; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione; T-AOC, total antioxidant capacity; TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin.

GTPs have exhibited antioxidant effects against oxidative stress and liver damage in different *in vivo* experimental models, such as lipopolysaccharide-induced inflammatory liver injury, azathioprine-induced hepatotoxicity, and carbon tetrachloride–induced hepatotoxicity (26–28). Furthermore, our data corroborate previous observations that exposure to D-Gal can induce hepatic oxidative stress associated primarily with a drastic decrease in the activity of antioxidant enzymes and content of non-enzymatic antioxidants in the liver of aged mice (29). We also found that GTPs effectively decreased oxidative products (MDA and 8-OHdG) in D-Gal-treated mice and increased the activity of endogenous antioxidant enzymes (T-SOD, CAT, GST) and content of antioxidants (T-AOC and GSH) in aging liver tissues, leading to the mitigation of D-Gal-induced impaired liver function. In a previous study with primary cultured rat hepatocytes exposed to 1,4-naphthoquinone, GTPs were not able to counteract the lipid oxidation (i.e., MDA levels) (30).

In an aging pathological state, oxidative stress can induce NF-κβ activation in the liver in addition to evoking direct cellular damage, thereby resulting in the release of proinflammatory cytokines (31, 32). The overexpression and secretion of proinflammatory cytokines negatively affect the hepatocyte's function and further enhance oxidative lesions. Thus, our results showed that the hepatic pro-inflammatory cytokines levels notably increased after D-Gal treatment in mice (**Figure 2D**), which was in accordance with the histopathological findings (**Figure 3**). Nonetheless, GTPs not only suppressed the overproduction of these cytokines but also significantly attenuated inflammatory cell infiltration caused by D-Gal in the liver, corroborating the data obtained by Xu et al. (33). Accordingly, the observed protective effect of GTPs on D-Gal-treated mice is likely to have significant beneficial effects on the inflammatory response.

Recent studies have revealed that inflammation-induced AGEs are accumulated in aging tissues of humans and animals, which can serve as a biomarker of organ function (34, 35). Furthermore, previous studies have verified that AGEs might enhance the homeostasis imbalance and age-related clinical diseases *via* increasing the production of ROS and

proinflammatory cytokines (36). Hence, it is hypothesized that GTPs could inhibit AGE production in D-Gal-treated mice and thus prevent liver aging. Notably, Nrf2/Keap1 signaling pathway is one of the major intracellular signaling pathways for attenuating oxidative stress–induced liver aging (24, 37). After signal stimulation, Nrf2 was isolated from Keap1, and activated the processing and synthesis of Nrf2 target antioxidative enzymes and non-enzymatic antioxidants, eventually initiating antioxidant response (24, 38, 39). Interestingly, this study found that GTPs increased the expression of Nrf2, HO-1, and NOQ1 proteins in the liver of D-Gal-treated mice. The phenomenon indicated that GTPs were able to mitigate D-Gal-induced liver aging *via* activation of the Nrf2/Keap1 pathway *in vivo*.

In the present study, a D-Gal-induced senescent mice model was used to investigate the protective effects of GTPs on the liver. GTPs upregulated the Nrf2 signaling pathway, maintained a balance in redox and inflammation, reduced cellular oxidative stress and AGE concentration, and improved the activities of antioxidant enzymes in D-Gal-treated mice, thus ameliorating the simulated aging process. Furthermore, histopathological observation revealed that GTPs effectively inhibited D-Gal-induced hepatic pathological changes, highlighting their potential use as a dietary supplement to decrease the effects of liver aging. Further research must be conducted on the potential mechanism by which GTPs activate Nrf2 translocation. Phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays a vital role in the regulation of proliferation, differentiation, and survival (40). Studies have shown that Nrf2 is a target of the PI3K/Akt signaling pathway and the nuclear translocation of Nrf2 requires the activation of the PI3K/Akt signaling pathway (41, 42). Our previous study found that GTPs can activate PI3K/Akt signaling pathway in mice (43); therefore, these results revealed that the PI3K/Akt signaling pathway was involved in Nrf2 activation induced by GTPs. Additionally, whether other redox-associated transcription factors, such as activator protein-1 and NF-κβ, are involved in the regulation of antioxidant enzymes by GTPs requires further studies. Moreover, the structure–activity relationship responsible for the anti-liver aging activity must be identified to explore its moderating effects on the Nrf2/Keap1 pathway.

Other polyphenols have also been reported to reduce D-Gal-induced liver aging through antioxidant and anti-inflammatory mechanisms, such as rambutan peel polyphenols, purple sweet potato polyphenols, ellagic acid, curcumin, and epigallocatechin-3-gallate (20, 44, 45). These results indicate that polyphenols exert beneficial effects on liver aging, and GTPs may be a source of bioactive polyphenols for protecting liver aging due to their rich content and biological activity *in vivo*. Although GTPs have a good protective effect on liver aging, some studies have found that the long-term consumption of tea polyphenol epigallocatechin-3-gallate can cause subacute liver failure in mice, including hepatocyte necrosis along with an abnormal change of blood transaminases, TBiL, and Alb (46). Among the active components of GTPs, anthocyanin, gallic acid, and epigallocatechin-3-gallate have been reported to have potential beneficial effects for the prevention and treatment of age-related liver diseases in mice or rats (47–49). Although we have observed beneficial effects of GTPs in a mouse model of liver aging, it is prudent to state that more studies using *in vitro* (i.e., cell cultures) and *in vivo* (i.e., piglets) protocols are highly required to demonstrate the toxicological safety, dose and time dependency effects, and overall outcomes of the supplementation of GTPs. To date, the consumption of GTPs in powder form or capsules that contain a dose of catechins more than 800 mg/day is not incentivized by international governmental agencies, such as the European Food Safety Authority (EFSA). From a practical standpoint, the dosage in future studies should be pondered: doses higher than those normally consumed in 1-3 teacups a day may cause damage to the outer mitochondrial membrane of hepatocytes (cell injury), and an uncoupling of oxidative phosphorylation may occur, which increases the ROS production and cytokines secretion (50, 51).

In the current study, we found that GTPs exert protective effects against D-Gal-induced liver aging in mice. These results show that GTPs can decrease D-Gal–induced liver dysfunction, histopathological changes, oxidative stress, pro-inflammatory cytokines production, and expression levels of 8-OHdG and AGEs in the liver through regulating Nrf2 signaling pathways. The results highlight the importance of GTP as a natural supplement of anti-aging compounds.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Ethics Committee at the Jiangsu University of Science and Technology (ethical approval code: 20200302), where the *in vivo* protocol was conducted.

## AUTHOR CONTRIBUTIONS

DW and DG contributed to the conceptualization and writing the original draft. TW contributed to data curation. DW was responsible for the investigation and resources. ZL was responsible for the methodology. YG visualized and supervised. ZL and DG reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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# Effects of Keemun and Dianhong Black Tea in Alleviating Excess Lipid Accumulation in the Liver of Obese Mice: A Comparative Study

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The chemical compositions of black teas differ greatly and may have different health benefits; however, systematic investigations into such benefits are lacking. Here, the chemical profiles of Keemun black tea (KBT) and Dianhong black tea (DBT), two common categories of tea in China, were analyzed, and their lipid-lowering effects in male C57BL/6 mice fed a high-fat diet (60% energy from fat) or the diet supplemented with 2% black tea powder for 15 weeks were investigated. The compounds most crucial in differentiating KBT and DBT were determined to be phenolic compounds, theanine, and D-psicose. DBT was more effective than KBT in preventing excess hepatic fat accumulation. Both black teas effectively and comparably altered the mRNA levels of hepatic lipid–metabolizing genes. DBT had more favorable effects in stimulating fecal fat excretion than did KBT. The differentiating compounds with the higher values of variable importance in the projection (VIP) might predominantly contribute to the different health benefits; however, the most essential compound or combination of compounds requires clarification.

**Keywords: black tea, diet-induced obesity, fatty liver, lipid metabolism, fecal excretion**

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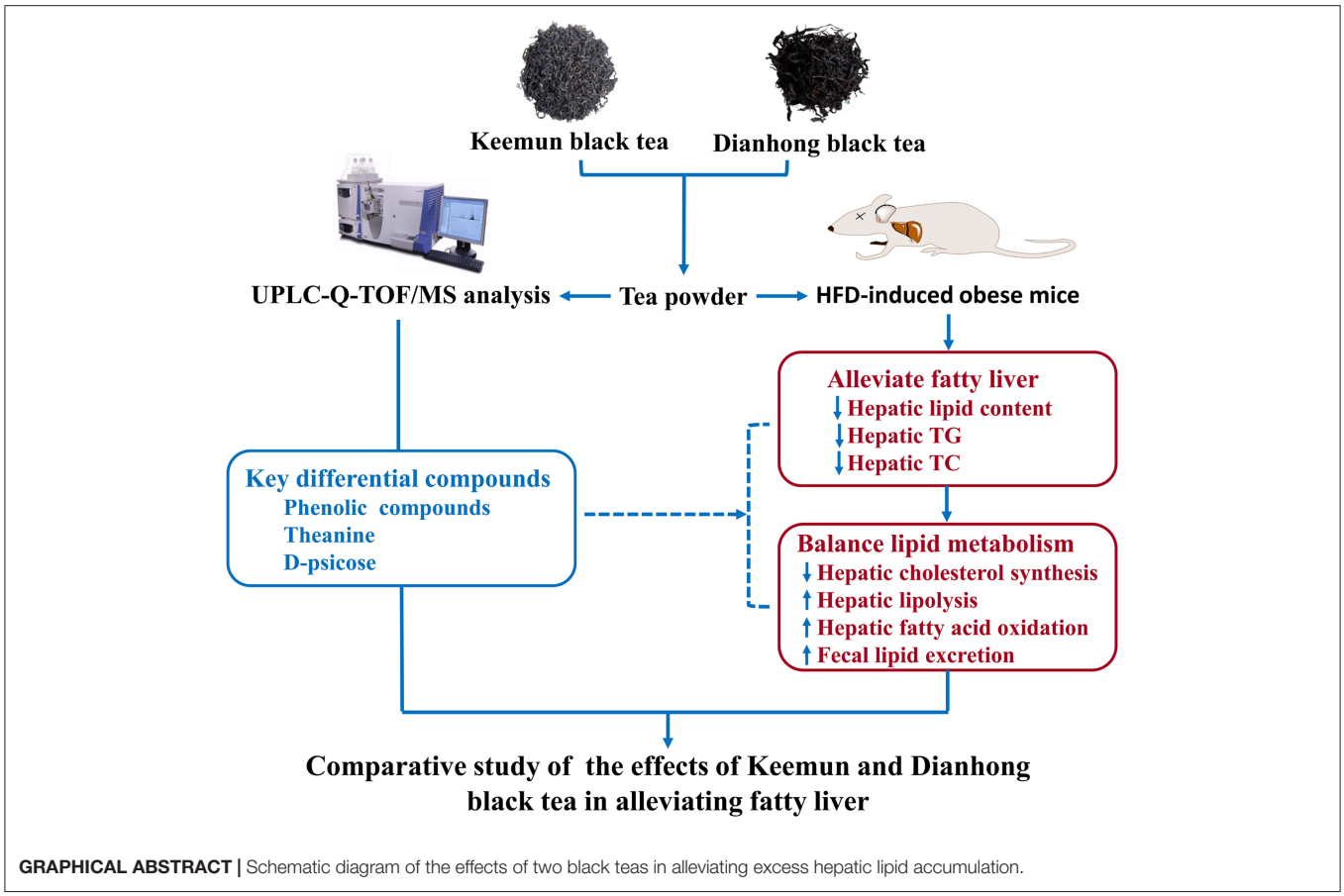
## INTRODUCTION

Fatty liver, which is characterized by excessive hepatic lipid storage, is closely related to high-fat diet (HFD)-induced obesity. Numerous pathophysiological mechanisms are involved in the development of obesity, and triglycerides and cholesterol in hepatic and intestinal tissues are crucial factors in the regulation of lipid metabolism and energy balance (1). Dietary control is considered a key strategy in the prevention of fatty liver (2). Natural phytochemicals, such as polyphenols, have demonstrated protective effects against fatty liver (3). Tea is popular worldwide, and its lipid-lowering and weight-reducing effects have been frequently reported in animal studies and human interventions (4).

Black tea is the most-consumed tea beverage in the world, and its health benefits, including anti-obesity anti-atherosclerosis properties and the prevention of fatty liver, have been frequently reported (5). As underlying mechanisms, tea inhibits fat synthesis and promotes fecal lipid excretion and fat oxidative decomposition (6). Black tea contains a large quantity of biologically active substances, such as catechins, theaflavins, thearubigins, theanine, alkaloids, phenolic acids,




**GRAPHICAL ABSTRACT |** Schematic diagram of the effects of two black teas in alleviating excess hepatic lipid accumulation.

and tea polysaccharides, which are the material basis of its efficacy. The chemical profiles of black tea categories differ greatly. They are influenced by the plant cultivar, garden environment, fresh leaf maturity, and manufacturing process among other factors (7). The genetic background of the tea plant is a vital. Taxonomically, the cultivated varieties of tea plant are generally classified into two groups: *Camellia sinensis* var. *assamica* and *C. sinensis* var. *sinensis* (8). Assam black tea from India and Dianhong black tea (DBT) from China are representative of the large-leaf cultivar *C. sinensis* var. *assamica*, and Keemun black tea (KBT) from China and Darjeeling black tea from India are produced by the small-leaf cultivar *C. sinensis* var. *sinensis*. Differences in the chemical compositions of black tea categories lead to different health benefits; however, few comparative studies have systematically investigated these differences in chemical profiles and efficacies.

In the present study, the chemical profiles of KBT and DBT and their effects in preventing HFD-induced fatty liver were examined. KBT is grown in Anhui province, China, and possesses unique floral and honey aromas (9). DBT is from Yunnan province, China, and has a higher phenolic content and strong, mellow aromas (10). Ultra performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF-MS/MS) was used to analyze the chemical compositions of KBT and DBT. Male C57BL/6 mice (7 weeks old) were fed different diets: a low-fat diet (LFD) or an HFD with or without black tea powder supplementation. The expression levels of key genes pertaining to lipid metabolism were measured using the PCR technique, and high-throughput sequencing was used to screen gut microbiota changes in fecal samples.

## MATERIALS AND METHODS

### Tea Samples

Fresh KBT and DBT leaves with the same maturity (one shoot and two young leaves) were picked in May 2019. The sample of KBT was processed and obtained from local tea factory (Qimen, Anhui, China), and DBT was processed and obtained from local tea factory (Yunnan, China). All samples were sealed stored at −20°C before analysis.

### Untargeted Metabolomic Analysis by LC-MS

Non-targeted metabolomic analysis was conducted as reported (11) with minor modifications. An Agilent 1290 liquid chromatography system connected to a time-of-flight mass spectrometer (Agilent, Palo Alto, CA) and an RP18 column (50 × 2.1 $mm^2$, 1.7 μm) (Waters, Milford, MA) was used to analyze the samples. The gradient elution, instrument parameters, and metabolomic analysis were as described by Guo et al.

(9). Qualitative and quantitative analyses were performed using the mass spectrometric data obtained. In brief, Optimus (Version 1.5.1) was employed to transform the original data obtained from LC-MS/MS. SMICA-P software (V14.1 Umetrics, Umea, Sweden), the Global Natural Products Social Molecular Networking platform, and Cytoscape (version 3.8.0) were used for multifactorial analysis and the identification of compounds. The final quantitative analysis was completed using MassHunter Qualitative Analysis (B.07.00).

## Animals, Diets, and Treatments

The animal experiment was conducted in compliance with institutional animal care guidelines and approved by the Committee of Anhui Agricultural University (approval number AHAU2019025). Forty-eight C57BL/6 mice (male, 6 weeks) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). Upon arrival, the mice were maintained in a specific-pathogen-free environment and reared in ventilated cages under controlled conditions ($25 \pm 2^{\circ}C$, $50 \pm 5\%$ relative humidity) with a 12-h light/dark cycle. All the animals had ad libitum access to diet and tap water during the entire rearing experiment.

The experimental groups received the following diets after acclimation for 1 week: (1) LFD (TP2330055BC), (2) HFD (TP2330055B, **Supplementary Table 1**), (3) HFD and 2% KBT powder (HFKB, HFD containing 2.0% KBT [w/w]), or (4) HFD and 2% DBT powder (HFDB, HFD containing 2.0% DBT [w/w]). The animal feed was provided by a commercial company (Trophic Animal Feed, Nantong, China) and kept under freezing temperature ($-20^{\circ}C$).

During the 15-week experiment, the body weight of the mice was measured weekly. Consumption of food and water was recorded every other day. Feces were collected every 2 weeks and stored at $-80^{\circ}C$. After 15 weeks of treatment, the animals were sacrificed under anesthesia with chloral hydrate (4%, w/w) by intraperitoneal (i.p.) injection. Blood was collected through cardiopuncture, and serum samples were obtained after centrifugation. Immediately afterward, liver and white adipose tissues were harvested and weighed. The small and large intestines were then collected, cut out, and rinsed in cold 0.9% saline, and the cecal contents were collected at the same time. All the samples were snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$ for subsequent analyses.

## Analysis of Blood Biochemical Parameters

Commercial kits (Jiancheng Technology, Nanjing, China) were used to determine the levels of serum lipids and aminotransferases. The measurements were conducted in strict accordance with manufacturer instructions.

## Hepatic Histochemical Analysis and Lipid Content Determination

For histochemical analysis, the liver samples were fixed in formalin and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed in compliance with the standard procedure, and the samples were examined at 200-fold magnification.

Lipids in the liver were extracted and measured according to our previous study (12), and the levels of hepatic triglycerides (TG) and total cholesterol (TC) were determined using the methods used with the serum samples.

## Total Fecal Bile Acid and Lipid Content Analysis

The total bile acids in the feces were extracted and determined according to the method described by Kim et al. (13). A commercial assay kit (Huili Biotech, Changchun, China) was used to determine the concentrations of bile acids extracted. The analysis of lipid content in feces was conducted identically to that for livers.

## Analysis of Gene Expression Through RT-qPCR

The total RNA of liver and small intestine tissues was extracted using an RNA extraction kit (Tiangen, Beijing, China), and reverse transcription was performed with a GoScript reverse transcription system (Promega, Madison, WI), following the manufacturer's instructions. The mRNA levels of hepatic and intestinal genes were measured with a PCR mix kit (Life Technologies, Carlsbad, CA), normalized to the expression level of β-actin, and calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of genes and primers were obtained and designed using the NCBI Gene Bank database and BLAS tools. The gene ID and primer sequences are presented in **Supplementary Table 2**.

## Short-Chain Fatty Acids and Gut Microbiota Analysis

The determination of fecal short-chain fatty acids (SCFAs) was done with reference to the method described by Tian et al. (14), with modifications. Briefly, an Agilent 7890A GC system connected to a flame ionization detector and an HP-INNOWAX column ($30\,m \times 0.25\,mm \times 0.25\,\mu m$, Agilent Technologies) was used. The column temperature was regulated as follows: $100^{\circ}C$ maintained for 1 min, then an increase of $5^{\circ}C$/min for 16 min to achieve a temperature of $180^{\circ}C$, which was maintained for 4 min.

The fecal genomic DNA was extracted with the TIANamp Stool DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The total bacterial DNA was sent to Novogene Co., Ltd. (Beijing, China) in dry ice. For high-throughput sequencing, the hypervariable region of the 16S rRNA (V3-V4) was selected for amplification. Sequences with a similarity of 97% according to UPARSE were clustered into operational taxonomic units, which were randomly subsampled (15). Alpha diversity analysis was performed to measure the complexity of species diversity. The species complexity in the samples was evaluated using beta diversity analysis. The alpha and beta diversities of both weighted and UniFrac results were assessed using R software (version 2.15.3 http://www.r-project.org/).

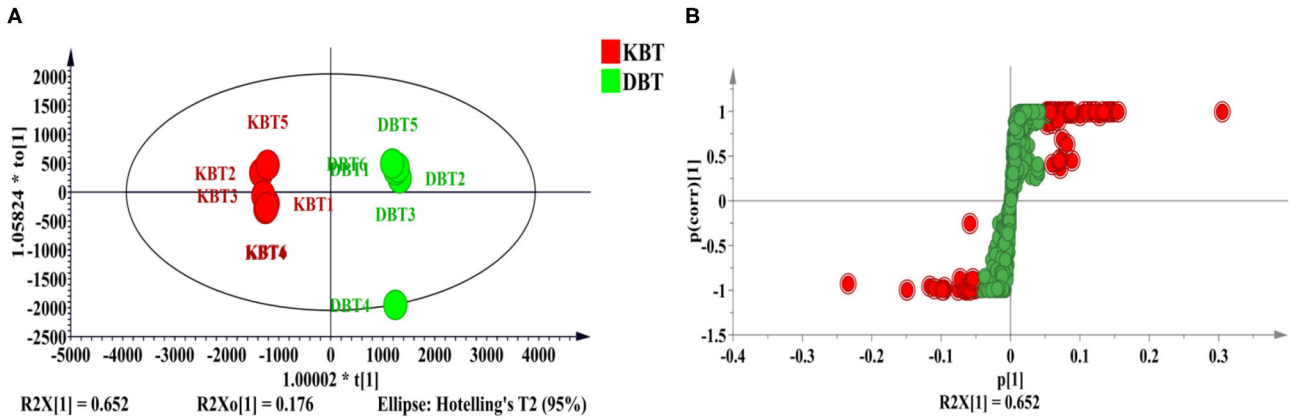



**FIGURE 1 |** Multivariate analysis of compounds detected in mass spectrometry. Keemun black tea (KBT), Dianhong black tea (DBT). **(A)** OPLS-DA; **(B)** S-plot.

## Statistical Analysis

The data are expressed as mean ± standard error of the mean. The multisample analysis was performed through one-way analysis of variance and Tukey's *post-hoc* test by using IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, NY). A difference was considered significant if $p < 0.05$.

# RESULTS

## Untargeted Metabolomic Analysis of KBT and DBT

To systematically examine the differences in the chemical profiles of KBT and DBT, UPLC-Q-TOF/MS was employed. Orthogonal partial least squares discriminant analysis (OPLS-DA) provided a clear classification for the two types of black tea (**Figure 1A**). Compounds with a VIP value ≥ 2 and $p[1]$ value in the S-plot > |0.05| were selected as the key differentiating compounds to distinguish KBT and DBT (**Figure 1B**). A total of 33 critical compounds in negative mode were screened and identified. As summarized in **Table 1**, three carbohydrates and carbohydrate conjugates, eight phenolic acids and contractive phenolic acids, three catechins, nine flavonoids, one tannin, two hydrolyzable tannins, two amino acids and derivatives, one gallic acid and derivatives, three citric acids or isomers, and one hyperoside were identified. Most of the 10 marker compounds with the highest VIP values were phenolic acids, flavonoids, theanine, hydrolyzable tannins, and D-psicose. A total of 25 compounds of the 33 critical ones were phenolic, indicating their vital roles in distinguishing these two categories of black tea.

## Effects of Dietary Black Tea on Visceral Fat Mass and Serum Lipids

UPLC-Q-TOF/MS analysis indicated that the contents of phenolic acids, catechins and flavonoids between KBT and DBT were different. To clarify the differences in the anti-obesity effects between KBT and DBT in HFD-fed mice, the body weight gain and visceral fat mass of the experimental animals were measured. As shown in **Figure 2A**, no significant differences were observed in the initial body weight of the four groups. After week three, HFD-treated mice had significantly higher body weight than did mice in the LFD group, and they eventually gained 81.4% more weight increase compared with the LFD-treated mice. Dietary DBT significantly decreased body weight by 30.7%. Although the body weight of HFKB-treated mice exhibited a decreasing trend compared with HFD mice at week 15, no significant difference was observed (food consumption of the four groups was comparable throughout the experimental period; **Supplementary Figure 1**). The HFD notably increased visceral fat mass, and HFKB and HFDB treatments significantly decreased perirenal adipose mass deposition by 22.8 and 32.8%, respectively (**Table 2**). No significant differences in mesenteric and epididymal adipose mass among the HFD, HFKB, and HFDB groups were noted (**Table 2**). Similarly, the HFD significantly enhanced the levels of serum lipid parameters (LDL-C, HDL-C, and TC), and black tea treatments failed to prevent these increases (**Supplementary Table 3**). Moreover, the serum TG levels of mice in the four groups were comparable.

## Effects of Dietary Black Tea on the Development of Fatty Liver

As shown in **Table 2**, the HFD significantly increased liver weight, and black tea treatments slightly but not significantly decreased the ratio of total liver weight to body weight. The levels of serum ALT in the mice in the HFD group were significantly (8.8-fold) higher than those in the LFD group, and HFKB and HFDB treatments significantly decreased this parameter by 32.3 and 40.9%, respectively (**Table 2**).

We also conducted liver histopathological analysis using slices stained with H&E. No sign of fatty liver was observed in the LFD mice (**Figure 2B**), and the hepatocytes of these samples were morphologically intact, with clear borders, and neatly arranged. In the HFD groups, numerous large fat vacuoles were observed, with the nucleus moving to one side and no clear boundary between cells (**Figure 2B**). Black tea treatments effectively prevented the development of HFD-induced fatty liver, especially in the HFDB-treated mice, whose liver slices

**TABLE 1 |** Compounds crucial in differentiating Keemun black tea and Dianhong black tea.

| No. | RT (min) | *m/z* | VIP | Identification | Classification |
|---|---|---|---|---|---|
| 1 | 0.84 | 191.059 | 11.41 | Quinic acid or it's isomer | Phenolic acids and contractive phenolic acids |
| 2 | 7.96 | 447.099 | 5.61 | Kaempferol-3-O-glucoside or it's isomer | Flavonoids |
| 3 | 0.5 | 173.092 | 5.61 | Theanine | Amino acids and derivatives |
| 4 | 7.69 | 463.091 | 5.54 | Isoquercitrin or it's isomer | Flavonoids |
| 5 | 4.32 | 633.078 | 5.35 | [(1R,21S,23R)-6,7,8,11,12,13,22,23-octahydroxy-3,16-dioxo-2,17,20-trioxatetracyclo[17.3.1.04,9.010,15]tricosa-4,6,8,10,12,14-hexaen-21-yl] 3,4,5-trihydroxybenzoate | Hydrolyzable Tannins |
| 6 | 7.71 | 739.214 | 5.24 | Kaempferol-3-O-galactoside-6"-rhamnoside-4"'-rha or it's isomer | Flavonoids |
| 7 | 3.09 | 337.098 | 5.01 | 3-O-Coumaroylquinic acid | Phenolic acids and contractive phenolic acids |
| 8 | 8.11 | 447.098 | 4.75 | Kaempferol-3-O-glucoside or it's isomer | Flavonoids |
| 9 | 6.49 | 635.095 | 4.62 | 1,2,3-Tri-O-galloyl-beta-D-glucose | Tannins |
| 10 | 0.54 | 179.06 | 4.51 | D-Psicose | Carbohydrates and carbohydrate conjugates |
| 11 | 0.5 | 145.065 | 4.35 | L-Glutamine | Amino acids and derivatives |
| 12 | 6.2 | 457.083 | 4.17 | Epigallocatechin gallate | Catechins |
| 13 | 1.24 | 169.018 | 3.93 | Gallic acid | Gallic acid and derivatives |
| 14 | 0.52 | 387.12 | 3.61 | Coniferyl alcohol + O-Hex | Carbohydrates and carbohydrate conjugates |
| 15 | 7.57 | 463.069 | 3.31 | Isoquercitrin or it's isomer | Flavonoids |
| 16 | 3.38 | 337.098 | 3.31 | 3-O-Coumaroylquinic acid or it's isomer | Phenolic acids and contractive phenolic acids |
| 17 | 4.95 | 337.098 | 3.28 | 3-O-Coumaroylquinic acid or it's isomer | Phenolic acids and contractive phenolic acids |
| 18 | 4.3 | 337.098 | 3.18 | Coumaroyl quinic acid | Phenolic acids and contractive phenolic acids |
| 19 | 3.77 | 353.093 | 3.01 | 3-O-Coumaroylquinic acid or it's isomer | Phenolic acids and contractive phenolic acids |
| 20 | 7.47 | 609.15 | 2.89 | Rutin or it's isomer | Flavonoids |
| 21 | 8.16 | 447.098 | 2.85 | Kaempferol-3-O-glucoside or it's isomer | Flavonoids |
| 22 | 6.91 | 457.08 | 2.85 | Epigallocatechin gallate | Catechins |
| 23 | 0.84 | 191.023 | 2.78 | Citric acid or it's isomer | Citric acid or it's isomer |
| 24 | 7.63 | 463.093 | 2.62 | Hyperoside | Flavonoids |
| 25 | 7.39 | 609.152 | 2.53 | Rutin or it's isomer | Flavonoids |
| 26 | 0.56 | 191.023 | 2.5 | Citric acid or it's isomer | Citric acid or it's isomer |
| 27 | 7.35 | 441.089 | 2.42 | Epicatechin gallate | Catechins |
| 28 | 0.56 | 191.06 | 2.39 | Quinic acid or it's isomer | Phenolic acids and contractive phenolic acids |
| 29 | 0.56 | 191.024 | 2.35 | Citric acid or it's isomer | Citric acid or it's isomer |
| 30 | 4.25 | 633.079 | 2.17 | [(1R,21S,23R)-6,7,8,11,12,13,22,23-octahydroxy-3,16-dioxo-2,17,20-trioxatetracyclo[17.3.1.04,9.010,15]tricosa-4,6,8,10,12,14-hexaen-21-yl] 3,4,5-trihydroxybenzoate | Hydrolyzable Tannins |
| 31 | 7.62 | 739.213 | 2.15 | Kaempferol-3-O-galactoside-6"-rhamnoside-3"'-rha or it's isomer | Flavonoids |
| 32 | 1.02 | 179.059 | 2.08 | Psicose | Carbohydrates and carbohydrate conjugates |
| 33 | 4.06 | 353.092 | 2 | Chlorogenic acid | Phenolic acids and contractive phenolic acids |

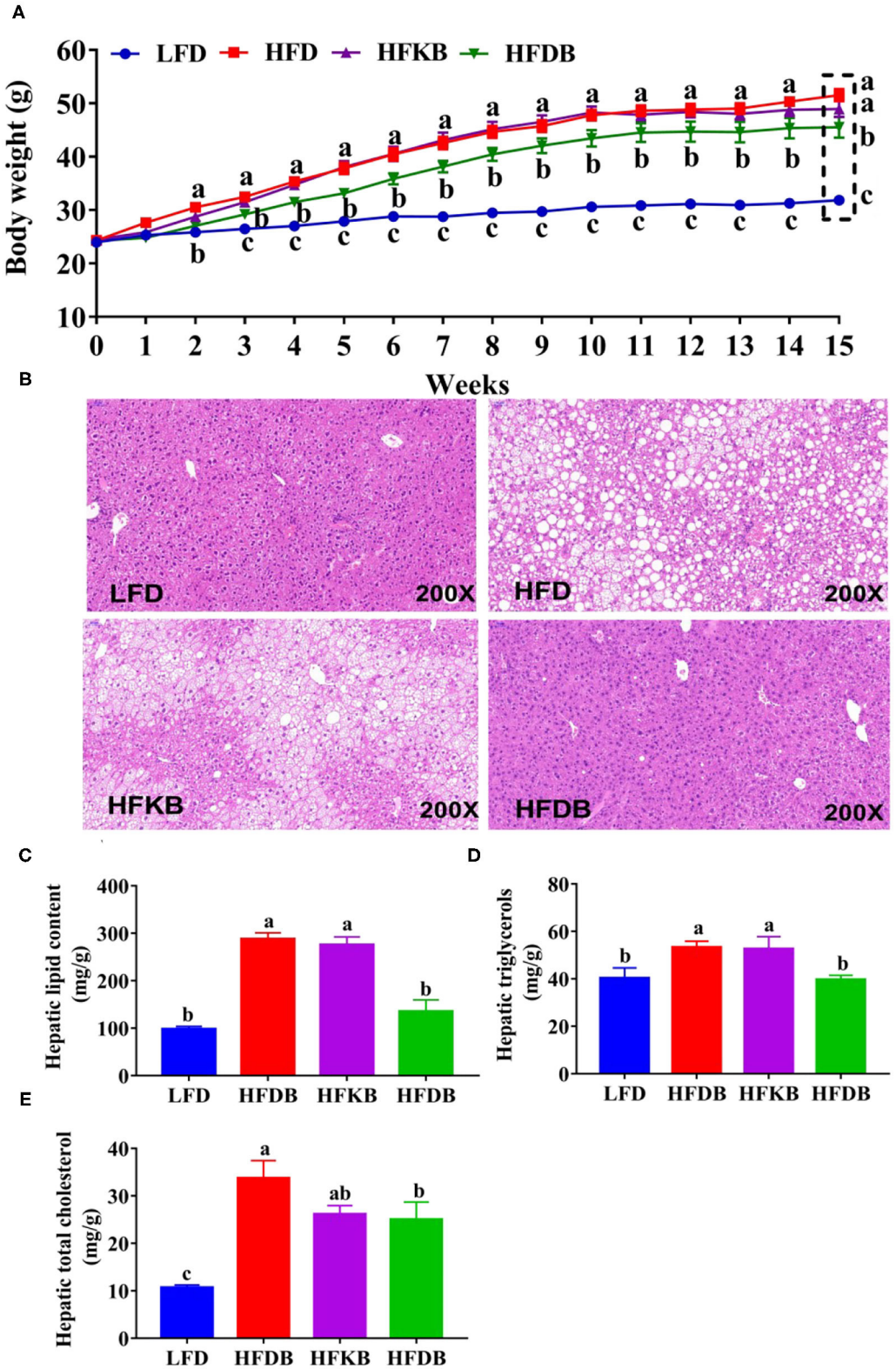



**FIGURE 2 |** Effects of black tea on body weight and fat accumulation in the liver. LFD, low-fat diet; HFD, high-fat diet; HFKB, HFD + KBT diet (containing 2.0% Keemun black tea); HFDB, HFD + DBT diet (containing 2.0% Dianhong black tea). **(A)** body weight; **(B)** hematoxylin and eosin–stained slices of liver; **(C)** total lipid content in the liver; **(D)** hepatic triglyceride content; **(E)** total cholesterol content in the liver. Data are presented as mean ± SEM ($n = 12$). $^{a,b,c}p$ represents significant differences among groups (ANOVA, $p < 0.05$).

**TABLE 2 |** Liver and visceral fat mass and concentrations of serum AST and ALT.

| | Percentage of body weight (%) | | | |
|---|---|---|---|---|
| | **LFD** | **HFD** | **HFKB** | **HFDB** |
| Perirenal adipose | $0.82 \pm 0.29^{c}$ | $3.41 \pm 0.47^{a}$ | $2.82 \pm 0.68^{b}$ | $2.56 \pm 0.71^{b}$ |
| Mesenteric adipose | $1.20 \pm 0.24^{b}$ | $3.08 \pm 0.56^{a}$ | $2.84 \pm 0.93^{a}$ | $2.80 \pm 0.96^{a}$ |
| Epididymal adipose | $2.70 \pm 0.77^{b}$ | $4.70 \pm 0.86^{a}$ | $4.32 \pm 1.34^{a}$ | $4.99 \pm 0.85^{a}$ |
| Liver | $4.02 \pm 0.67^{b}$ | $7.00 \pm 1.67^{a}$ | $6.97 \pm 1.71^{a}$ | $6.12 \pm 1.24^{a}$ |
| AST (U/L) | $29.11 \pm 2.91$ | $24.24 \pm 3.32$ | $19.39 \pm 4.65$ | $27.23 \pm 5.26$ |
| ALT (U/L) | $5.17 \pm 1.89^{c}$ | $50.67 \pm 16.06^{a}$ | $35.99 \pm 9.43^{b}$ | $32.04 \pm 8.13^{b}$ |

*AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values are expressed as mean ± SEM (n = 12). Different letters indicate significant differences (ANOVA, $p < 0.05$).*

were similar in appearance to those of LFD mice. Furthermore, total hepatic lipid, TG, and TC levels were significantly increased in the HFD groups relative to the LFD group (**Figures 2C–E**). Similar to the implications of the histopathological data, DBT treatment completely prevented the HFD-induced increase of hepatic total lipids and TG. DBT treatment also significantly decreased hepatic TC content by 37.9%. KBT treatment slightly but not significantly prevented lipid accumulation in the livers of experimental mice.

## Effects of Dietary Black Tea on Total Bile Acid and Lipids in Fecal Samples

To examine the effects of dietary black tea on intestinal fat absorption, we measured total bile acid and lipid levels in murine feces. The level of fecal total bile acids in the HFD groups was significantly lower than that of the LFD group. HFKB and HFDB slightly increased the fecal excretion of bile acids; however, no significant differences were observed (**Figure 3A**). Higher levels of fecal total lipids, TGs, and cholesterol were also observed in mice in the HFD group. Mice who underwent DBT treatment exhibited higher excretion of fecal lipids and TGs compared with the mice in the HFD group (**Figures 3B–D**). Similar to the aforementioned parameters, the fecal lipid–promoting effects of KBT were lower than those of DBT.

## Effects of Dietary Black Tea on Lipid-Metabolizing Gene Expression in the Liver

To further examine the prevention of excessive fat deposition in the liver by dietary black tea, the expression of lipid-metabolizing genes in the liver was measured. The mRNA level of hepatic HMG-CoA reductase (HMGR) was significantly decreased by DBT, which neutralized the effects of the HFD treatment (**Figure 4A**). However, neither black tea treatment altered the mRNA expression of stearoyl-CoA desaturase 1 (SCD1), fatty acid synthase (FAS), sterol regulatory element binding protein-1c (SREBP1c), acetyl-CoA carboxylase A (ACACA), or acetyl-CoA carboxylase B (ACACB; **Figure 4A**). The expression of SCD1, FAS, ACACA, and ACACB among the four groups was comparable. Notably, the HFD treatment enhanced the mRNA level of lipoprotein lipase (LPL) and suppressed the expression of adipose triglyceride lipase (ATGL) in the liver; the black tea treatments completely reversed the HFD-induced changes of these two genes. The gene expression levels of peroxisome proliferator-activated receptor-alpha (PPARα), carnitine palmitoyl transterase-1 (Cpt1α), and acyl-CoA oxidase (ACOX) in mice undergoing black tea treatments were significantly enhanced relative to those of the HFD group (**Figure 4B**). Unlike the effects in alleviating fatty liver, the alterations of the mRNA expression of hepatic lipid–metabolizing genes by KBT or DBT treatment exhibited no significant differences.

## Effects of Dietary Black Tea on Expression of Genes Related to Lipid Transport and Metabolism in the Small Intestine

Given the notable effect of dietary black tea in promoting fecal lipid excretion, we quantified the mRNA expression levels of genes involved in lipid transport and metabolization in the small intestine. The HFD significantly increased the mRNA levels of acyl-CoA cholesterol acyltransferase 2 (ACAT2), Cpt1α, and ACOX and decreased the gene expression of ATP-binding cassette subfamily G 5 (ABCG5), ATGL, and Niemann–Pick C1-like 1 (Npc1l1; **Figure 4C**; **Supplementary Figure 3A**). Moreover, DBT treatment significantly decreased the gene expression of ACAT2 and increased the mRNA levels of ABCG5 and ATGL, indicating its neutralizing effects on the alterations resulting from high-fat feeding. The alterations of these genes through the KBT treatment exhibited similar trends; however, no significant differences were observed. Neither black tea treatment changed the gene expression of Npc1l1. Moreover, the mRNA levels of mitochondrial functional protein (MTP) and ATP-binding cassette transporter A1 among the four groups were comparable (**Figure 4C**). Similarly, neither black tea treatment altered the mRNA expression levels of genes involved in fatty acid re-esterification, prechylomicron assembly and secretion, or fatty acid metabolism in the small intestine (**Supplementary Figures 2A–C**).

## Effects of Dietary Black Tea on Gut Microbiota

That gut microbiota are closely related to HFD-induced obesity and non-alcoholic fatty liver disease (NAFLD) has been well documented (16). The concentration of fecal SCFAs was analyzed using gas chromatography, and the colonic microbiota were profiled using 16S rDNA gene sequencing. Our data indicated that total SCFA content, acetate, propionate, and butyrate were markedly decreased by high-fat feeding; however, neither black tea treatment prevented the changes induced by the feeding (**Supplementary Table 4**). Chao 1 and ACE estimators and the Shannon and Simpson indexes were used to assess community richness and diversity, respectively. No differences in Chao 1 and ACE estimations or the Shannon and Simpson indexes were evident among the four groups (**Supplementary Table 5**). However, the HFD induced a

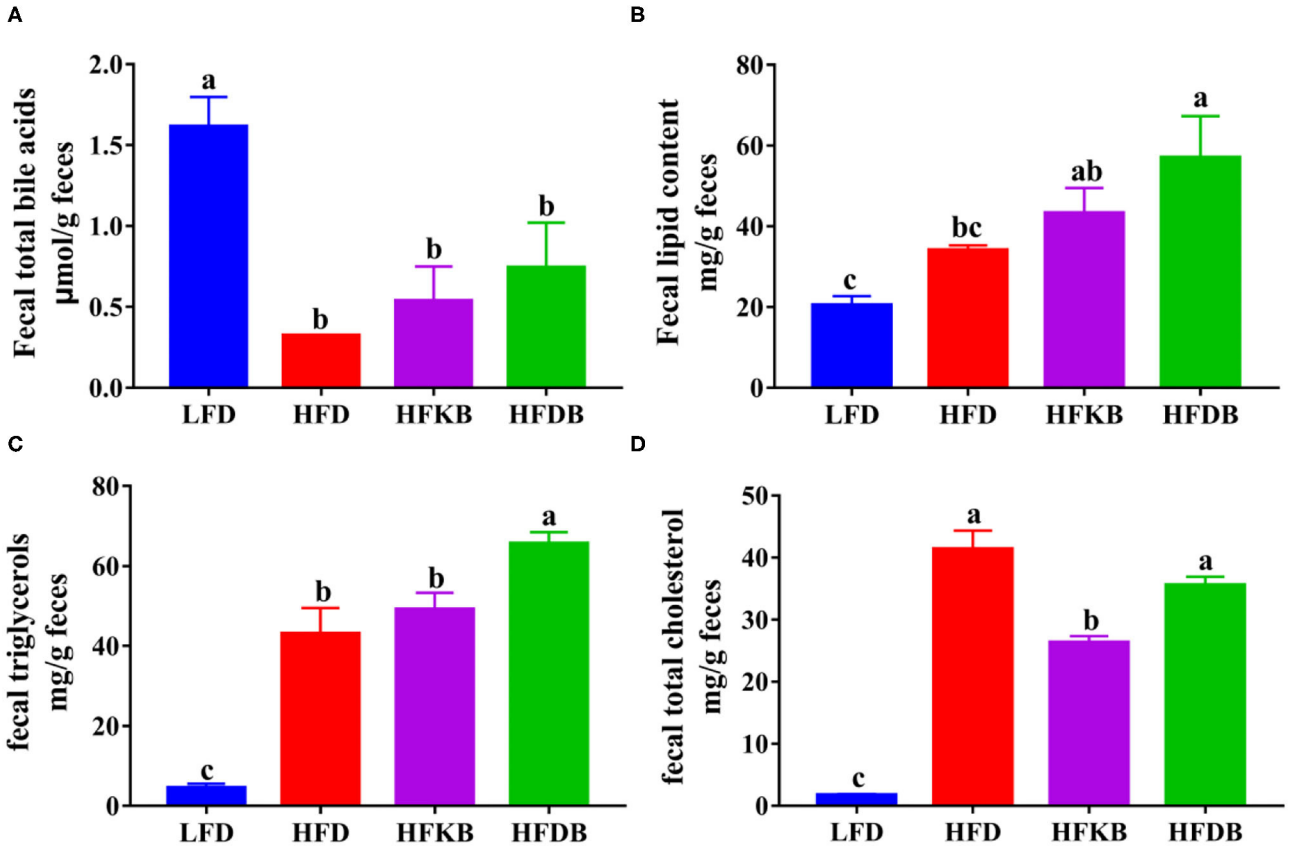



**FIGURE 3 |** Effects of black tea on fecal total bile acids and lipids. **(A)** fecal total bile acids; **(B)** fecal total lipids; **(C)** fecal triglycerides; **(D)** fecal total cholesterol. Data are presented as mean ± SEM ($n = 12$). $^{a,b,c}p$ represents significant differences among groups (ANOVA, $p < 0.05$).

dramatic shift in gut microbiota; the proportion of Firmicutes increased and that of Bacteroidetes decreased, and the Firmicute to Bacteroidete ratio was significantly increased in HFD mice compared with the LFD animals. However, black tea treatment did not significantly affect the ratio (**Supplementary Figure 3D**). Similarly, in the results of principal coordinates analysis and linear discriminant analysis effect size, black tea supplementation exhibited little impact on the modulation of intestinal microbiota (**Figure 5**).

## DISCUSSION

The health benefits of tea have become a hot topic in food science research (17). Black tea is the most consumed tea beverage worldwide. The major phenolic compounds of black tea are oxidized and dimerized to form theaflavins, which are of large molecular weight and not easily absorbed by the small intestine. The effects of black tea on fatty liver prevention in HFD-induced obese animals are well-established (18). Significant differences in chemical compositions are evident among categories of black tea; however, few studies have been conducted to systematically clarify the differences of chemical profiles and the potential health benefits of types of black tea. In the present study, two common categories of black tea in China were selected, and their chemical profiles and effects in alleviating excessive hepatic lipid depositions in mice fed an HFD were compared.

KBT and DBT are produced from a small-leaf tea plant cultivar (*C. sinensis* var. *sinensis*) and a large-leaf tea plant cultivar (*C. sinensis* var. *assamica*), respectively. In previous studies, the shoots and fresh leaves of large-leaf tea plant cultivars had higher contents of inclusion and more phenolic compounds than did small-leaf cultivars (19). Wang et al. (7) reported that tea polyphenols, flavonoids, and amino acids might play key roles in the differences between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*. In the present study, mass spectrographic analysis indicated that the marker compounds with the largest VIP values (≥2) between KBT and DBT were generally phenolic acids, catechins, and flavonoids, which is consistent with previous studies. Moreover, our former results (**Supplementary Table 6**) indicated that the amounts of total phenols, crude fiber, and tea polysaccharides in DBT were significantly higher than those in KBT, although the crude protein content did not differ. Therefore, in addition to small-molecule compounds (mainly phenolic compounds and theanine), tea polysaccharides and other macromolecular

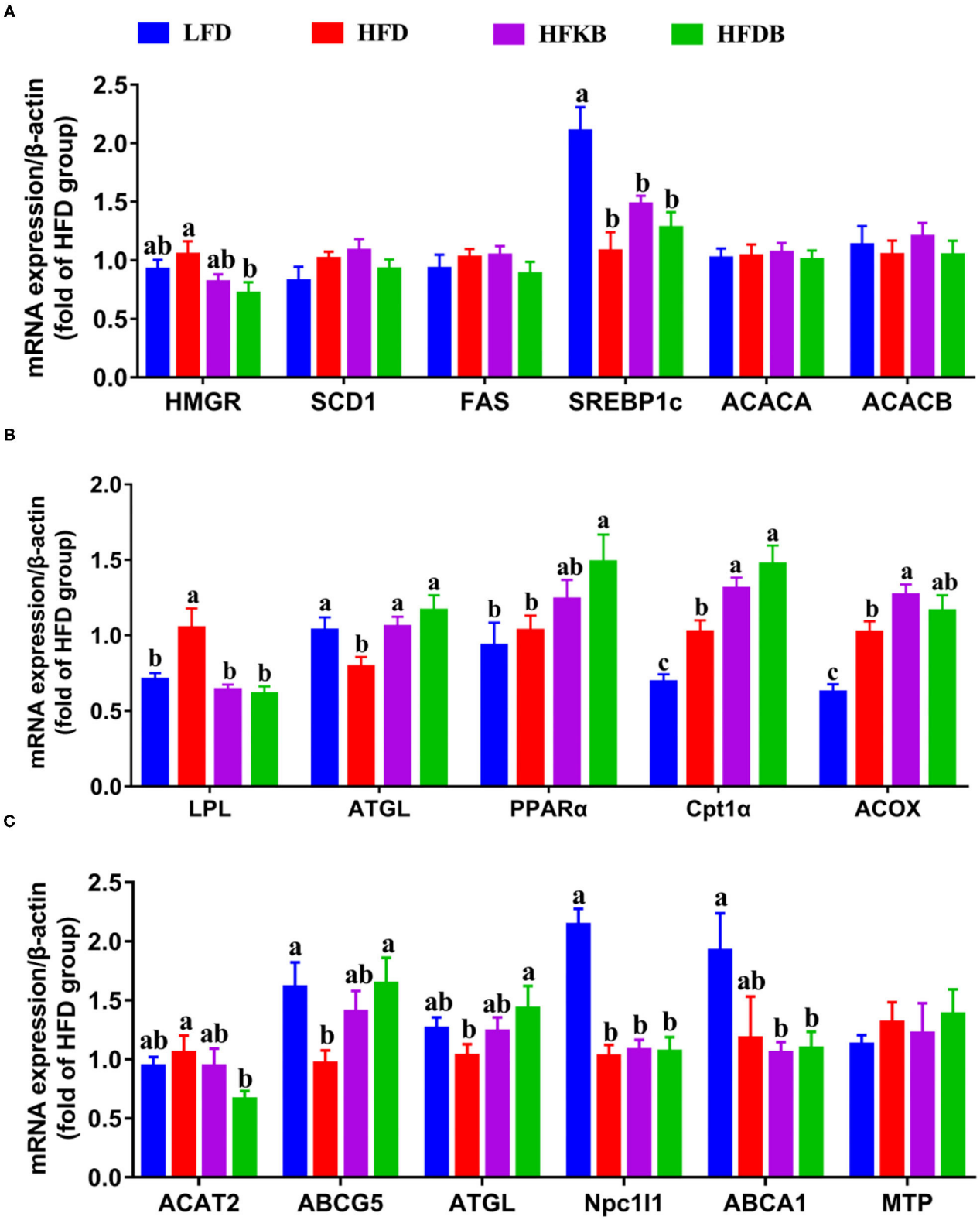



**FIGURE 4 |** Effects of black tea on mRNA levels of genes in the liver and the small intestine. **(A,B)**, hepatic genes; **(C)** genes in the small intestine. Data are presented as mean ± SEM ($n = 12$). $^{a,b,c}p$ represents significant differences among groups (ANOVA, $p < 0.05$).

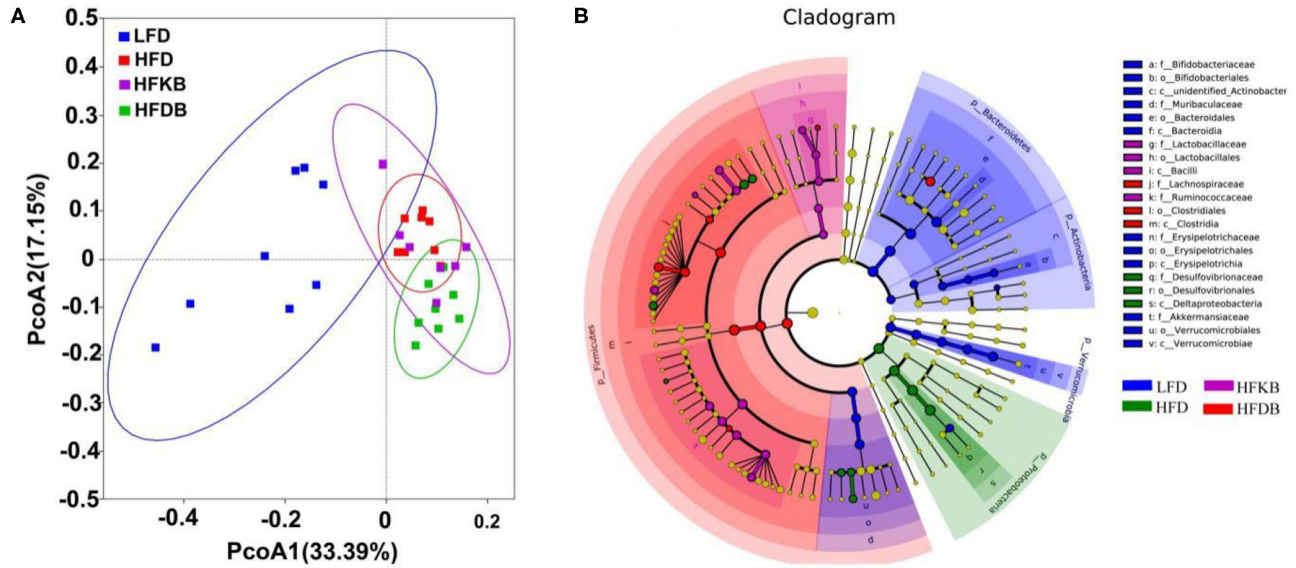



**FIGURE 5 |** Effects of black tea on overall microbial structure. **(A)** principal coordinates analysis; **(B)** linear discriminant analysis effect size comparison of microbiota for LFD, HFD, HFKB, and HFDB.

substances play vital roles in distinguishing between KBT and DBT.

Excessive fat consumption contributes to the development of obesity and NAFLD and causes hepatocellular damage and gut microbiota dysbiosis (20). The putative anti-obesity effects of tea have been most commonly ascribed to its rich bioactive compounds, especially the phenolic compounds (21). In the present study, HFD-treated mice exhibited the symptoms typical of obesity and NAFLD, and the results were consistent with those of other reports (20). Only dietary DBT significantly reduced body weight gain, perirenal adipose mass, serum ALT levels, and hepatic excess fat accumulation in mice fed an HFD, but these effects did not appear in the KBT group, with the exception of effects on perirenal adipose mass and serum ALT levels. Consistently, the significant alleviation of excessive fat accumulation and steatosis severity in the liver was observed only in the DBT-treated mice. We speculate that this phenomenon may be related to the differences in the levels of phenol compounds, theanine, and tea polysaccharides in the two types of tea.

Hepatic lipid metabolism disorder is a main cause of the development of fatty liver disease (22). We examined the expression of hepatic lipid–metabolizing genes. Both KBT and DBT significantly decreased the expression of hepatic HMGR, a rate-limiting enzyme in cholesterol biosynthesis (23), and the alteration of this gene might contribute to the decreased levels of hepatic cholesterol in black tea–treated mice. The excessive accumulation of hepatic cholesterol critically contributes to the pathogenesis of fatty liver (24). Our data demonstrated that dietary black tea was an effective approach to fatty liver prevention. Moreover, excess hepatic lipid accumulation usually reflects imbalances of fatty acid import and export, catabolism, and lipogenesis in the liver (25). In our study, the mRNA levels of genes involved in hepatic fatty acid *de novo* synthesis (FAS, ACACA, ACACB, and SCD1) were not significantly altered by black tea treatments. However, KBT and DBT significantly increased the mRNA expression of ATGL, PPARα, Cpt1α, and ACOX, which are related to fat lipolysis and fatty acid beta-oxidation. This result is consistent with a study in which Puerh tea treatment significantly increased mRNA levels of the transcription factor and enzymes involved in fatty acid oxidation, including PPARα, Cpt1α, and ACOX in HFD-induced obese mice (26). Notably, both black tea treatments completely restored the HFD-induced increase of LPL gene expression. LPL is an enzyme vital in catalyzing the hydrolysis of triglycerol from circulating chylomicrons or very low-density lipoproteins (27). The enhanced mRNA level of LPL in hepatic stellate cells increases the uptake of cholesterol from serum lipoproteins, which induces an increase in TLR4 signaling and the exacerbation of liver fibrosis (28). The decreased LPL mRNA levels evident with dietary black tea treatment indicated the role of LPL mRNA level in restraining the entry of free fatty acids and cholesterol into hepatic cells. Overall, the hepatic gene results suggested that dietary black tea might prevent the development of fatty liver by decreasing the synthesis of cholesterol, stimulating fat lipolysis and fatty acid oxidation, and inhibiting the absorption of cholesterol and free fatty acids from circulation. The alteration of hepatic lipid metabolism by KBT was comparable to that by DBT.

Our data also indicated that black tea treatments significantly increased fecal lipid excretion. We measured the expression of key genes related to the absorption, re-esterification, transport, and metabolism of lipid molecules in the small intestine. ACAT2 is a key enzyme for the re-esterification of cholesteryl ester, and the loss of ACAT2 results in defective cholesterol absorption (29). ABCG5 is a heterodimer involved in the transport of

cholesterol from hepatocytes into the bile and from enterocytes into the intestinal lumen (30). Black tea treatments, especially DBT, significantly decreased the mRNA level of intestinal ACAT2 and increased the expression of ABCG5, which was notably suppressed by the HFD. DBT also effectively restored the reduced mRNA levels of intestinal ATGL in high-fat-feeding mice. These results implied that dietary DBT is more effective than dietary KBT in decreasing cholesterol absorption and promoting cholesterol efflux and lipolysis in the small intestine. However, the expression of most genes involved in the re-esterification, transport, and metabolism of intestinal lipid molecules was not altered by black tea supplementation. Our results indicated that black tea treatments did not alter the absorption of fat, especially the fatty acids that originate from neutral fat, which accounts for ∼95% of dietary lipids. The inhibition of lipid digestion in the small intestine might be the main mechanism for the enhanced fecal fat excretion in black tea–treated mice. Previous studies have shown that dietary tea powder, tea polyphenols, and epigallocatechin gallate can decrease the activity of pancreatic lipase and alter the emulsification of dietary lipids (31). Therefore, black tea supplementation significantly stimulated fecal fat excretion but did not alter the absorption of lipid molecules in the small intestine. The stimulatory effects of DBT on fecal lipid excretion were significantly stronger than those of KBT, and this might be the primary contributor to the differences between the two black teas in preventing the development of fatty liver.

In the past decade, the relationship between the development of fatty liver and the alteration of the gut microbiota has been frequently reported in several lines of studies (32). As the metabolites of microorganisms, the composition and amounts of fecal SCFAs can reveal the status of the gut microbiota (33). Our data demonstrated that the HFD significantly decreased the amount of intestinal SCFAs, and black tea supplementation did not recover the production of fecal SCFAs. Similarly, the amelioration of the Firmicutes to Bacteroidetes ratio was not observed in black tea–treated mice. Furthermore, black tea supplementation did not change the α- and β-diversity of intestinal microbiota, indicating that intestinal microbes made no significant contribution to the improvement of fatty liver observed in our study. The reason for these intricate results may be related to the cycle of the high-fat model and the dosage of the black teas.

Our results demonstrated that DBT was more effective than KBT in alleviating excessive hepatic lipid accumulation in mice fed an HFD. In UPLC-Q-TOF-MS/MS data, the compounds crucial in differentiating KBT and DBT were quinic acid, kaempferol-3-O-glucoside or its isomer, theanine, isoquercitrin or its isomer, and others, and these may be key factors in the differences in the health benefits of the teas. Quinic acid and chlorogenic acid are phenolic acids, and either can effectively prevent fatty liver disease through the alteration of fat metabolism in the liver and other organs (34). Kaempferol-3-O-glucoside, isoquercitrin, rutin, and hyperoside are active flavonoids in black tea and can prevent fatty liver by inhibiting lipogenesis and facilitating fatty acid metabolism (35). Theanine and L-glutamine are amino acids that effectively ameliorated lipid metabolism disorders in male Sprague–Dawley rats and C57BL/6J mice (36). D-psicose is a new-generation sugar substitute that suppressed lipogenesis and stimulated fatty acid oxidation in Wistar rats (37). As one of the widely studied active compounds in black tea, phenolic acids are considered to be the crucial compounds of health benefits, including catechins (38), gallic acid (39) and other compounds found in black tea, is likely to be the most crucial compounds responsible for the stronger beneficial effects of DBT than KBT in the prevention of fatty liver, which is a limitation that need to be addressed in additional studies. Besides, tea polysaccharide is also an important functional ingredient in black tea, and whether the content and structure of tea polysaccharide play an important role in promoting the gut health warrants further studied. Collectively, these results indicate that most of the compounds crucial to the differentiation of the KBT and DBT samples play vital roles in the prevention of fatty liver. However, the compound or combination of compounds that is the key factor in the differences in the health benefits of the two black teas is not yet clear.

## CONCLUSION

In conclusion, most of the compounds differentiating KBT and DBT are phenolic compounds, theanine, and D-psicose. DBT was more effective than KBT in preventing excess fat accumulation in the liver of mice fed an HFD. Both black tea treatments effectively and comparably altered the mRNA levels of hepatic genes involved in cholesterol synthesis, fat lipolysis, fatty acid beta-oxidation, and the absorption of free fatty acid and cholesterol from circulation. DBT treatment exhibited more favorable effects in stimulating fecal fat excretion than did KBT treatment, and this may be the primary factor in the different health-promoting effects of the two tea treatments in this study. The different compounds with the higher VIP values might make the main contributions to the different health benefits; however, the most important compound or combination of compounds requires clarification.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI Trace Archive NCBI Sequence Read Archive repository, accession number PRJNA804701.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Anhui Agricultural University.

## AUTHOR CONTRIBUTIONS

WL: investigation, methodology, data curation, and writing - original draft. SL: investigation, methodology, and data curation.

YC and YK: investigation and methodology. DW and TL: methodology and writing-review and editing. YW and IK: writing-review and editing. ZX: funding acquisition and writing-review and editing. JH: conceptualization, supervision, funding acquisition, and writing-review and editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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REVIEW
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# L-Theanine: A Unique Functional Amino Acid in Tea (*Camellia sinensis* L.) With Multiple Health Benefits and Food Applications

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Tea (*Camellia sinensis L.*) is a very popular health drink and has attracted increasing attention in recent years due to its various bioactive substances. Among them, L-theanine, a unique free amino acid, is one of the most important substances in tea and endows tea with a special flavor. Moreover, L-theanine is also a bioactive compound with plenty of health benefits, including antioxidant, anti-inflammatory, neuroprotective, anticancer, metabolic regulatory, cardiovascular protective, liver and kidney protective, immune regulatory, and anti-obesity effects. Due to the unique characteristics and beneficial functions, L-theanine has potential applications in the development of functional foods. This review summarized the influencing factors of L-theanine content in teas, the main health benefits and related molecular mechanisms of L-theanine, and its applications in food, understanding of which can provide updated information for the further research of L-theanine.

**Keywords: L-theanine, tea, health benefits, mechanisms of action, food applications**

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## INTRODUCTION

Tea (*Camellia sinensis* L) is originated from China and is one of three major popular beverages in the world (1). Fresh tea leaves need to go through various processing procedures to be made into tea products prior to consumption. The processing operations, such as fermentation and baking, can change the color, aroma, taste, and chemical composition of tea (2, 3). Based on the degree of fermentation, tea can be divided into six categories, including green, yellow, white, oolong, black, and dark teas (4). Tea is rich in diverse chemical components, endowing tea with multiple beneficial functions (5, 6).

L-theanine, a non-protein water-soluble amino acid, is characteristically found in tea plants (7, 8). It is a unique taste component with caramel flavor, which can alleviate the bitterness of caffeinel

(9). As a unique secondary metabolite in tea, L-theanine is the main source of tea flavor (10). L-theanine was proved to contribute to the generation of tea volatiles, which may be the main source of the crispy-rice-like smell and the chestnut-like balminess (11, 12). It can be used as one of the significant indexes to estimate the freshness of tea (13). In addition, it has many health benefits, such as antioxidant, anti-inflammatory, neuroprotective, anti-cancer, anti-anxiety, metabolic regulatory, cardiovascular protective, liver and kidney protective, and immune regulatory effects (14–16). Due to its flavor and diverse health benefits, L-theanine has wide applications, such as being used as a beverage ingredient or dietary supplement (17).

In our current review article, high-quality literature published in recent 5 years was collected from the Web of Science Core Collection and PubMed databases. The influence factors of L-theanine content in tea and its effect on tea fragrance were first introduced, and its health benefits were then summarized, with intensive discussion about the molecular mechanism of actions, and finally, its practical applications in foods were briefly introduced. We hope that this review paper can provide an updated understanding of L-theanine and support its wide applications in the development of L-theanine-based functional foods.

## INFLUENCING FACTORS OF L-THEANINE CONTENT IN TEA

L-theanine is widely distributed in different parts of the tea plants, and the content is different. L-theanine could be first produced in the roots of tea plants and then transported to the shoots (18). Its content in roots could be up to 6% of dry weight (19). Another study reported that tea leaves and roots had higher L-theanine contents than stems (20). Different influencing factors of L-theanine content in tea leaves are summarized in **Figure 1**.

Firstly, L-theanine content is variable among different tea categories. Through the quantitative analysis of 37 different varieties of tea, the average content of L-theanine in green, white, oolong, and black teas were 6.56, 6.26, 6.09, and 5.13 mg/g, respectively (21). Moreover, the content of L-theanine in albino yellow tea was higher than that in normal green tea, and the accumulation mechanism of albino yellow tea was associated with the slow catabolism of L-theanine (22). Secondly, L-theanine content was related to the expression of its metabolism-related genes. Among 17 identified genes related to L-theanine metabolism, the transcription levels of *GsTS2*, *GsGS1*, and *GsGDH2* were positively correlated with L-theanine content, while most other genes were negatively correlated (20). Thirdly, temperature and season also affect L-theanine content to a certain extent. It was found that melatonin could accelerate the photosynthesis of tea plants and increase the biosynthesis of L-theanine in tea leaves under sub-high temperature (35/30°C) (23). A combination of transcriptomics and metabolomics analysis showed that L-theanine content in spring was significantly higher than that in summer and autumn (24). Consistently, quantitative determination of 58 Chinese white tea showed that L-theanine content in the early spring-produced silver needle white tea was higher than that in the late spring-produced white peony white tea and autumn produced Shoumei white tea (25). Further analysis showed that the change of L-theanine content in different seasons was due to the effects of sunshine intensity on the photosynthesis of tea plants and then the expression of main transcription factors and structural genes (24). Finally, the content of L-theanine was affected by the growth period. Taking the leaves of tea at different stages (bud, 1st leaf, 2nd leaf, 3rd leaf, and old leaf) as the research object, it was found that the content of bud and 1st leaf was the highest and the content of L-theanine in leaves decreased gradually with the leave maturity (20). In addition, by comparing the fresh Jukro tea leaves at the growth stage of 40, 60, and 90 days, it was found that the content of L-theanine was the highest in the 60-day leaves (13).

To sum up, the content of L-theanine in tea leaves was higher. Even the content of L-theanine in the same tissue would vary due to several influencing factors. Among the six categories of tea, the content of L-theanine in albino yellow tea was the highest. Even in the same category of tea, L-theanine content also showed significant differences among genetic background, temperature and season, and growth periods.

## HEALTH BENEFITS OF L-THEANINE

L-theanine exhibits a variety of health benefits, such as antioxidant, anti-inflammatory, neuroprotective, anticancer, metabolic regulatory, cardiovascular protective, liver, and kidney protective, immune regulatory, as well as urogenital and intestinal protective effects, which are summarized in **Supplementary Table 1** and briefly discussed below, with highlights about the potential mechanism of action (**Figure 2**).

### Antioxidant Activity

Recent studies reported that L-theanine exhibited good *in vitro* and *in vivo* antioxidant activities. In a neuronal-like rat pheochromocytoma cell model stimulated by cadmium oxide, L-theanine could reduce the synthesis of reactive oxygen species (ROS) and enhance the activity of antioxidant enzymes to weaken oxidative damage (26). It was reported that L-theanine showed antioxidant effects through adjusting the non-enzymatic activities, enhancing the activities and mRNA expression of catalase (CAT), and increasing superoxide dismutase (SOD) and glutathione peroxidase 1 (Gpx1) in enterotoxigenic *Escherichia coli* (ETEC)-infected mice (17). In a haloperidol (HAL)-induced rat model of orofacial dyskinesia (OD), the main feature of tardive dyskinesia (TD), it was found that L-theanine might have protective effects on OD due to its formidable antioxidant properties (27). In another study, L-theanine treatment reduced the levels of lipid peroxide and nitric oxide (NO) in OD rat models induced by HAL, thus improving the antioxidant capacity of the striatum (28). Combined with the existing evidence of animal studies, L-theanine may be able to treat human TD clinically through its antioxidant activity and regulating the activity of NO.

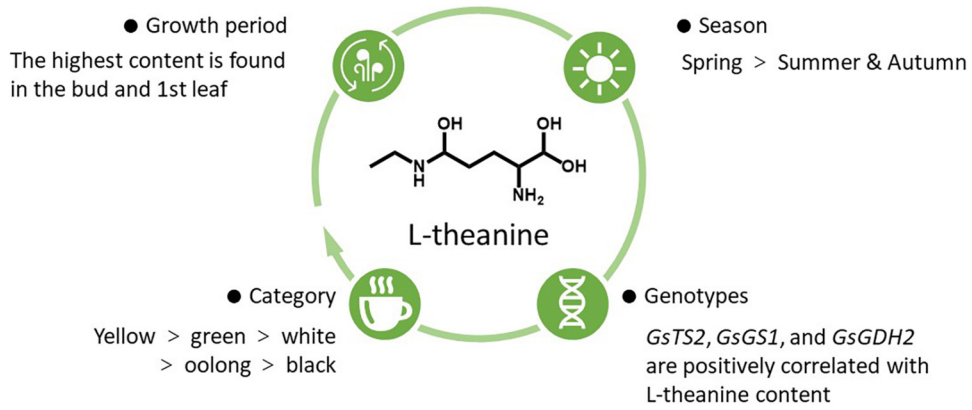


**FIGURE 1 |** Influencing factors of L-theanine content in tea leaves.

Generally, L-theanine has strong antioxidant activity, which can be associated with the regulation of the expression and activities of antioxidant-related enzymes.

## Anti-inflammatory Activity

The anti-inflammatory activity of L-theanine has been verified *in vitro* and *in vivo*. Through establishing an interleukin (IL)-1β-stimulated chondrocytes, it was found that L-theanine could inhibit the nuclear factor kappa B (NF-κB) pathway, thereby reducing the expression of proinflammatory factors, including cyclooxygenase-2 (COX-2), prostaglandin E2, inducible nitric oxide synthase, as well as NO, and protect the degradation of extracellular matrix (29). At the same time, L-theanine also significantly relieved osteoarthritis (OA) lesions in the anterior cruciate ligament transection-induced OA rat models (29). In addition, it was found that in 12-O-tetradecanoylphorbol-13-acetate (2.5 μg/ear)-induced ear edema mouse models, L-theanine could downregulate the expression of platelet endothelial adhesion molecule-1 (PECAM-1) and decrease the production of pro-inflammatory factors, including IL-1β, tumor necrosis factor-alpha (TNF-α), and COX-2, which were significantly expressed in neutrophils, thus improving the infiltration and activation of neutrophils (30). L-theanine was found to inhibit inflammation in rats with inflammatory bowel disease (IBD) induced by dextran sulfate sodium (DSS), and L-theanine treatment (200 mg/kg/day) could improve DSS-induced IBD through the molecular mechanisms related to cholesterol and retinol metabolism (31). In addition, the study on DSS-induced colitis in C57BL/6J male mouse models also confirmed that L-theanine could effectively inhibit intestinal inflammation (32). In rat models with intestinal stress induced by enterotoxigenic ETEC infection, the combined treatment of L-theanine with L-glutamine significantly decreased the expression of inflammatory factors, such as IL-1β, IL-6, and TNF-α (33). L-theanine was also found to reduce inflammation in lipopolysaccharide-induced mouse models, by normalizing the hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis and reducing the expression of inflammatory factors, including IL-1β, TNF-α, and IL-6, via inhibiting the NF-κB pathway (34). In ovalbumin-induced mouse asthma models, L-theanine treatment could reduce the transport of inflammatory cells to bronchoalveolar lavage fluid (BALF) and inhibit the infiltration of inflammatory cells via blocking the activation of NF-κB pathway and its downstream production of ROS, monocyte chemoattractant protein-1 (MCP-1), IL-4, IL-5, IL-13, TNF-α, and interferon (IFN)-γ in BALF (35).

In summary, the anti-inflammatory activity of L-theanine can be associated with inhibiting the expression of inflammatory factors and inflammation-related signaling pathways.

## Neuroprotective Effect

L-theanine has been reported with excellent neuroprotective effects on neuro injury. In a cell-based model induced by excessive dopamine, L-theanine exhibited neuroprotective effects on neuronal injury through the release of body fluid molecules from astrocytes, such as glutathione (36). Pretreatment of multipotential neural stem cells (NSCs) and C57BL/6J mice with L-theanine showed that L-theanine could alleviate the injury of NSCs induced by isoflurane and cognitive dysfunction of young mice, and the mechanism was related to the Akt/glycogen synthase kinase 3 beta (GSK3β) signaling pathway (37). In addition, since L-theanine was shown to have a relaxing effect and gamma-aminobutyric acid (GABA) was an important inhibitory neurotransmitter, the mixture of L-theanine and GABA had a positive synergistic effect on sleep behavior, including sleep quality and duration in caffeine-induced awake rats, and the mechanism might be that the mixture could promote the expression of GABA receptor, which was conducive to sleep (38). In another study, the mixture of L-theanine and Neumentix proprietary spearmint extract also regulated sleep disorders, prolonged sleep duration, significantly increased brain acetylcholine (Ach) and GABA concentrations, and decreased serotonin (5-HT) concentrations (39). Using the rat models of spinal cord injury (SCI), it was found that L-theanine could promote the recovery of behavioral motor function after SCI, and its potential neuroprotective mechanism may be related to the inhibition of posttraumatic oxidative reaction, neuroinflammation, and apoptosis (40). In rat

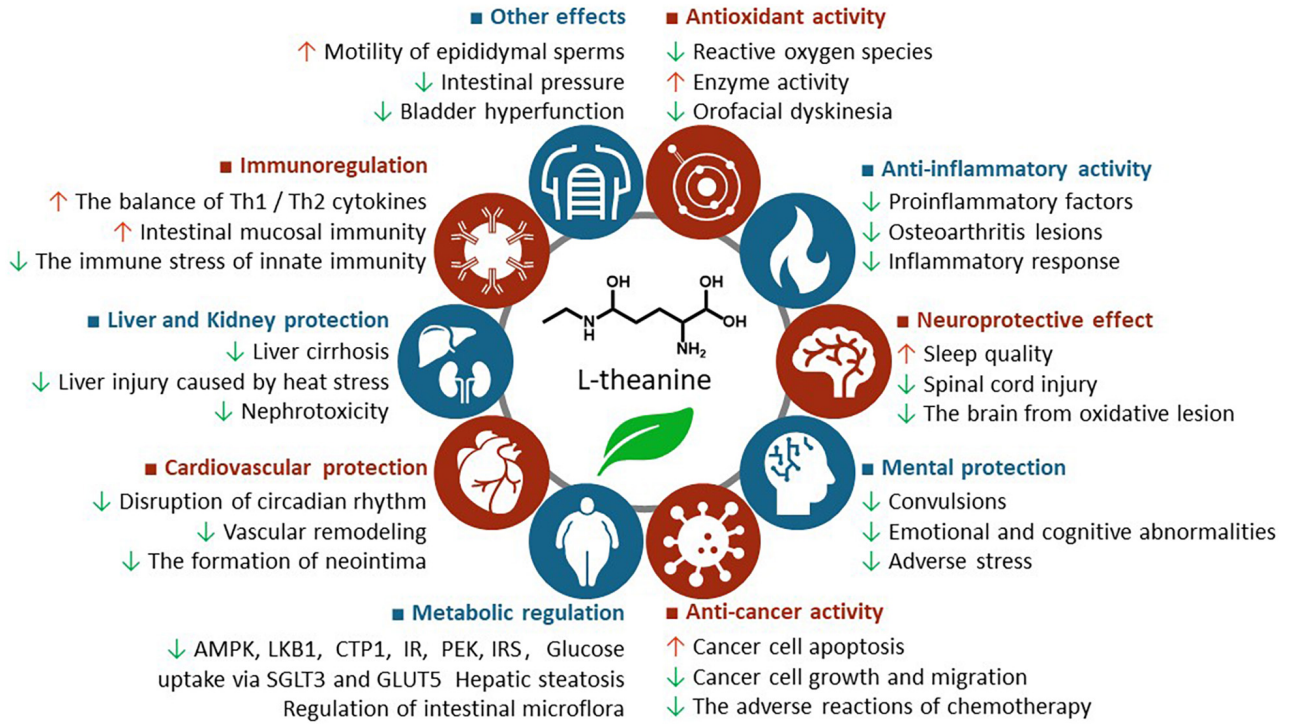


**FIGURE 2 |** The health benefits of L-theanine. AMPK, adenosine 5′-monophosphate-activated protein kinase; CPT1, carnitine palmitoyltransferase 1; IR, insulin receptor; IRS, insulin receptor substrate; LKB1, liver kinase B1; PFK, phosphofructokinase.

models of orofacial dyskinesia induced by reserpine, L-theanine showed potential neuroprotective activities by reducing oxidative damage, neurotransmitter deficiency, neuroinflammation, and apoptosis (41). Moreover, by intraperitoneal injection of Aroclor 1254, brain oxidative stress and neurobehavioral changes were induced in rats (42). On this basis, the oral administration of L-theanine (200 mg/kg BW) could repair the normal brain structure, downregulate the expression of inflammatory cytokines, so as to protect the brain from the oxidative lesion (42). Further study on the brain injury model of mice induced by Cadmium (Cd) showed that L-theanine could protect mice from Cd-induced neurotoxicity, which was achieved by reducing the level of Cd in the brain and plasma, inhibiting the death of neurons in the cortex and hippocampus, improving the activities of SOD, GSH, and CAT in the brain, and most importantly, significantly alleviating the hyperphosphorylation of tau protein Ser199, Ser202, and Ser396 (26).

In addition, L-theanine could be cooperated with other substances to protect from neuro injury. A composite membrane was prepared by chemical grafting of L-theanine with graphene oxide, and it promoted the survival, proliferation, and neuronal differentiation of neural stem cells, suggesting that it might be used in the treatment of central nervous system injury (43). L-theanine and cystine, as supplements, performed well in the prevention of oxaliplatin-induced peripheral neuropathy in mouse models (44). This effect was further verified in human studies. Through the treatment of 28 patients with colorectal cancer, it was shown that daily oral intake of L-theanine and cystine could effectively reduce the damage of oxaliplatin-induced peripheral neuropathy. This was mainly because that oral intake of L-theanine and cystine could promote the synthesis of glutathione, which was a potential substance to prevent neuropathy (45). Furthermore, in a model of brachial plexus root avulsion created in Sprague Dawley (SD) rats, L-theanine combined with NEP1-40 observably accelerated nerve regeneration after brachial plexus root avulsion (46).

On the other hand, L-theanine has also been shown to be effective in treating neurodegenerative diseases. L-theanine could alleviate the memory impairment of the aging mouse by upregulating janus kinase 2 (JAK2)/activator of transcription 3 (STAT3), M1 muscarinic cholinergic receptor (mAChR), and extracellular signal-regulated kinase (ERK) signaling (47). L-theanine combined with luteolin could prevent symptoms similar to Alzheimer's disease (AD) in rat models injected with amyloid-β (25–35) into the hippocampal CA1 region, and this was mainly associated with the improvement of hippocampal insulin signaling, norepinephrine metabolism, and the mitigation of neuroinflammation (48). L-theanine could also relieve the memory impairment and save the damage of hippocampal long-term potentiation in AD mice by activating the dopamine D1/5 receptor-protein kinase A pathway (49). Similarly, in the rat models of human Huntington's disease (HD) induced by quinolinic acid, L-theanine alone could reduce the changes caused by QA

(50). In rat HD models, neuropathological changes in the rat striatum were induced by 3-nitropropionic acid, and L-theanine exhibited neuroprotective effects, which mainly depended on not only inhibiting the production of harmful NO but also preventing the change of neurotransmitters in the striatum (51).

Therefore L-theanine has a good neuroprotective effect not only on improving cognitive and memory impairment, but also on preventing peripheral neuropathy and repairing nerves, and has a prominent influence on some neurodegenerative diseases, like AD and HD.

## Mental Protection

L-theanine also has positive effects on mental health. In mouse models, it was found that L-theanine could improve the anticonvulsive effect of pentobarbital sodium in a dose-dependent manner (52). In a mouse model of psychosocial stress, it was found that green tea had an anti-stress effect, which was due to the synergistic effect of L-theanine, epigallocatechin, and arginine, thus eliminating the antagonistic effect of caffeine and epigallocatechin gallate on psychological stress-induced adrenal hypertrophy (53). In adolescent male rat models exposed by the Delta-9-tetrahydrocannabinol (THC), L-theanine could strongly block the development of emotional and cognitive abnormalities associated with adolescent THC exposure, since L-theanine pretreatment could intercept THC-induced downregulation of local GSK-3 and Akt signaling pathway in the prefrontal cortex (PFC) (54). In addition, through behavioral tests and cerebrospinal fluid analysis in rats, L-theanine might change the levels of glutamate and methionine in the brain to improve the hippocampal activity, showing an antianxiety effect (55). Besides, the 30-day test score of 33 cats showed that L-theanine could alleviate all stress-related symptoms and eliminate the adverse stress performance after 15 days, and the effect was better after 30 days (56). In chronic unpredictable mild stress (CUMS) rat models, L-theanine could effectively improve the depressive-like behaviors of rats, which was regulated by monoamine neurotransmitters in the limbic-cortical-striatal-pallidal-thalamic-circuit related brain regions (57). L-theanine intake (6 mg/kg) could prevent brain atrophy and stress vulnerability in senescence-accelerated mice prone 10 (SAMP10) mice, with the mechanism of intaking L-theanine could block the expression changes of the transcription factor neuronal PAS domain protein 4 (*Npas4*) and Lipocalin 2 (*Lcn2*) in hippocampus and PFC of SAMP10 (58). Another study suggested that the administration of L-theanine ameliorated the depression-like behavior of stress-challenged SAMP10 mice (59).

Some clinical trials have also been carried out to verify the role of L-theanine in mental health. In terms of attention, a study involving 27 healthy adults showed that a high dose of L-theanine could improve the neurophysiological indexes of attention processing in a dose-dependent manner (60). Another study confirmed that L-theanine and caffeine had additive effects on cognition and attention in 20 healthy men (61). Furthermore, the test results of nine healthy adult males showed that L-theanine could reduce the allocation of neural resources to distractors, so attention would be more efficient in focusing on goals, and L-theanine and caffeine could cooperate to reduce mind wandering (62). In addition, in the study of Japanese men and women aged 50–69, L-theanine showed excellent performance in improving attention, and the working memory and executive function of the subjects were also enhanced (63). Furthermore, five boys (8–15 years old) with attention deficit hyperactivity disorder (ADHD) were treated with L-theanine and caffeine, and the result showed that L-theanine combined with caffeine could effectively treat ADHD-related injuries in sustained attention, inhibitory control, and overall cognitive performance (64). L-theanine has also shown good clinical effects in psychological and mental related aspects. For the purpose of assessing the influence of L-theanine on the mental and physical health of athletes, 20 college athletes were chosen for the study, and it was found that small amounts of L-theanine, caffeine, and tyrosine could boost the movement accuracy of athletes before and after exhaustive exercise (65). In another study, 30 subjects (9 men and 21 women, with the age of 48.3 $\pm$ 11.9 years old) without major mental illness were selected to evaluate the stress-related symptoms, sleep status, and cognitive function, and the result showed that L-theanine could facilitate the mental health of normal persons with stress-related diseases and cognitive disorder (66). On the other hand, 20 patients (4 males and 16 females, with the age of 41.0 $\pm$ 14.1 and 42.9 $\pm$ 12.0 years old, respectively) with major depressive disorders were selected as the research objects, and it was found that L-theanine (250 mg/day) treatment for 8 weeks was safe and effective to significantly mitigate the symptoms of depression, anxiety, somnipathy, and cognitive disorder (67). Besides, after taking L-theanine-containing beverage, the subjective stress response of 34 healthy adults aged 18–40 who were subjected to a multitasking cognitive stressor was significantly decreased, and the response of salivary cortisol to stressors was also decreased after positive treatment (68).

In summary, L-theanine shows excellent therapeutic effects on mental health, such as depression, stress, as well as emotional and cognitive function, and can also improve sleep condition and physical fitness to some extent.

## Anti-cancer Activity

Recent studies have demonstrated the anticancer activity of L-theanine in cell and animal models. Firstly, L-theanine could contribute to preventing the reproductive system cancers. In previous study, L-theanine and its derivatives, ethyl 6-bromocoumarin-3-carboxylyl L-theanine (TBrC), could effectively prevent the growth and migration of highly metastatic human cervical cancer cells, which was confirmed by *in vitro* and *in vivo* studies (69). They could decrease the expression and phosphorylation of epidermal growth factor receptor (EGFR), Met, Akt, and NF-κB in cervical cancer cells, and totally inhibit the EGFR/Met-Akt/NF-κB signaling pathway activated by hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (69). Meanwhile, L-theanine and TBrC obviously inhibited the growth of cervical cancer in nude mice bearing tumors but showed no toxicity to mice. A recent study found that L-theanine had the therapeutic potential for metastatic

prostate cancer (PCa), since L-theanine inhibited the epithelial-mesenchymal transition process of PCa by downregulating matrix metallopeptidase 9 (MMP9), N-cadherin, Vimentin, and Snail, and upregulating E-cadherin (70). Moreover, L-theanine also inhibited the transcription of MMP9 and Snail through weakening the ERK/NF-κB signaling pathway and p65 binding activity with MMP9 and Snail promoter region (70).

Secondly, L-theanine could induce or inhibit the digestive system cancers. It was shown that L-theanine (600 μg/mL) could induce apoptosis of tumor cells through the mitochondrial pathway in human HepG2 hepatoblastoma cells and HeLa adenocarcinoma cells (71). Furthermore, L-theanine and its semi-synthesized derivative (R)-2-(6,8-dibromo-2-oxo-2H-chromene-3-carboxamido)-5-(ethylamino)-5-oxopentanoic ethyl ester (DTBrC) also restrained the growth and migration of human hepatocellular carcinoma (HHC) cells in *in vitro*, *ex vivo*, and *in vivo* HHC models, and the mechanism of this effect was that L-theanine and DTBrC blocked the Met/EGFR/vascular endothelial growth factor receptor (VEGFR)-Akt/NF-κB pathways (72). Then, L-theanine alone or in combination with theobromine could effectively inhibit tumor production in male Wistar rats with colon cancer induced by dimethylhydrazine, and the mechanism of action was related to downregulating the Akt/mTOR (mammalian target of rapamycin) and JAK2/STAT3 pathways and increasing the mRNA and protein expression of tumor suppressor Smad2 (73).

In addition, L-theanine can also be used as an auxiliary measure to reduce some side effects of cancer treatment. L-theanine and cystine pretreatment (280 mg/kg for 5 days) could significantly enhance the weight loss and survival rate of rats after the irradiation, which may be connected with the inhibition of apoptosis and the enhancement of the proliferation of bone marrow cells (74). It was noteworthy that oral L-theanine could also weaken the adverse reactions of S-1 adjuvant chemotherapy (75).

In general, L-theanine could induce cancer cell apoptosis through the mitochondrial pathway, inhibiting the EGFR, NF-κB, and other signaling pathways, downregulating MMP9, or upregulating Smad2 in cancer treatment. In addition to acting directly on cancer cells, L-theanine also has beneficial effects in radiotherapy and chemotherapy.

## Metabolic Regulation

The absorption of nutrients is very important to human health, and L-theanine can effectively regulate metabolism. Pretreatment of RIN-m5F pancreatic beta-cell line with L-theanine increased the beta-cell mass and insulin production in a dose-dependent manner (76). In addition, L-theanine (50 μM) promoted the proliferation of human Sertoli cells (SCs) and increased its glucose metabolism (77). L-theanine can regulate metabolism in animal models. By observing serum insulin secretion and blood glucose concentration in rats, it was found that L-theanine downregulated the expression of *SGLT3* and *GLUT5* in the intestinal tract, leading to the inhibition of glucose uptake in the small intestine (78). In addition, L-theanine (100 mg/kg) could effectively regulate the metabolism of glucose, lipids, and proteins in SD rats, and the main mechanism was that L-theanine could upregulate the mRNA expression of phosphofructokinase (PFK), carnitine palmitoyltransferase 1 (CPT1), insulin receptor (IR), insulin receptor substrate (IRS), and liver kinase B1 (LKB1), and enhance the phosphorylation of adenosine 5′-monophosphate-activated protein kinase (AMPK) (79). The effects of L-theanine on metabolism were also supported by human studies. For example, serum ethylamine level was used as an indicator of L-theanine consumption, and the monitoring of 2,253 Japanese residents aged 40–79 without diabetes found that a higher level of serum ethylamine was significantly correlated with a lower risk of type 2 diabetes, suggesting a negative association between L-theanine and diabetes (80). L-theanine also played an effective role in diet-induced obesity. After oral administration of L-theanine, the metabolic activity of brown fat and subcutaneous white fat were increased, which significantly improved the obesity and hepatic steatosis of mice fed a high-fat diet (HFD), and the composition of intestinal microflora was also reasonably regulated (81).

These results suggest that L-theanine can regulate the metabolism of glucose, lipid, and protein by downregulating *SGLT3* and *GLUT5* expression and upregulating the mRNA expression of IR, PFK, IRS, especially since it has a positive health effect against diabetes and obesity.

## Cardiovascular Protection

L-theanine showed a positive effect on the cardiovascular system. It was reported that L-theanine could significantly inhibit the proliferation and migration of cultured vascular smooth muscle cells (VSMCs) induced by angiotensin II (82). The JAK2/STAT3 and ERK pathways were involved in the possible molecular mechanism. In addition, the pathogenesis of cardiovascular diseases (CVD) was also related to the dysregulation of circadian rhythm (82). In dexamethasone-induced rat VSMCs circadian gene expression models, L-theanine treatment showed that the expression of clock genes Bmal1, Cry1, Reverb alpha, and Per2 increased (83). At the same time, L-theanine could also upregulate a bunch of the rhythm genes and differential expression genes involved in vasoconstriction and actin cytoskeleton regulation pathways. Moreover, L-theanine could significantly inhibit the formation of neointima and prevent the transformation of VSMCs from contractile type to synthetic type in rat carotid artery balloon injury models (84). Further research showed that L-theanine had a potential preventive effect on neointimal hyperplasia and related vascular remodeling, mainly by inhibiting the phosphorylation of Elk-1 and activating mitogen-activated protein kinase-1 (MAPK-1) (84).

Collectively, L-theanine can block the JAK2, STAT3, and ERK1/2 pathways, regulate the expression of clock genes and rhythm genes and inhibit the formation of neointima, which makes L-theanine a potential cardiovascular beneficial substance.

## Liver and Kidney Protection

A number of studies have proved the positive effects of L-theanine on the liver. By adding L-theanine to the drinking water of cirrhotic rats established by carbon tetrachloride, it

was found that L-theanine inhibited the expression of NF-κB, downregulated the pro-inflammatory cytokines (e.g., IL-1 and IL-6), and profibrotic mediators (e.g., transforming growth factor β and connective tissue growth factor), and promoted the expression of anti-inflammatory cytokine IL-10 and fibrinolytic enzyme metalloproteinases-13 (85). Therefore, L-theanine could effectively restrain liver cirrhosis in rats due to its anti-inflammatory and anti-fibrosis effects. In addition, L-theanine distinctly reduced the elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (86) activities in ETEC infected mouse models (87). Further study showed that L-theanine could obviously increase the expression of Bcl-2 mRNA and protein, decrease the expression of Bax, a pro-apoptotic molecule, and decrease the phosphorylation of ERK1/2 and c-Jun NH2-terminal kinase (JNK1/2) MAPKs (87). In D-galactose-induced aging rats, L-theanine could protect the liver not only by reducing the levels of pro-inflammatory factors, such as IL-1β, TNF-α, and IL-6 but also by efficiently reducing the production of edema and vacuole induced by D-galactose (88). Liver injury is a side effect of heat stress. After intragastric administration of L-theanine before systemic heat exposure, heat-induced liver injury was also reduced in mice (89). In LPS-induced inflammatory mice, L-theanine treatment reduced the acute liver injury by inhibition of the concentrations of ALT and AST, the level of hepatic total superoxide dismutase (T-SOD), and malondialdehyde (MDA) (34). The molecular mechanism might be that L-theanine significantly reduced the release of IL-1β and TNF-α, and the phosphorylation of NF-κB, and increased the ratio of IL-10 to interferon (IFN)-γ.

With regard to kidney protection, in the doxorubicin (DOX) induced acute nephrotoxicity rat models, it was found that the treatment with L-theanine could attenuate the decrease of creatinine clearance, inhibit the production of lipid peroxidation in the kidney, and inhibit the reduction of glutathione content and SOD activity after DOX administration (90). Moreover, another study of DOX-induced nephrotoxicity in SD rats proved that L-theanine could protect the kidney by reducing the levels of oxidized glutathione (GSSG), gamma-glutamyltransferase 1 (GGT1), NF-κB p65, and the percentage of apoptosis indexes in the tissue and plasma, and increasing the levels of GSH and the activities of GPx, glutathione reductase (GR), and glutathione S-transferase (GST) (86). Furthermore, cecal ligation and perforation could lead to sepsis and damage the liver and kidney of SD rats, and L-theanine had a significant inhibitory effect on this kind of liver and kidney injury in a dose-dependent manner (91).

In general, the protective mechanism of L-theanine for the liver and kidney is to inhibit the NF-κB pathway, regulate pro-inflammatory cytokines, such as IL-1, IL-6, IL-10, and TNF, and finally regulate the activities of AST, ALT, T-SOD, and other related enzymes, to effectively protect the liver and kidney and deal with liver and kidney injury caused by different reasons.

## Immunoregulation

L-theanine has an excellent performance in immune regulation. In the SD rat models, daily intragastric administration of L-theanine solution (400 mg/kg) could increase the weight of the spleen, modify the balance of Th1/Th2 cytokines, reduce the level of serum corticosterone, increase the level of dopamine and 5-HT in the brain, and regulate the mRNA expression of phospholipase C isomers in the heart, finally improving the immune function (92). Moreover, in another study, L-theanine effectively regulated the secretion of cytokines such as IFN-γ, IL-2, IL-4, IL-10, IL-12, and TNF-α except for IL-6 by activating the mRNA and protein expression of Ras-related protein Rap-1A (Rap1A), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), and farnesyl diphosphate synthase (FDPs) in the mevalonate synthesis pathway of rat splenic lymphocytes (93). In addition, L-theanine treatment decreased the mRNA expression of Toll-like receptors (e.g., TLR-2 and TLR-4) and cytokines (e.g., IFN-α, IFN-γ, and IL-2) in broilers (94). Furthermore, a 28-day feeding study of SD rats showed that L-theanine could increase the content of total short-chain fatty acids and regulate intestinal mucosal immunity based on dietary fiber feeding (95). Whereafter, L-theanine could regulate the innate immunity of mice with immune stress induced by ETEC, mainly by inhibiting the overexpression of nucleotide-binding oligomerization domain, IL-1β, and TNF-α, partially reducing the protein level of NF-κB p65, and suppressing the phosphorylation of ERK1/2 (96). In another study, it was found that L-theanine antagonized cannabinoid receptor 1 and inhibited its activity, relieved the inhibition of cannabinoid receptor 1 on COX-2 expression, reduced the pro-inflammatory factor TNF-α, and enhanced the anti-inflammatory factor IL-10, making L-theanine show a significant regulatory effect on the immune function of normal and E44813-stressed rats (97). Additionally, in the study of Polish rowing team members, it was found that L-theanine supplementation could contribute to the reduction of IL-10 concentration after exercise, which had an advantageous influence on the destruction of Th1/Th2 balance of top athletes (98).

In a word, L-theanine can regulate the balance of Th2/Th1 cytokines and the content of related substances. The main mechanism is closely related to the expression of protein and mRNA of action factors.

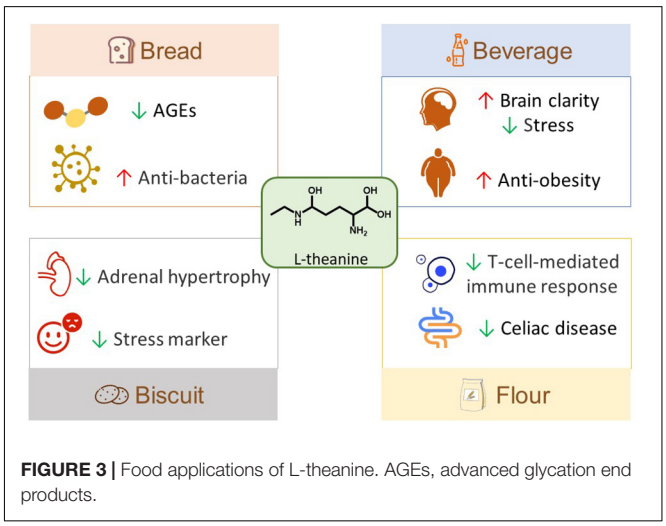


**FIGURE 3 |** Food applications of L-theanine. AGEs, advanced glycation end products.

### Urogenital Protection

L-theanine also exhibits protection in the urogenital system *in vitro* and *in vivo*. In urethane-anesthetized female Wistar rats, L-theanine could reduce substance P-induced bladder hyperfunction through inhibiting pro-inflammatory protein kinase C (PKC)/ERK/NF-κB/intercellular adhesion molecule 1 (ICAM-1)/IL-33 signaling, oxidative stress, apoptosis, autophagy (99). It was noted that when L-theanine, caffeine, and EGCG were supplemented in the culture medium, the motility of rat epididymal sperms was increased after 72 h of incubation at room temperature (100). More than that, studies on SCs found that L-theanine (50 μM) could promote the proliferation and glucose metabolism of human SCs to maintain the Krebs cycle, which was very important to prevent spermatogenesis disruption (77). On the whole, L-theanine can block bladder hyperfunction and protect spermatogenesis.

### Intestinal Protection

Recent studies suggest that L-theanine exhibits intestinal protective effects in animal models. When L-theanine was added to the diet of broilers, L-theanine had a beneficial effect on the intestinal microbiota, with increases in beneficial microorganisms, such as Lactobacillus, while inhibiting harmful microorganisms, such as Clostridium (94). Another study also validated that L-theanine could improve the intestinal development and health status of broilers, and the relative weight of duodenum, jejunum, and ileum increased, and the villus height and glutathione peroxidase activity of jejunum showed a linear or quadratic increase, and also enhanced the mRNA levels of intestinal amino acid and peptide transporters (101). It was also found that L-theanine could alleviate the intestinal pressure and stabilize the healthy intestinal tract in the stressed rat models established by E44813 infection, mainly by significantly enhancing the synthesis of glutamine and increasing the villus height and crypt depth of the intestinal tract (33). Overall, the intestinal protection of L-theanine can mainly be associated with the regulation of intestinal microbiota and the reduction of enterotoxin-mediated intestinal damage.

## FOOD APPLICATIONS OF L-THEANINE

L-theanine has some applications in foods and this kind of functional food has a positive effect on health and is very popular with consumers (**Figure 3**). L-theanine powder was obtained from decaffeinated tea and then made into theanine bread and other baked foods (102). Tea powder was used to prepare L-theanine enriched fractions (TEF), which could prevent the formation of fluorescent advanced glycation end-products, therefore, TEF was used to make healthy and delicious L-theanine bread (103). In addition, the concentrated L-theanine was added to wheat bread, which could inhibit E. coli and extend the shelf-life of bread for 1 day (40). Besides, due to the anti-stress effect of L-theanine, a nutritional beverage was made based on L-theanine, and the test results showed that the beverage could significantly reduce the subjective stress of 36 participants responding to multi-task cognitive stressors (68). Moreover, the cold-water brewed green tea, a new type of functional low-calorie beverage, was made at 30°C and contained a large number of L-theanine, catechin, gallic acid, and other bioactive ingredients, and it was found to significantly alleviate obesity and regulate the intestinal flora of HFD mice (104). A nootropic beverage containing L-theanine, pine bark, and blackcurrant could reduce mental fatigue in the sports environment, maintain brain clarity, improve the total score and accuracy, and effectively control the tension of athletes (105). In order to alleviate pressure, L-theanine was added into mango sorbet to make functional food, and the study proposed that the influence of food matrix should be considered when establishing functional food ingredients (106). Moreover, eating Matcha biscuits containing L-theanine also verified the pressure-reducing effect of L-theanine through animal experiments and clinical trials (107). Furthermore, a wheat flour rich in L-theanine was developed, and it could forcefully weaken the immune response mediated by T cells stimulated by gluten in the intestine of patients with celiac disease, and retain the function of gluten (108). This suggests its potential application in gluten-containing food products.

At present, the food application of L-theanine is mainly in beverages and bread. L-theanine has attracted much attention due to its various biological activities, suggesting its potential for formulating functional food products. In the future, more applications of L-theanine in foods should be explored, and its dosage and processing in products should be further studied.

## CONCLUSION AND PERSPECTIVES

L-theanine is a special free amino acid in tea, which is widely distributed in tea plants. The content of L-theanine in different kinds of tea is varying, with the climate and growth period affecting its content. As one of the main components of tea, L-theanine also has a variety of health benefits and some applications in foods as mentioned above. In the future, the following points are worthy of attention. Firstly, the mechanism of L-theanine on tea flavor should be further studied, and the changing trend of L-theanine content in different fermentation stages should be explained. Secondly, although a number of studies have confirmed the health benefits of L-theanine *in vitro* and *in vivo*, human-based research is still limited, and more clinical trials should be guaranteed to evaluate the health benefits of L-theanine. Overall, L-theanine exhibits plenty of beneficial functions and can be a promising functional additive or supplement in the food and nutritional industry.

## AUTHOR CONTRIBUTIONS

R-YG and D-TW: conceptualization, supervision, and funding acquisition. M-YL and H-YL: writing—original draft preparation. D-TW, AK, H-BL, AG, FG, HL, and R-YG: writing—review and editing. R-YG: project administration.

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## SUPPLEMENTARY MATERIAL

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REVIEW
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# Potential Application of Tea Polyphenols to the Prevention of COVID-19 Infection: Based on the Gut-Lung Axis

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Coronavirus disease 2019 (COVID-19) disrupts the intestinal micro-ecological balance, and patients often develop the intestinal disease. The gut is the largest immune organ in the human body; intestinal microbes can affect the immune function of the lungs through the gut-lung axis. It has been reported that tea polyphenols (TPs) have antiviral and prebiotic activity. In this review, we discussed TPs reduced lung-related diseases through gut-lung axis by inhibiting dysbiosis. In addition, we also highlighted the preventive and therapeutic effects of TPs on COVID-19 complications, further demonstrating the importance of research on TPs for the prevention and treatment of COVID-19 in humans. Based on this understanding, we recommend using TPs to regulate the gut microbiota to prevent or alleviate COVID-19 through the gut-lung axis.

**Keywords: tea polyphenols, COVID-19, gut microbiota, gut-lung axis, antiviral**

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## INTRODUCTION

Human pathogenic coronavirus, including severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2, it binds to the angiotensinogen-converting enzyme 2 (ACE2), a recently discovered mono-carboxypeptidase and the first ACE homolog, and then enters the cell (1). SARS-CoV S1 contains a receptor-binding domain (RBD) that explicitly recognizes ACE2 as its receptor (2), and tea polyphenols (TPs) have been found to bind to RBD to inhibit virus invasion (3). Numerous studies have demonstrated TPs to prevent obesity, diabetes, cardiovascular disease, cancer, and antiviral activity and fight diseases caused by oxidative stress and inflammation (4, 5). For instance, when the balance between the accumulation of reactive oxygen species (ROS) and the body's antioxidant process is disturbed, oxidative stress can be induced, causing damage to cells and tissues, thus leading to various diseases (6). However, when TPs enter the body, the activity of antioxidant enzymes increases, the inhibition of lipid peroxidation, and the production of ROS in the body can be promoted to achieve the antioxidant effect (7). These effects are also likely to help alleviate a range of complications caused by the new coronavirus.

Tea is the most popular beverage besides water and the most widely used (8). In China, tea consumption has been more than 5,000 years. TPs are a mixture of phenolic compounds extracted from tea leaves. In terms of concentration, tea catechins are one of the most important bioactive substances in tea leaves, accounting for 60–80% of total polyphenols. Catechins are the main polyphenol compounds in tea, including epigallocatechin-3-gallate (EGCG), epigallocatechin-3-gallate, epicatechin-3-gallate and epicatechin, the content and activity of EGCG was the highest (9).

It is known that the dysbiosis of the human gut microbiota is associated with various health conditions, including respiratory tract infections (RTI) via the gut-lung axis. The gut microbiota is involved in various physiological responses, including nutrient absorption, energy regulation, glucose metabolism, and immune system regulation (10). Perhaps only 1.9% of the gut microbiome is heritable, while more than 20% of the biodiversity of the microbiome is derived from the environment (including diet). TPs can effectively modulate gut microbiota composition, thereby effectively improving gut microbiome and host health. For many, COVID-19 brings few symptoms, but others are life-threatening due to SARS-CoV-2. While certain gut microbes have been linked to adverse outcomes from viral infections, some researchers suggest using these bacteria as biomarkers. If gut health affects the prognosis of COVID-19, we should use it to better control and prevent COVID-19. TPs have been used in research in the fields of immunity, psychiatric diseases, cardiovascular and metabolic diseases, and have achieved certain achievements. It can be seen that it is reasonable to use tea polyphenols to regulate intestinal microecology and prevent and intervene in COVID-19 (11). Therefore, improving the nutritional status of patients and enhancing the body's immunity by regulating the microbiota is of great significance for the treatment of novel coronavirus pneumonia. In this review, we summarized the possible use of TPs to prevent viral infections. In addition, the mechanism of action of TPs against COVID-19 was discussed from the perspective of the gut-lung axis.

## THE IMPORTANT ROLE OF GUT MICROBES IN COVID-19

Patients with COVID-19 show signs of intestinal flora imbalance, which can cause intestinal damage and damage to the lung and vital organ systems in the event of a pathogenic SARS-CoV-2 infection. Therefore, it is essential to maintain a healthy gut microbiome to optimize the immune system in order to prevent excessive inflammation (12).

### Intestinal Flora and the Gut-Lung Axis

Intestinal microorganisms can interact with the immune system, and the immune cells generated by the immune function between the intestine and the lungs move through the lymphatic system and blood circulation. The interaction network between the intestine and lung tissue mediated by microorganisms and immune cells is called the "gut-lung axis" (13). The imbalance of intestinal flora interacts with lung diseases and respiratory infections. When a deadly influenza virus invades, intestinal flora such as endogenous *Bifidobacteria* will increase, enhancing the host's resistance to influenza (14). The main bacterial phyla of the lungs are the same as the intestines, mainly *Firmicutes* and *Bacteroides*, followed by *Proteobacteria* and *Actinomycetes*, the interaction between the lung microbiota and immunity is also a two-way process (15). "Gut-lung axis" refers to the intestinal flora that can affect and regulate the immunity and function of the lungs, and may be related to acute and chronic lung diseases (16). And patients with chronic gastrointestinal inflammation and other diseases have a higher prevalence of lung diseases. Respiratory influenza infection can cause intestinal injury when lung injury occurs, and influenza infection changes the composition of intestinal microflora (17).

Patients with respiratory infections usually have intestinal dysfunction, and some COVID-19 patients experience gastrointestinal (GI) symptoms, including diarrhea and vomiting (18). The proportion of 651 COVID-19 patients with gastrointestinal symptoms was 11.4%; trends in fever and severity (severe/critical, mechanical ventilation, and ICU admission rates) were significantly higher in COVID-19 patients with gastrointestinal symptoms (19). Experiments in mice have shown that depletion or loss of the intestinal microbiota can lead to impaired immune response and worsen the prognosis of bacterial or viral respiratory infections (20). The gut-lung axis results from complex interactions between microbial components in the gut and lung flora and local and long-term immunity. Mice infected with the H1N1 flu in the nose developed lung infections, a marked change in the composition of the intestinal flora, and an increase in *Bacteroides* (21). Using mouse models of respiratory tract influenza infection found that respiratory tract influenza infection can cause intestinal damage and change the composition of the intestinal microbiome with the increase of *Enterobacteriaceae* bacteria and the decrease of *Lactobacillus* and *Lactococcus* (22). In a meta-analysis, the gut microbiota of 30 COVID-19 subjects, 24 H1N1 patients, and 30 healthy controls were evaluated. It was found that the intestinal bacterial diversity of subjects infected with SARS-CoV-2 was significantly reduced, and the relative abundance of beneficial microorganisms, such as *Bifidobacterium* was also reduced (23). Therefore, it is speculated that SARS-CoV-2 may indirectly affect the intestinal flora related to the intestine-pulmonary axis and damage human immunity, and it can prevent and treat lung infections caused by SARS-CoV-2 by regulating the relevant intestinal flora.

### Changes in the Intestinal Flora of COVID-19 Patients

The intestinal microflora is closely associated with respiratory viral infections and causes various infections through the gut-lung axis (24). In addition, influenza infection will affect the composition of the intestinal flora, and the disorder of the intestinal flora will reduce the host's antiviral immune response, thereby aggravating the lung damage caused by these infections (25). Among them, changes in the intestinal environment and immune factors caused by actinomycetes may aggravate the damage caused by inflammatory bowel disease. Compared with healthy individuals, the fecal microbiome of COVID-19 patients has significantly changed. The baseline abundance of *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hatheway* correlates with the severity of COVID-19; the abundance of *Faecalibacterium prausnitzii* (an anti-inflammatory bacteria) the degree is negatively correlated with the severity of the disease (26). Sequenced 274 feces samples (including feces from 100 COVID-19 patients) and found that members of the *Bacteroidetes* phylum in patients with COVID-19 were

relatively abundant, and the compositional differences in the gut microbiota of COVID-19 were mainly caused by the enrichment of *Ruminococcus gnavus*, *Ruminococcus torques* and *Bacteroides dorei*, and the depletion of *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii* and *Eubacterium rectale* (27). In conclusion, the gut microbiota of SARS-CoV-2-infected patients is altered by a decrease in commensal microorganisms, a loss of bacterial diversity, and an increase in opportunistic pathogens.

Patients with metabolic and GI are considered to be at moderate to high risk of SARS-CoV-2 infection, suggesting that gut dysbiosis directly affects the severity of COVID-19 (28). RNA metagenomics sequencing was performed on the continuous fecal virus extracts of 15 COVID-19 hospitalized patients. Feces with high SARS-CoV-2 infectivity have higher microbiome functions, and demonstrated the increased relative abundance of *Collinsella aerofaciens*, *C. tanakaei*, *Morganella morganii*, and *Streptococcus infants* (29). Based on COVID-19 patient data, a blood proteomics risk score was constructed, and it was found that gut microbial characteristics can highly predict the susceptibility and severity of COVID-19 (30). Therefore, intestinal microbial characteristics and related metabolites can be used as potential prevention/treatment targets for intervention, especially for those who are susceptible to SARS-CoV-2 infection.

### Gut Flora Regulates Immunity Through the Gut-Lung Axis

The gastrointestinal tract hosts a complex and highly diverse microbial ecosystem that interacts with the host to ensure the establishment and persistence of immune homeostasis (31). These complex microbial communities provide important genomic and enzymatic capabilities and play critical roles in the immune system's induction, development, and function, protection from pathogens and sustained tolerance to innocuous antigens, and protection of the ecology of the microbiota (32). The gut microbiome is the protective agent during pneumococcal pneumonia, and the gut microbiome enhances primary alveolar macrophage function (20). In an acute lung infection model, oral administration of segmented filamentous bacteria stimulates pulmonary T helper cell responses and reduces *S. pneumoniae* infection and mortality (33). Studies have shown that patients with COVID-19 have lower levels of probiotics (such as *Lactobacillus* and *Bifidobacterium*) (34). Because of the critical role of the intestinal flora and its metabolites in regulating the host's immune and inflammatory response, the regulation of the intestinal flora can be used to prevent and treat COVID-19 and related diseases (such as viral and/or bacterial pneumonia, acute respiratory infections, or flu) has attracted considerable attention.

The interaction between the gastrointestinal tract and the respiratory tract is achieved through a common mucosal immunity. There is persistent crosstalk between the intestine and the pulmonary mucosa through the mesenteric lymphatic system and the pulmonary lymph nodes (35). Short chain fatty acids (SCFAs) induce the expression of dendritic cell and macrophage pattern recognition receptors and regulate cytokine secretion and antibody synthesis (sIgA and IgM) (36). Dysbiosis in the lung affects the immune system and decreased immune cell recruitment leads to increased viral load in the lungs and reduced IFN-α and -β production, which negatively affects T cell priming (37). Germ-free mice display defects in several specific immune cell populations, such as impaired innate lymphocyte function, lack of IgA-producing plasma cells, and, more generally, increased susceptibility to infection (38). Using each of 53 separate bacterial species to single-colonize mice, it was found that the diversity of microbes in the gut ensures the ability of the microbiota to produce consistent immune regulation (39). When circumventing responses to pathogenic infections such as coronaviruses, a healthy gut microbiome may be vital to maintain an optimal immune system, preventing a cascade of excessive immune responses that ultimately damage the lungs and vital organ systems (**Figure 1**).

## POTENTIAL APPLICATIONS OF TPS TO ALLEVIATE COVID-19

The gut-lung axis plays an important role in SARS-CoV-2 infection, so targeting the gut-lung axis to treat COVI-19 is particularly important. TPs are considered to be multifunctional bioactive molecules, which have antiviral effects in addition to antibacterial and intestinal flora regulation to enhance immune function (40). Therefore, TPs are considered to have potential preventive and therapeutic effects on COVID-19.

### Antibacterial Effect of TPs

In addition to various pharmacological effects such as antioxidant, lowering blood sugar, and immune regulation, TPs also have potent antibacterial effects, especially for Gram-positive and Gram-negative bacteria (41). Although the current research results on the antibacterial mechanism of TPs are not very clear, researchers generally believe that the mechanism involves many aspects, such as destroying the cell wall membrane structure, interfering with cell growth and division, and inducing oxidative stress (42). Catechins can inhibit bacterial toxins directly by binding to bacterial toxins or indirectly by preventing bacterial toxin secretion or promoting bacterial protease breakdown (43).

The influence of the catechin structure in TPs on the antibacterial effect mainly includes: (1) The complexation of the ortho-phenolic hydroxyl group with the metal ion. The metal ions in bacteria are partly prosthetic groups of enzymes and partly essential bacteria elements. Multiple ortho-phenolic hydroxyl groups of TPs molecules can be used as multi-base ligands to undergo complex reactions with iron, calcium, and other ions to produce precipitation. Induce oxidative stress while depriving bacteria of essential nutrients, thereby affecting bacterial activity, growth, and reproduction (44, 45); (2) The phenolic hydroxyl group and benzene ring structure are combined with proteins. The phenolic hydroxyl group and benzene ring structure of TPs can be combined with bacterial proteins through hydrogen bonds or hydrophobicity, affecting the physiological functions of proteins, thereby inhibiting bacterial infection and metabolic activity (46, 47); (3) The effect of polymerization degree on the bacteriostatic effect of

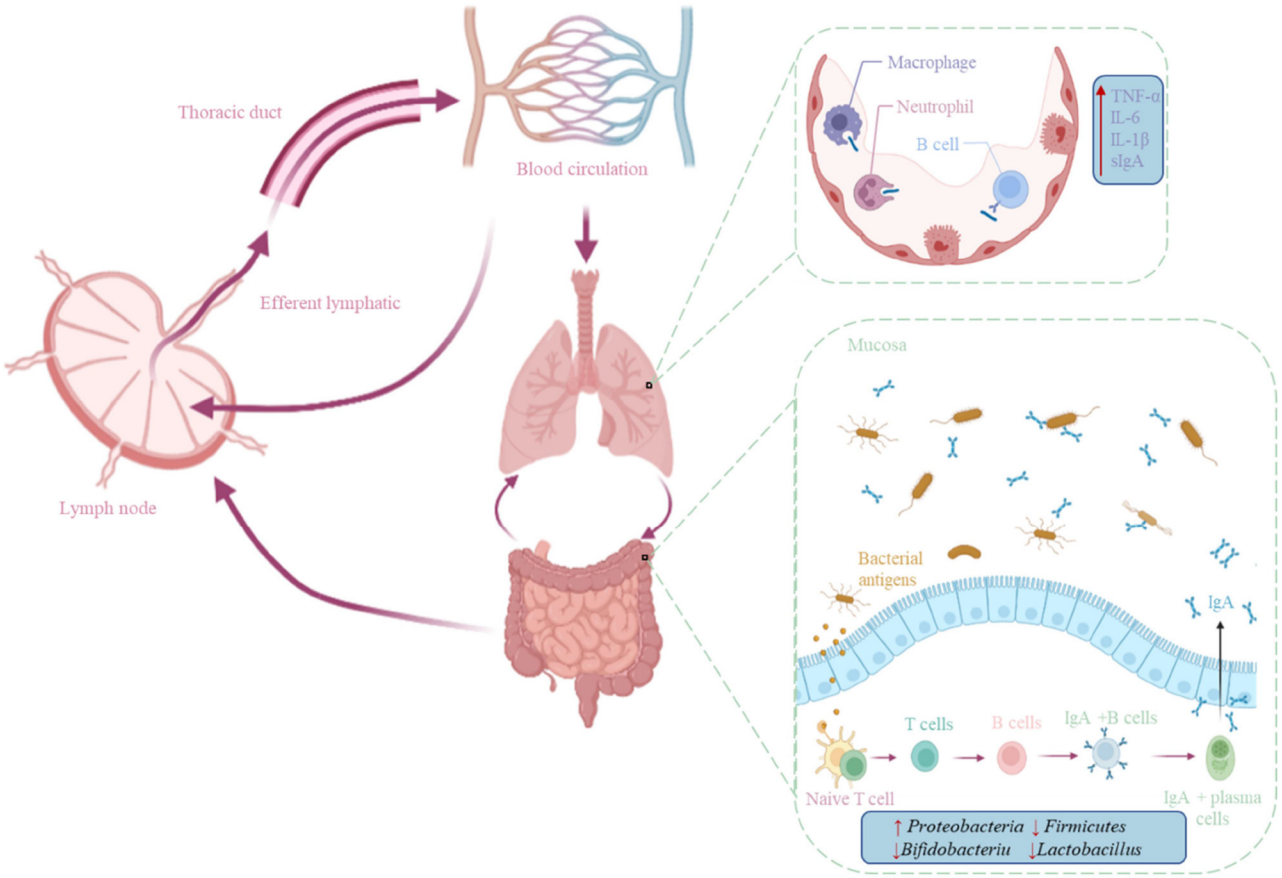


**FIGURE 1 |** Mechanisms of the gut-lung axis in the immune system. The previous crosstalk between the gut and lung mucosa is through the mesenteric lymphatic system and the lymph nodes in the lungs, and immune cells generated by the immune function between the gut and the lungs move through the lymphatic system and blood circulation.

catechins. For example, compared with catechin monomers, its oligomers have higher antibacterial properties, which may be attributed to the polymers having more phenolic hydroxyl groups and benzene ring structures and having a more substantial binding ability with proteins (48). However, compared with catechin oligomers, catechin polymers and polymers have weaker bacteriostatic effects, which may be because the molecular weight of catechin polymers increases with the increase of the degree of polymerization, making it challenging to penetrate bacterial cell membranes (49). In addition, affected by the steric hindrance effect of macromolecules, the activity of the phenolic hydroxyl group is weakened, resulting in a decrease in the antibacterial ability.

## Regulation of Gut Microbes by TPs

TPs can promote the growth of beneficial bacteria in the intestinal tract and inhibit the growth of pathogenic microorganisms in the intestinal tract from regulating the composition of intestinal flora (50). Intestinal microbes are an essential component of the intestinal environment. Intestinal microbes can enhance the function of the intestinal barrier by interacting with the body's metabolism to produce various metabolites and promote mucosal immune homeostasis (51). The intestinal mucosal barrier is a defense system against external infections and self-maintenance and plays an essential role in maintaining intestinal homeostasis and body health. Zhang et al. (52) studied TPs' therapeutic and preventive effects on ileal injury and intestinal flora disorder. The results showed that TPs could reduce inflammatory and oxidative stress markers, increase the levels of antioxidant enzymes and tight junction proteins, effectively improve the intestinal flora imbalance, reduce the damage to the intestinal mucosa and boost the body's immunity.

Ten volunteers who did not drink green tea drank it for 10 consecutive days, and the proportion of *Bifidobacteria* showed an overall increasing trend (53). A mouse model was established to explore the regulatory effect of TPs on the intestinal flora. The study found that after feeding green TPs, specific bacterial communities such as *Bacteroidetes* and *Proteobacteria* still increased, and *Firmicutes* showed a decreasing trend (54). This result indicates that TPs in green tea can improve the diversity of intestinal flora and regulate the composition of flora, thereby improving and maintaining the ecological balance of intestinal flora, which is beneficial to human health. The addition of TPs to calf feed reduces *Clostridium perfringens* in

the gut, which is associated with a lower incidence of digestive and respiratory diseases (55). Green tea consumption decreased relative abundance at the phylum level of *Bacteroidetes*. In addition, SCFAs-producing bacteria, including *Faecalibacterium*, *Coprococcus*, and *Bifidobacterium longum*, increased, while species from *Prevotella* decreased. And SCFAs are important factors regulating cytokine secretion and antibody synthesis (56). In conclusion, the protective effect of TPs on intestinal microflora has been supported by a large number of experimental results. Therefore, the reconstruction of immune homeostasis through the normalization of the intestinal microbiome is considered an effective method to treat COVID-19.

## Inhibitory Mechanism of TPs on SARS-CoV-2

Recent studies have demonstrated that TPs, particularly EGCG, inhibit coronavirus enzymes as well as coronavirus replication *in vitro* (57). However, laboratory and clinical studies have been performed to study the efficacy of green tea consumption in COVID-19 treatment, and the results are promising. SARS-CoV-2 has a high affinity for ACE2, which acts as a receptor for the spike glycoprotein on the surface of coronaviruses to facilitate virus entry. Nrf2 is a cytoprotective transcription factor that regulates the expression of a wide range of genes involved in detoxification, inflammatory, immune and antiviral responses (58). EGCG, *via* activating Nrf2, can suppress ACE2 (a cellular receptor for SARS-CoV-2) and TMPRSS2 (the cell entry that mediates the virus) (59). 3CL protease is required for the maturation of SARS-CoV-2, and numerous experiments have demonstrated that TPs (EGCG and theaflavins) have inhibitory effects on SARS-CoV-2 3CL protease (60). Mice with COVID-19 had lower levels of coronavirus RNA in their lungs when fed EGCG and TPs containing more than 60% catechins (61). Results demonstrated that EGCG treatment decreases viral RNA and viral protein production in the media, therefore, EGCG can inhibit coronavirus replication.

Data from docking simulations and *in vitro* assays suggest that EGCG is capable of inhibiting the SARS-Cov-2 major protease activity and thus can be used to interfere with SARS-Cov-2 infection (62). In a recent study reviewing the antiviral activities of EGCG and theaflavins, the authors suggest that both polyphenols are able to interact with receptors present in the structure of the SARS-CoV-2 virus, thereby inhibiting its replication. In particular, theaflavin-3,3'-digallate (TF3) can be employed as prophylactic agents due to their capacity to bind spike RBD the main binding domain of the S protein located on the S1 subunit of the SARS-CoV-2 virus; and TF3 can directly bind to viral M protease and ACE2 receptors, helping to fight SARS-CoV-2. EGCG can be used as a potential preventive agent because of its ability to dock various active sites of the SARS-CoV-2 virus (63).

Evidence suggests that patients infected with RNA viruses are in a chronic oxidative stress state, which is induced by the activation of phagocytes to produce and release ROS, and leads to the depletion of antioxidant defense systems (64). The increase in reactive oxygen species and the loss of antioxidant defense mechanisms increase the incidence of SARS-CoV-2 infection and the risk of immune dysfunction and death (65). Catechins activate antioxidant enzymes, and the antioxidant power of human plasma increases with the continued intake of green tea. These antioxidant defense systems also protect against oxidative damage in the brain, long-term intake of green tea catechins may be important because cells are often exposed to oxidative stress (66). TPs inhibits certain enzymes involved in reactive oxygen species ROS production by upregulating other endogenous antioxidant enzymes (such as glutathione peroxidase, superoxide dismutase and catalase); while promoting heme oxygenation enzyme 1 expression to reduce ROS production (67). Therefore, it is necessary to supplement dietary antioxidants to improve immunity when managing COVID-19. The use of exogenous antioxidants such as TPs can significantly influence the clinical outcome of COVID-19 by improving patients' health, speeding up the immune process, and thus shortening hospital stay.

Overall, TPs have antiviral solid and antioxidation properties that may help reduce the risk of developing severe COVID-19 symptoms; these findings highlight the potential for TPs to prevent and treat COVID-19 (**Figure 2**).

# REDUCTION OF COVID-19 COMORBIDITY RISK BY TPS

In a retrospective study of 1,591 severely ill patients with COVID-19, hypertension was the most common comorbidity (49%), followed by cardiovascular disease (21%), hypercholesterolemia (18%), and diabetes (17%) (68). Patients with COVID-19 can develop complications of lung disease (cough, decreased lung diffusivity, sleep apnea, and pulmonary fibrosis), cardiovascular disease (diabetes, arrhythmia, and myocarditis), and neurological disorder (depression, anxiety, and attention disorders) (69). TP can effectively prevent and treat some complications.

## Pulmonary Fibrosis

COVID-19 patients may have the sequelae of pulmonary fibrosis, with symptoms such as dry cough, fatigue, and dyspnea, leading to weight loss, worsening physical condition, long-term disability, and affecting the patient's quality of life (70). EGCG strongly inhibited neutrophil, inhibited reactive oxygen species activity and inhibited apoptosis of activated neutrophil, enhanced the regression of pulmonary inflammation model, and significantly reduced subsequent fibrosis (71). EGCG reduces NF-κB, TNF-α, and IL-1β, and this blockade may be critical for the upregulation of proinflammatory and fibrotic cytokine genes in models of pulmonary fibrosis (72). But so far, there are no clear and reliable data on the frequency and severity of pulmonary fibrosis in COVID-19 patients.

During treatment of pulmonary fibrotic rats with EGCG, the rats exhibited reduced inflammation, alveolar damage, and vascular congestion, which were associated with the membrane-stabilizing and antioxidant properties of EGCG, demonstrating that EGCG can act as a potential anti-fibrotic drug (73). Oral administration of green tea extract (equivalent to EGCG doses of 300–400 mg/kg) in drinking water to mice almost wholly

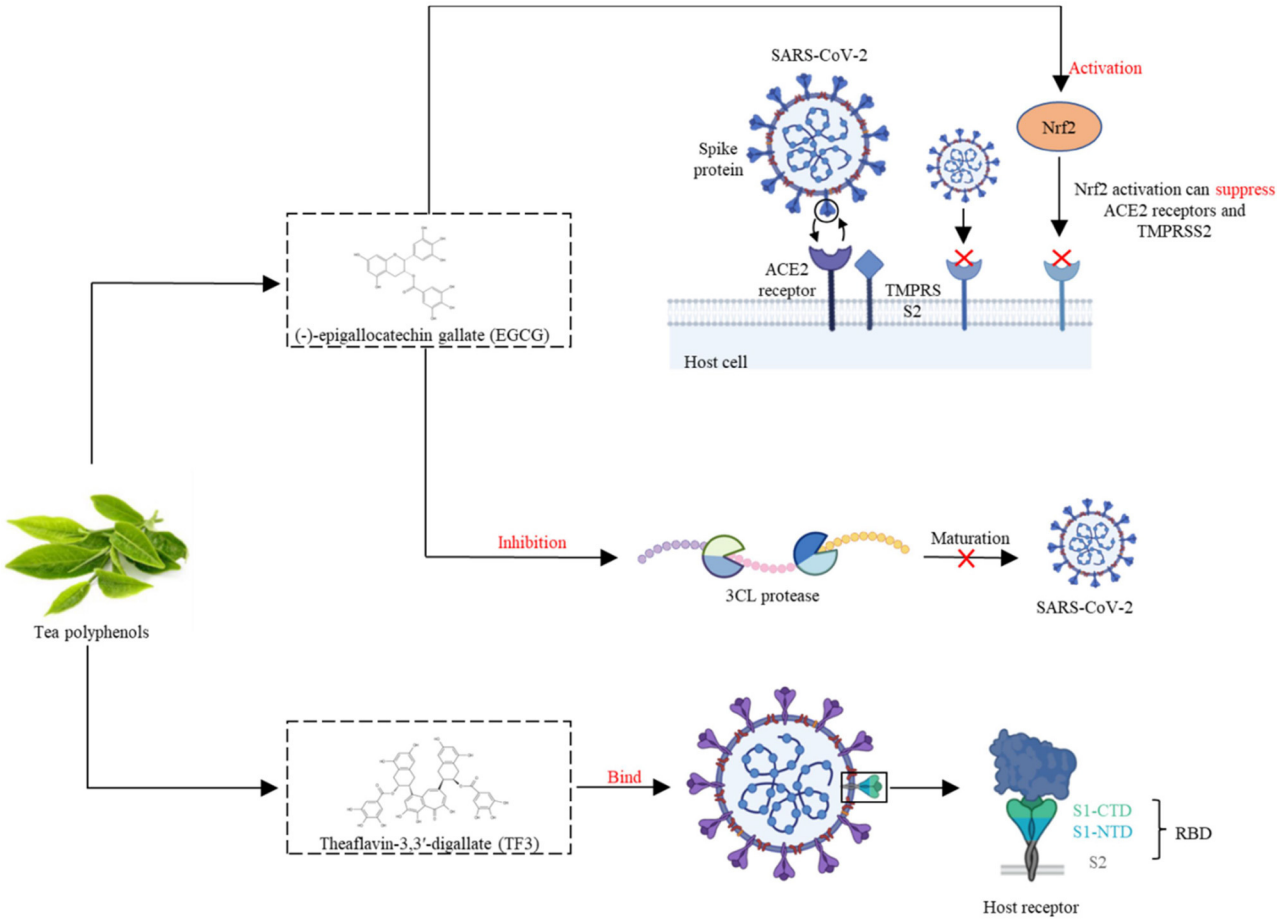


**FIGURE 2 |** EGCG, *via* activating Nrf2, can suppress ACE2 receptors and TMPRSS2 during SARS-CoV-2 infection. TF3 can bind the SARS-CoV-2 spike receptor-binding domain, and help to fight SARS-CoV-2.

prevented interstitial and peribronchial fibrosis, >99% reduction in interstitial and peribronchial fibrosis and ∼50% reduction in perivascular fibrosis (74). After EGCG (600 mg given orally for 14 days) treatment in 20 patients with pulmonary fibrosis, reverses profibrotic biomarkers in their diagnostic biopsies and serum samples, EGCG treatment was associated with a reduction in fibrogenesis (75). These inhibitory activities of EGCG in rodent models and humans suggest that EGCG may be beneficial for preventing and treating pulmonary fibrosis in COVID-19 patients.

## Diabetes

High-risk patients with severe COVID-19 or death have a variety of characteristics, including advanced age and masculinity, as well as potential health problems such as cardiovascular disease, obesity, and diabetes (76). Preliminary studies have found that diabetes increases the risk of infection with SARS-CoV-2 and increases the severity of COVID-19 (77). In human monocytes, an increase in glucose levels leads to an increase in SARS-COV-2 replication, which is maintained by glycolysis through the production of mitochondrial reactive oxygen species and activation of hypoxia-inducible factor 1α (78). Therefore, high blood sugar may support virus proliferation. Patients with diabetes usually have higher levels of SARS-CoV-2 infection than patients without diabetes. ACE2 knockout mice are more susceptible to high-fat diet-induced pancreatic beta-cell dysfunction than wild-type mice (79); and SARS-CoV infection can lead to hyperglycemia in people with no history of diabetes (80). This finding suggests that coronaviruses might specifically damage islets, potentially leading to hyperglycemia.

Drinking 3–4 cups of tea per day (600–900 mg/day) is often considered to prevent personal obesity metabolic syndrome or reduce disease risk (81). In mice fed a high-fat (60% calorie) diet; we found that EGCG (0.32% of the diet) significantly reduced weight gain, body fat, and visceral fat at 16 weeks (82). A retrospective study of 17,413 Japanese adults aged 40–65 showed compared with people who drank <1 cup of green tea a week, drinking more than six cups a day reduced the risk of diabetes by 33% (83). TPs in reducing plasma cholesterol levels, prevention of hypertension and improving endothelial function in the role of helping to prevent cardiovascular diseases. Weight loss and improved metabolic health may help better cope with COVID-19, whether regular drinking tea (and the required amount) can reduce the risk of COVID-19 infection

**TABLE 1 |** The reduction of COVID-19 comorbidity risk by TPs.

| Comorbidity | Risk | Experimental model | Results | Reference |
|---|---|---|---|---|
| Pulmonary fibrosis | 33% | EGCG doses of 300–400 mg/kg | >99% reduction in interstitial and peribronchial fibrosis and ∼50% reduction in perivascular fibrosis | (74) |
| Diabetes | 17% | Drink ≥ 6 cups a day | 33% lower risk of diabetes | (83) |
| Depression | 25% | Drink ≥ 4 cups a day | 51% lower prevalence of depressive symptoms | (88) |

and related syndromes requires a large number of experiments to prove.

## Depression

In addition to posing a significant threat to physical health, the COVID-19 pandemic also poses a threat to the population's mental health due to increased fear and uncertainty; and disruption to social and economic systems. The prevalence of depression in the general population during the COVID-19 outbreak is 25% (84). Alterations in the composition of the gut microbiota can increase the permeability of the gut barrier, activate systemic inflammatory and immune responses, modulate the release and efficacy of monoamine neurotransmitters, alter the activity of the hypothalamic-pituitary-adrenal axis and function, and alters the abundance of brain-derived neurotrophic factor (BDNF) (85). A deficiency of BDNF may lead to neuroplasticity impairment and depression. The mechanism of the anti-depression effect of TPs is related to the inhibition of HPA axis hyperactivity by reducing serum corticosterone and ACTH levels (86). TPs also have an anti-anxiety effect (similar to anti-anxiety drugs) at lower doses.

An investigation involving 2,011 Finnish general individuals found that daily consumption of tea was negatively correlated with the risk of depression (87). In addition, participants who drank ≥4 cups of green tea per day had a 51% lower prevalence of depressive symptoms compared to those who drank ≤1 cup of green tea per day (88). The antidepressant mechanism of TPs may be related to scavenging brain free radicals, regulating monoamine neurotransmitters in the brain tissue of depressed animals, and increasing the activity of brain antioxidant enzymes. Via establishing a mouse model of depression, it was found that the content of 5-HT and norepinephrine in the brain tissue of normal mice was significantly higher than that of depressed mice (89). After TPs were given, the content of 5-HT and norepinephrine in the brain tissue was significantly higher than that in the original depression mice (90). The antidepressant effect of TPs has been proven, prompt prevention of mental health status is also necessary for COVID-19 patients, whether regular drinking tea can reduce the risk of COVID-19 infection and related syndromes needs to be further investigated (**Table 1**, **Figure 3**).

# DOSAGE AND INSTRUCTIONS FOR CORRECT USE OF TPS

Tea is rich in polyphenols, inexpensive, readily available, and most importantly, safe for long-term use regardless of the patient's age (91). However, it is essential to note that taking EGCG containing preparations during pregnancy may increase the risk of fatal leukemia (92). According to the review of toxicological evidence, the liver is the target organ, and hepatotoxicity is the critical effect, strongly associated with certain dosing conditions (such as mode of administration, fasting) and positively correlated with catechin and EGCG content (93). In the case of oral therapy, changes in hepatotoxicity and serum lipid profiles were evident only at the highest dose of 108 mg/kg/p.o. However, EGCG treatment to achieve appreciable plasma concentrations may concomitantly increase serum lipids, thereby increasing the severity of the liver injury (94). At present, we have not accurately explained the association mechanism between EGCG, liver, and blood lipids, which must be clarified further. Regular consumption of green tea appears to be safe, but high doses of green tea extract in dietary supplements may affect drug metabolism and efficacy (95). Because these products contain concentrated bioactive agents, the doses consumed extensively exceed the doses available from food. Compared to the control group (about 10 mg per day), short-term (3 days) overconsumption of green tea catechins (about 8 grams per day) resulted in a significant increase in liver enzyme activity (by 35–80%) (96). Interestingly, short-term overdose of green tea extract has a more significant effect on drug biotransformation enzymes than long-term small doses. There is no clear information on how much catechins should be taken to achieve the best results. Therefore, it is necessary to evaluate the specific amount of catechin and its possible adverse effects.

According to the United States Department of Agriculture (USDA), the average total catechin and EGCG per 100 mL of brewed green tea was 126.6 mg and 77.8 mg, respectively, based on 1 g tea leaf 100 mL infusion (93). Therefore, every 240 mL serving of brewed green tea provides about 304 mg of total catechin and 187 mg of EGCG. Thirty-six healthy male volunteers took 800 mg of EGCG orally for 10 days and were tested for safety, tolerability, and plasma kinetic behavior; the researchers found that the dose was safe and well-tolerated (97). For adults with normal liver function, the safe intakes limit of 338 mg of EGCG per day in solid form (under-eating or fasting conditions) may be considered. The observed safe level of EGCG equivalent dose (ingestion or fasting) for green tea preparations consumed in beverage form is 704 mg/day (93). In a randomized, double-blind trial of 200 healthcare workers, six capsules per day (including 378 mg of catechin and 270 mg of EGCG) for 5 months were better at preventing the flu virus than a placebo (98).

Numerous experiments are still needed to confirm the specific drug administration (green tea beverage, powdered

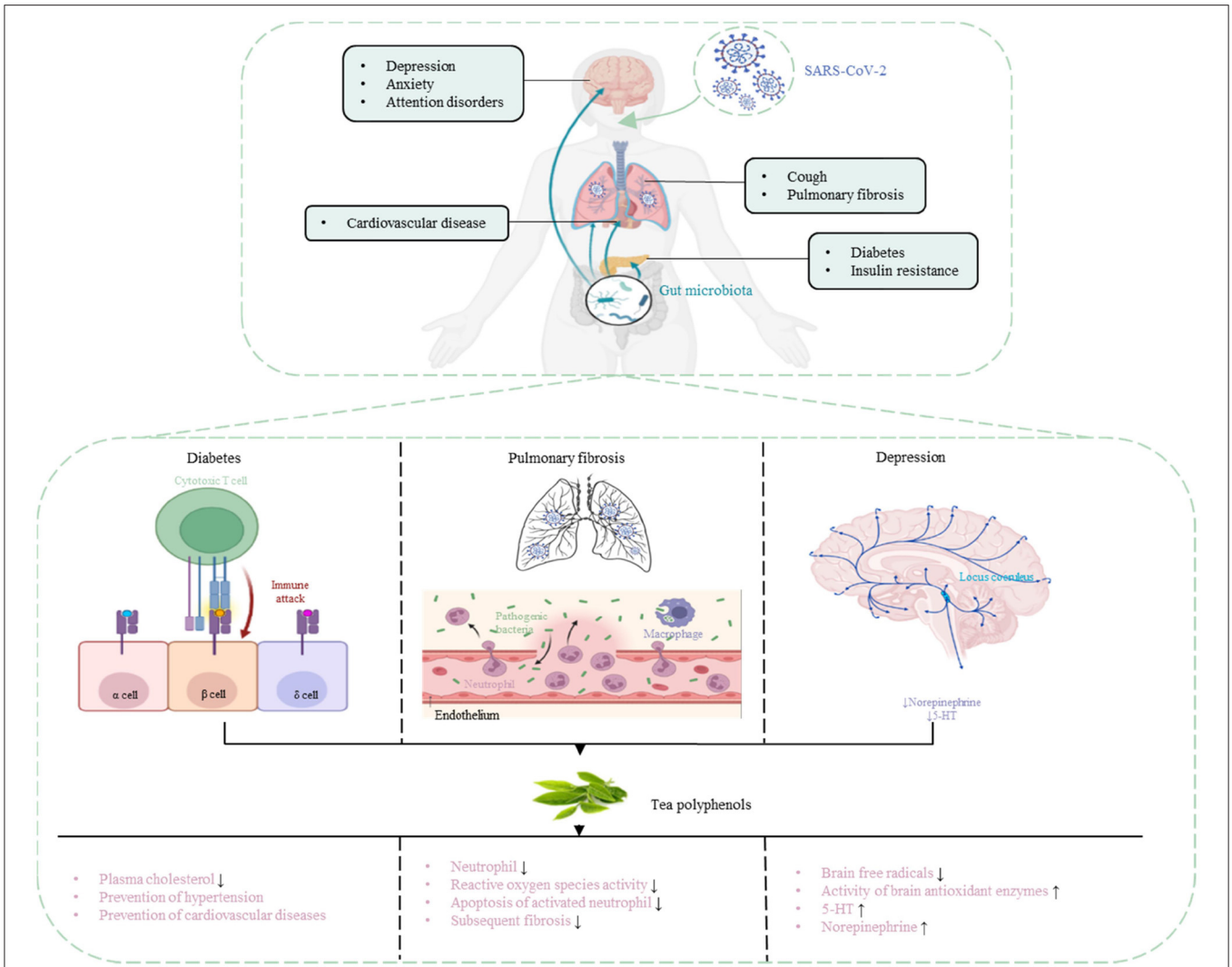


**FIGURE 3 |** Prevention and treatment of COVID-19 complications with TPs.

green tea extract, catechin mixture, catechin alone), dose regimen (different doses, different duration of treatment), and administration pathway management (oral in diet, oral in a beverage) before determining the use of TPs for the treatment of COVID-19.

## SUMMARY AND FUTURE DIRECTION

Many experiments have confirmed the safety of tea, and an appropriate amount of TPs will not cause harm to the human body. EGCG is one of the most important catechins in tea, enhancing the body's antiviral ability and gradually being regarded as a potential therapeutic agent for novel coronavirus infection. Although numerous epidemiological and clinical studies have shown that TPs have preventive and therapeutic effects on COVID-19, we lack specific dosages of TPs as dietary supplements or nutraceuticals for the prevention and treatment of COVID-19. To obtain more specific information, well-designed extensive cohort studies and human intervention trials are necessary.

## AUTHOR CONTRIBUTIONS

LX: conceptualization, validation, and writing–original draft. C-TH: supervision. YL: validation and writing–original draft. ZW: editing. XZ: supervision, writing–review, and editing. All authors contributed to the article and approved the submitted version.

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# Green and Oolong Tea Extracts With Different Phytochemical Compositions Prevent Hypertension and Modulate the Intestinal Flora in a High-Salt Diet Fed Wistar Rats

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Green tea (GT) and oolong tea (OLT) are widely consumed beverages, and their preventive and regulatory effects on hypertension have been reported. However, the interventional effects of GT and OLT on hypertension induced by a high-salt diet and its mechanism have not been fully explored. This study evaluated the anti-hypertensive effects of GT and OLT and their underlying mechanisms. The *in vivo* anti-hypertensive effects of GT and OLT and their capability to prevent hypertension and regulate the intestinal microbiota in Wistar rats fed with a high-salt diet were evaluated. Our results show that GT and OLT supplementations could regulate oxidative stress, inflammation, gene expression, and parameter levels related to blood pressure (BP) and prevent the increase in BP induced by a high-salt diet. Furthermore, both GT and OLT boosted the richness and diversity of intestinal microbiota, increased the abundance of beneficial bacteria and reduced the abundance of harmful bacteria and conditionally pathogenic bacteria, and regulated the intestinal microbial metabolism pathway related to BP. Among them, OLT presented better effects than GT. These findings indicate that GT and OLT can prevent hypertension caused by high-salt diets, which may be due to the regulation of intestinal flora by GT and OLT.

**Keywords: green tea, oolong tea, high-salt diet, hypertension, intestinal flora**

## INTRODUCTION

High-salt intake in the western diet is an important risk factor for many cardiovascular diseases (1). It is becoming increasingly difficult to ignore the adverse effects of a high-salt diet on cardiovascular health. Emerging evidence reveals that the potential cardiovascular hazards of high-salt diets are mainly related to arterial hypertension and are significantly and positively correlated with its morbidity and mortality (2). Besides, previous intervention studies have revealed that reducing sodium salt in the diet would reduce the occurrence of cardiovascular events (3). A long-term

high-salt diet can also cause other unfavorable conditions, such as the imbalance of the intestinal microecology (4). So far, most studies have focused on the direct effects on the blood vessel and kidney system (5). However, some studies have shown that gut microbes are involved in these processes, and changes in their composition and structure will affect the occurrence and development of hypertension (1).

The intestinal microorganism is an essential part of the human micro-ecological environment and crucial in maintaining human health. It is a dynamically balanced system. Intestinal microbes respond to fluctuations in the composition of the diet, resulting in transient or persistent changes in the composition of the gut microbiome (6). The intake of high-salt food can cause significant changes in the composition of the microbial community and induce hypertension, thus producing a profound impact on the host's health (1). Accumulating evidence shows that hypertension is associated with host intestinal microflora and its metabolic disorders (7). In addition, many animal experimental models also show that hypertension causes intestinal flora imbalance (8, 9), and transplanting the dysbiological intestinal microbiota from hypertensive subjects and animal models into normotensive animals would increase the recipient's BP (7, 10). Given the relationship between hypertension and intestinal microflora, adjusting the intestinal microflora is still a potential and effective way to reduce hypertension. The environment plays a vital role in regulating the composition and structure of intestinal microbes, especially diet (11).

Since diet has a noticeable impact on intestinal microbes, it is an executable strategy to use dietary intervention to restore the destroyed intestinal flora and ameliorate hypertension and its complications. Tea is one of the three largest non-alcoholic beverages globally, and its drinking has a history of nearly a thousand years. Tea contains many active ingredients, such as tea polyphenols, polysaccharides, proteins, and catechins, which are considered to have various health benefits (12). Many types of teas and extracts can intervene or influence the intestinal microflora and microenvironment, thus exerting its prebiotic effect (13). So far, however, there has been little discussion on tea's impact on reducing BP by regulating the composition of intestinal microorganisms. Accumulating studies have indicated that tea has shown remarkable effects in preventing and managing hypertension (12). The epidemiological and population-based cohort results show that drinking GT or OLT can significantly reduce the risk of hypertension (14). Moreover, intervention studies of many hypertensive patients and animal models have shown that black tea and GT have a significant anti-hypertensive effect and can protect the cardiovascular system (15, 16). However, research has consistently shown that the mechanism of tea lowering BP has not been adequately investigated. To solve these issues, more related works need to be carried out.

In this work, we investigated the effects of GT and OLT on BP, metabolic disorders, and gut microbial structure and composition in high-salt-fed rats. Also, we initially explored the possible mechanism of GT and OLT to prevent hypertension. These outcomes will contribute to the development of functional hypotensive foods.

# MATERIALS AND METHODS

## Materials and Reagents

Green and oolong teas were obtained from Enshi Selenium Impression Agricultural Development Co., Ltd., (Enshi, China) and Guangdong Baixiang Tea Co., Ltd., (Guangdong province, China), respectively. The cultivar of GT came from the local area of Enshi, named 'Enshi Taizi', contained four or five leaves with fully mature. The cultivar of OLT was semi-treerescent form and sexual group and it contained one bud and four or five leaves with fully mature. Glutathione peroxidase (GPX), superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO), creatinine (Cre), aldosterone (ALD), angiotensin-converting enzyme II (Ang II), and c-reactive protein (CRP) detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All catechin standards used in liquid chromatography were purchased from Chengdu RefMedic Biotech Co., Ltd., (Chengdu, China). All 21 amino acid standards were purchased from Sigma Co., (St Louis, MO, United States). All other chemicals were of analytical grade unless otherwise specified.

## Tea Aqueous Extract Preparation

Tea leaves were crushed and then extracted (1:10 and 1:9, w/v) twice with boiling water for 4 h. After filtering through 500 mesh nylon cloth, the extracts were combined and centrifuged, and then the supernatant was concentrated and lyophilized.

## Chemical Profile Analysis

The polysaccharide content was measured by the phenol sulfuric acid method (17). The polyphenol content was determined by using Folin–Ciocalteu method regarding the national standard of China (GBT8313-2018). The flavonoid content was investigated according to the description of the national standard of China (SNT4592-2016). The element distribution was percormed according to the previous report (18). The catechin, alkaloid, and phenolic acid contents were measured according to the previous report by our lab (19). A high-performance liquid chromatography LCC-AT20 system (Shimadzu, Tokyo, Japan) was used to analyze the amino acid content in the samples. All kinds of amino acid standards (dissolved in 0.1 mol/L HCl) were prepared into a 1 mg/mL solution, and then each standard solution was mixed equally and diluted to each concentration gradient with 0.1 mol/L HCl. The samples were prepared in the same way. A total of 200 μL combined standard solution or sample was dissolved in mixed solution (200 μL OPA derivatization reagent + 600 μL boric acid buffer) and derivatized for 15 min in the dark. Next, HPLC analysis was performed. The HPLC system was as follows: C18 column (250 mm × 4.6 mm, 5 μm), 35°C for column temperature, 1 mL/min for flow rate, 338 nm for detection wavelength, 20 μL for injection, mobile phase A: 20 mmol/L dihydrogen phosphate sodium solution; mobile phase B: a mixed solution (methanol: acetonitrile: distilled water = 45: 45: 10).

## Animals and Experimental Design

Twenty-four 8-week-old cleaning Wistar male rats were obtained from Slaccas Laboratory Animal Co., Ltd., (Shanghai, China) and divided into four groups, including model control (MC), GT, OLT, and normal control (NC) groups. All animal experiments were carried out according to the Experimental Animal Ethics Standards of the Experimental Animal Ethics and Use Committee of Shanghai Jiao Tong University (approval A2020080) and the Laboratory animal-Guideline for ethical review of China to maximize animal welfare. All experimental rats were housed at the Animal Experiment Center of Shanghai Jiao Tong University with free access to food and water in a controlled animal room (25 ± 1°C, 70–75% humidity, and a 12 h light-dark cycle). After acclimatization for 1 week, the NC group received Shoobree common standard feed (No. 1010009, Jiangsu Synergy Pharmaceutical Bioengineering Co., Lt d., Jiangsu, China) for 9 weeks on a regular diet; MC, GT, and OLT groups received with high-salt chow (92.45% common standard feed + 7.55% sodium chloride, 20210308(x), Suzhou Hongxin Biotechnology Co., Ltd., Jiangsu, China) for 9 weeks to induce hypertension. The Shoobree typical standard feed composition was presented in our previous report (20). In addition, the rats in GT and OLT groups were given 500 mg/kg GT and OLT aqueous extracts added into drinking water daily, respectively, and the rats in MC and NC groups were given distilled water. At the start and end of treatment, the body weight and systolic pressure reflecting BP of rats were measured.

## Histology Analysis

After dehydration, the heart and kidney tissues were embedded in paraffin and sliced (3 μm of thickness) and then placed in an oven at 60°C for 30–60 min. Next, gradient staining was performed according to the following steps: xylene I for 5 min, xylene II for 5 min, xylene III for 5 min, absolute ethanol for 1 min, 95% alcohol for 1 min, and 75% alcohol for 1 min. After washing, the sections were stained with hematoxylin staining solution for 2 min. After washing and returning to blue, the gradient dyeing was continued according to the following steps: eosin staining solution for 1 min, 75% alcohol for 30 s, 95% alcohol for 30 s, absolute alcohol for 30 s, xylene transparent for 1 min. Finally, the sections were mounted, dried, and observed under an optical microscope (× 400).

## Real-Time Reverse Transcription-Quantitative PCR (qRT-PCR) Analysis

The total mRNA in kidney tissue was extracted and reverse transcribed into cDNA according to the Servicebio®RT First Strand cDNA Synthesis Kit instructions (Service, Wuhan, China). The mRNA expression level was detected by SYBR qPCR Master Mix (High ROX, Wuhan, China) according to the light quantitative PCR kit instructions. The specific primers used were as follows: ACE, 5′-TCATCCAGTTCCAGTTCCACG-3′ (F), 5′-CGTGTTTGGTGTCCAGG-3′(R); endothelin-1 (ET-1), 5′-TTGCTCCTGCTCCTCCTTGAT-3′(F), 5′-CTGTTCCCTTGG TCTGTGGTC-3′(R); endothelial nitric oxide synthase (eNOS), 5′-GGTATTTGATGCTCGGGACTGC-3′(F), 5′- GTGATGG CTGAACGAAGATTGC-3′ (R). β-actin, 5′-TGCTATGTTGC CCTAGACTTCG-3′ (F), 5′-GTTGGCATAGAGGTCTTTAC GG-3′ (R). The gene β-actin was employed as an internal reference, and the relative mRNA level of target genes was calculated using the $2-\Delta\Delta$ Ct method.

## Biochemical Analysis

The blood of rats was collected by cardiac puncture. Then, blood was immediately managed and centrifuged at high speed (10,000 rpm) at 4°C for 10 min. Serum was collected and stored at −80°C. The levels of GPX, SOD, MDA, NO, Cre, ALD, Ang II, and CRP in serum were measured by commercially available kits.

## Microbiome Profiling of Fecal Samples

The tail of the fixed rats was lifted, and the lower abdomen of the rats was gently pressed. After that, fresh feces were collected aseptically and stored at −80°C until detection. Detailed DNA extraction analysis and sequencing steps were summarized in **Supplementary Information**.

## Statistical Analysis

The data were expressed as the arithmetic mean ± standard deviation. The student's *t*-test assessed comparisons between groups for two groups and one-way ANOVA for multiple groups using the Tukey test. A level of $p < 0.05$ was considered statistically significant. All statistical analysis was performed by SPSS 20.0 (SPSS Inc., Chicago, IL, United States) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, United States).

# RESULTS

## Chemical Composition

As shown in **Table 1** and **Supplementary Figure 1**, both GT and OLT extracts contained appreciable contents of tea polysaccharides, polyphenols, epigallocatechin gallate (EGCG), epigallocatechin (EGC), total flavonoids, and L-theanine. Therein, the higher contents of EGCG, EGC, and L-theanine were found in OLT extracts, and the higher contents of tea polysaccharides, polyphenols, and total flavonoids were found in GT extracts. In addition, the two tea extracts also contained a high content of free amino acids, alkaloids and element distributions (**Supplementary Table 1**). However, most of the components of the two tea extracts had significant differences, which might be related to their sources and processing techniques. These results indicate that both GT and OLT extracts contain beneficial nutrients, which may be the main contributors to the regulation of BP.

## Effect of Green Tea and Oolong Tea on Body Weight, Blood Pressure, and Histology of the Heart and Kidney

To assess the implication of a high-salt diet on the body weight and BP of Wistar rats before and after drinking tea were measured. Overall, a long-term high-salt diet did not significantly affect the body weight, and the intervention of teas

**TABLE 1 |** Comparison of GT and OLT extracts for the main components ($n$ = 3).

| Taxonomy | Category | GT | OLT |
|---|---|---|---|
| Polysaccharides (mg/g) | Tea polysaccharides | 283.02 ± 7.09 | 176.23 ± 5.14** |
| Tea polyphenols (mg/g) | Total polyphenols | 221.30 ± 1.82 | 192.49 ± 1.12** |
| Flavone (mg/g) | Total flavonoids | 30.61 ± 0.63 | 20.49 ± 0.11** |
| Catechins (mg/g) | Catechin | 5.31 ± 0.07 | 11.07 ± 0.02** |
| | EC | 16.29 ± 0.39 | 9.00 ± 1.42** |
| | ECG | 12.06 ± 0.19 | 11.18 ± 0.20** |
| | GC | 48.36 ± 0.85 | 8.62 ± 0.35** |
| | EGC | 52.46 ± 0.98 | 59.36 ± 2.80* |
| | GCG | 18.24 ± 0.30 | 10.24 ± 0.53** |
| | EGCG | 57.24 ± 0.98 | 86.31 ± 0.75** |
| Alkaloids (mg/g) | Caffeine | 62.69 ± 0.35 | 8.40 ± 0.59** |
| | Theobromine | 13.50 ± 0.23 | 21.64 ± 0.07** |
| Phenolic acids (mg/g) | Gallic acid | 3.78 ± 0.05 | 20.05 ± 0.13** |
| | Caffeic acid | 0.81 ± 0.01 | 0.83 ± 0.09ns |
| | p-Coumaric acid | 1.40 ± 0.02 | 1.24 ± 0.05* |
| | Ferulic acid | 0.59 ± 0.01 | 0.69 ± 0.03* |
| Amino acids (mg/g) | Asp | 6.72 ± 0.05 | 3.38 ± 0.02** |
| | Glu | 6.50 ± 0.17 | 0.65 ± 0.07** |
| | Asn | 1.99 ± 0.01 | 0.87 ± 0.01** |
| | Gln | 0.35 ± 0.01 | 0.62 ± 0.01** |
| | Gly | 0.35 ± 0.01 | 0.65 ± 0.32** |
| | His | 0.44 ± 0.02 | 0.51 ± 0.04* |
| | Thr | 1.27 ± 0.05 | 1.04 ± 0.09** |
| | Pro | 0.15 ± 0.01 | 0.15 ± 0.07ns |
| | Ala | 1.14 ± 0.01 | 0.58 ± 0.04** |
| | Ser | 0.36 ± 0.05 | 1.34 ± 0.08** |
| | L-theanine | 20.68 ± 0.02 | 21.53 ± 0.03* |
| | Tyr | 0.08 ± 0.01 | 3.51 ± 0.07** |
| | Arg | 2.79 ± 0.12 | 2.12 ± 0.18ns |
| | Val | 0.17 ± 0.01 | 0.36 ± 0.02** |
| | Met | 1.40 ± 0.13 | 1.65 ± 0.15** |
| | Trp | 1.95 ± 0.13 | 2.61 ± 0.15** |
| | Ile | 0.78 ± 0.05 | 2.47 ± 0.30** |
| | Phe | 0.21 ± 0.03 | 0.44 ± 0.02** |
| | Leu | 0.70 ± 0.07 | 0.64 ± 0.12ns |
| | Lys | 1.75 ± 0.13 | 4.27 ± 0.33** |
| | Cys | ND | ND |

*ND, not detected. Asterisk indicates a significant difference compared to GT. *p < 0.05, **p < 0.01. EC, epicatechin; ECG, epicatechin gallate; GC, gallocatechin; EGC, epigallocatechin; GCG, gallocatechin gallate; EGCG, epigallocatechin gallate.*

had limited effects on the body weight (**Figure 1A**). However, the treatment of GT or OLT prevented the increase in BP caused by a high-salt diet, and the BP of the GT and OLT groups was significantly different from that of the MC group after 8 weeks of intervention (**Figure 1B**). Moreover, the effect of OLT on preventing the increase of BP was better than GT (**Figure 1C**). Further histological analysis shows that both GT and OLT reversed the structural damage of the heart and kidney tissue caused by a high-salt diet (**Figure 1D**), including hypertrophy and necrosis of cardiomyocytes, thickening of the arterial walls of small blood vessels in the myocardium, and glomerular capillary dilation, as well as vacuolization, degeneration and necrosis of renal tubular epithelial cells.

## Effect of Green Tea and Oolong Tea on the Gene Expression

Studies have shown that a long-term high-salt diet causes an increase in BP (1). Thus, we investigated the effect of GT and OLT on the gene expressions closely related to BP regulation in the kidneys, including ACE, ET-1, and eNOS. From the data in **Figures 2A–C**, compared with the NC group, the mRNA expression of ACE and ET-1 of the MC group increased significantly, while the eNOS expression decreased significantly. However, compared with the MC group, GT and OLT treatments significantly down-regulated the mRNA expressions of ACE and ET-1 and significantly up-regulated the mRNA expression of eNOS. In particular, OLT exhibited a stronger regulatory effect than GT.

## Effect of Green Tea and Oolong Tea on the Serum Biochemical Parameters

Ang II, ALD, and NO are important regulators to maintain the balance of BP in the body, and their aberrant level will have a meaningful impact on BP (21, 22). As shown in **Figures 2D–F**, the MC group reported significantly more Ang II and ALD levels and lowered NO level than the NC groups. GT or OLT administration remarkably reduced the Ang II and ALD levels and considerably elevated the NO level. Therein, only a limited regulation by GT on the ALD level was presented. Besides, the regulation effect of OLT on ALD was also better than GT.

The continuous increase of BP will increase the degree of oxidative stress and inflammation, which will lead to vascular dysfunction and kidney damage (23). **Figures 2G–J** shows a significant decrease in SOD and GPX enzyme activities and a significant increase in MDA and CRP levels in the MC group. For SOD and GPX enzyme activities, GT and OLT treatments significantly enhanced the enzyme activities of SOD and GPX, respectively. GT and OLT treatments noticeably decreased the MDA level but had a limited effect on CRP level for MDA and CRP levels. CRE is one of the markers of renal injury, and damaged kidneys are usually accompanied by elevated serum CRE levels (24). As shown in **Figure 2K**, a long-term high-salt diet caused a significant increase in the Cre level in the MC group, but this could be significantly reversed by GT or OLT intervention. Interestingly, GT and OLT showed similar effects on the regulation of oxidative stress, inflammation, and kidney damage (no significant difference between groups).

## Effect of Green Tea and Oolong Tea on the Diversity of Intestinal Flora

16S rRNA high-throughput sequencing technology was used to sequence the microbiota in fecal samples of rats on the Illumina novaseq platform. A total of 2,322,739 valid sequences and 4,801 different OTUs were provided from 24 samples ($n$ = 6 in each group). OTU and Shannon rarefaction curves (**Supplementary Figure 2**) indicate that the number of sequencing samples is sufficient, and the species richness and community uniformity are both high. The results obtained from the preliminary analysis of α-diversity, including the Chao and Shannon indices, are presented in **Figures 3A,B**. As can be seen, the high-salt

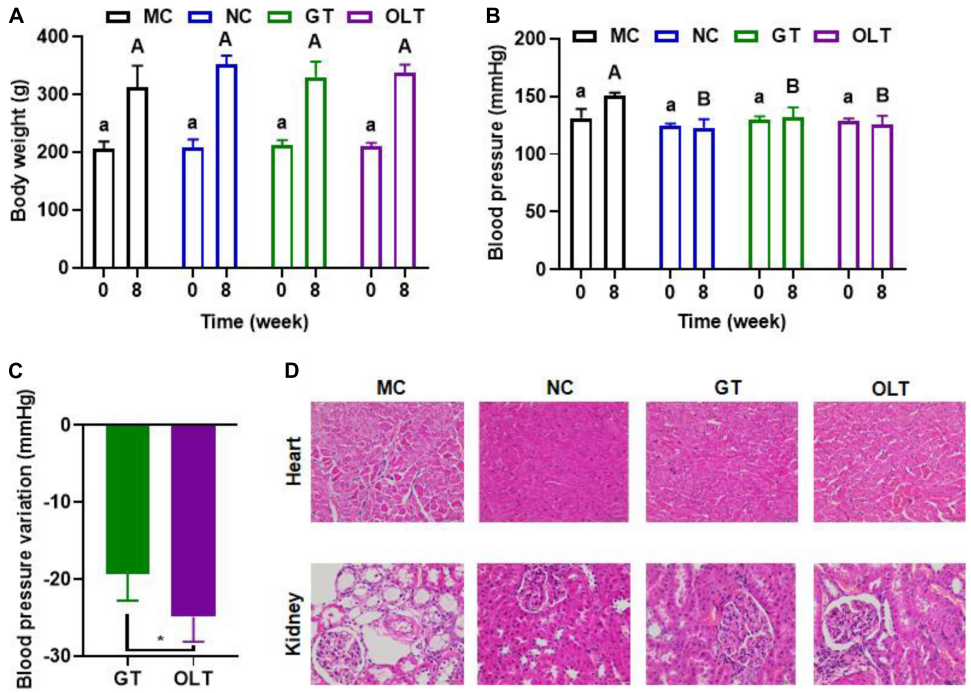


**FIGURE 1 |** GT and OLT affected the body weight, blood pressure, and tissue condition. **(A)** Body weight. **(B)** Blood pressure. **(C)** Blood pressure variation after GT and OLT supplementation, **(D)** Histological analysis of heart and kidneys. Different uppercase or lowercase letters represent a significant difference among multiple groups ($p < 0.05$). Asterisk represent a significant difference between groups. *$p < 0.05$. One-way ANOVA analysis followed by a Tukey test was employed to estimate the statistical significance.

diet caused a significant decrease in the OTU richness and community α-diversity of the intestinal flora. At the same time, the intervention of GT and OLT alleviated the decline in the OTU richness and α-diversity caused by the long-term high-salt diet. Among them, OLT showed a better alteration effect. The flower diagram of OTUs (**Figure 3C**) presented 446 mutual OTUs of all groups. The peculiar OTUs in MC, NC, GT, and OLT groups were 707, 962, 1,196, and 637. PCoA analysis (β-diversity) (**Figure 3D**) showed a complete separation of gut microbiota community between the MC and NC groups. The intervention of GT and OLT reshaped the gut microbiota destroyed by the high-salt diet and brought it close to a healthy state, particularly OLT, with more effectiveness.

## Effect of Green Tea and Oolong Tea on the Gut Microbiota Composition at the Phylum Level

At the phylum level (**Figure 3H**), the intestinal flora structure of rats was dominated by Firmicutes and Bacteroidetes. Compared with the NC group, the continuous high-salt diet feeding caused a significant increase in Firmicutes and a significant decrease in Bacteroidetes in the intestinal microbes of the MC group (**Figures 3E,F**). As a result, an elevated ratio of Firmicutes to Bacteroides in the MC group was found (**Figure 3G**). Conversely, OLT supplementation significantly decreased Firmicutes and significantly increased Bacteroidetes. Accordingly, OLT supplementation could substantially reduce the ratio of Firmicutes to Bacteroides, while GT supplementation had a limited effect.

## Effect of Green and Oolong Teas on the Gut Microbiota Composition at the Genus Level

The intestinal microbes (top 20 genera in relative abundance) at the genus level in different treatment groups also had obvious distinctions (**Figure 4A**). Additionally, Spearman correlation analysis based on the relative abundance showed more or less antagonistic or synergistic effects among various bacteria in rats of each group (**Figure 4B**). The cladogram in **Figure 4C** and linear discriminant analysis (LDA) histogram in **Figure 4D** indicate that the GT group specifically and significantly enriched the *Enterococcus* genus compared with other groups. In contrast, the OLT group was characterized by specific and significant enrichment for the *Allobaculum*, *Paraprevotella*, *Oscillospira*, *Bifidobacterium*, and *Ruminococcus* genera. Likewise, a significant selective enhancement for *Turicibacter*, *Treponema*, *Ralstonia*, and *Coprococcus* genera in the NC group was found. However, the most unexpected result from this data is that the MC group specifically and significantly enriched the abundance of *Lactobacillus*.

## Difference in Composition and Enrichment of Bacteria Genera With High Abundance

Just like the ecosystem, not all species are equal. The number of main microorganisms in the intestinal micro-ecosystem may have a crucial impact on the intestinal microenvironment.

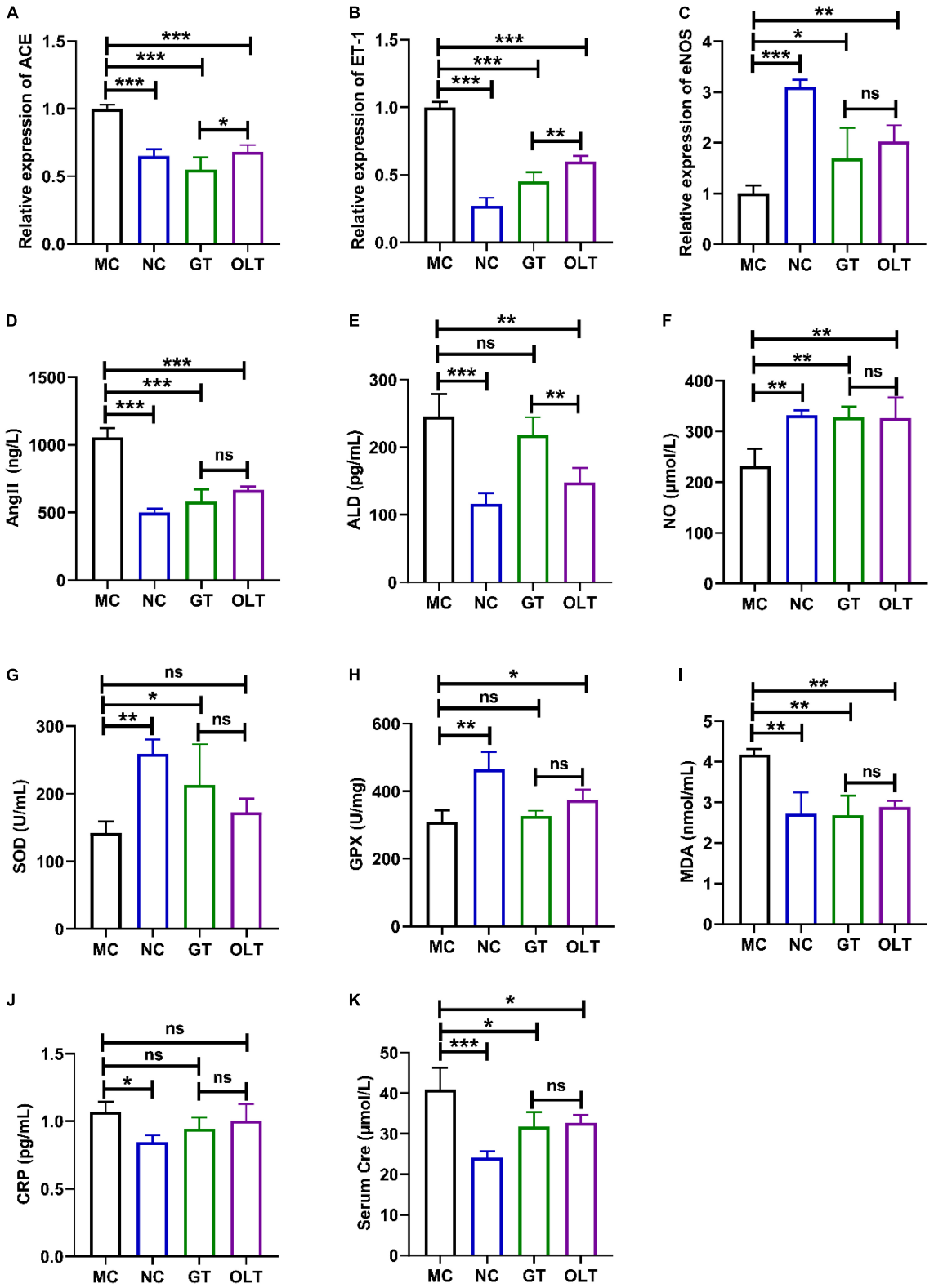


**FIGURE 2 |** GT and OLT adjusted the gene expression related to blood pressure and the serum biochemical parameters related to blood pressure in rats fed a high-salt diet. The relative expression of ACE **(A)**, ET-1 **(B)**, and eNOS **(C)**. The levels of Ang II **(D)**, ALD **(E)**, NO **(F)**, SOD **(G)**, GPX **(H)**, MDA **(I)**, CRP **(J)**, and Cre **(K)**. Asterisk represent a significant difference between groups. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. One-way ANOVA analysis followed by a Tukey test was employed to estimate the statistical significance.

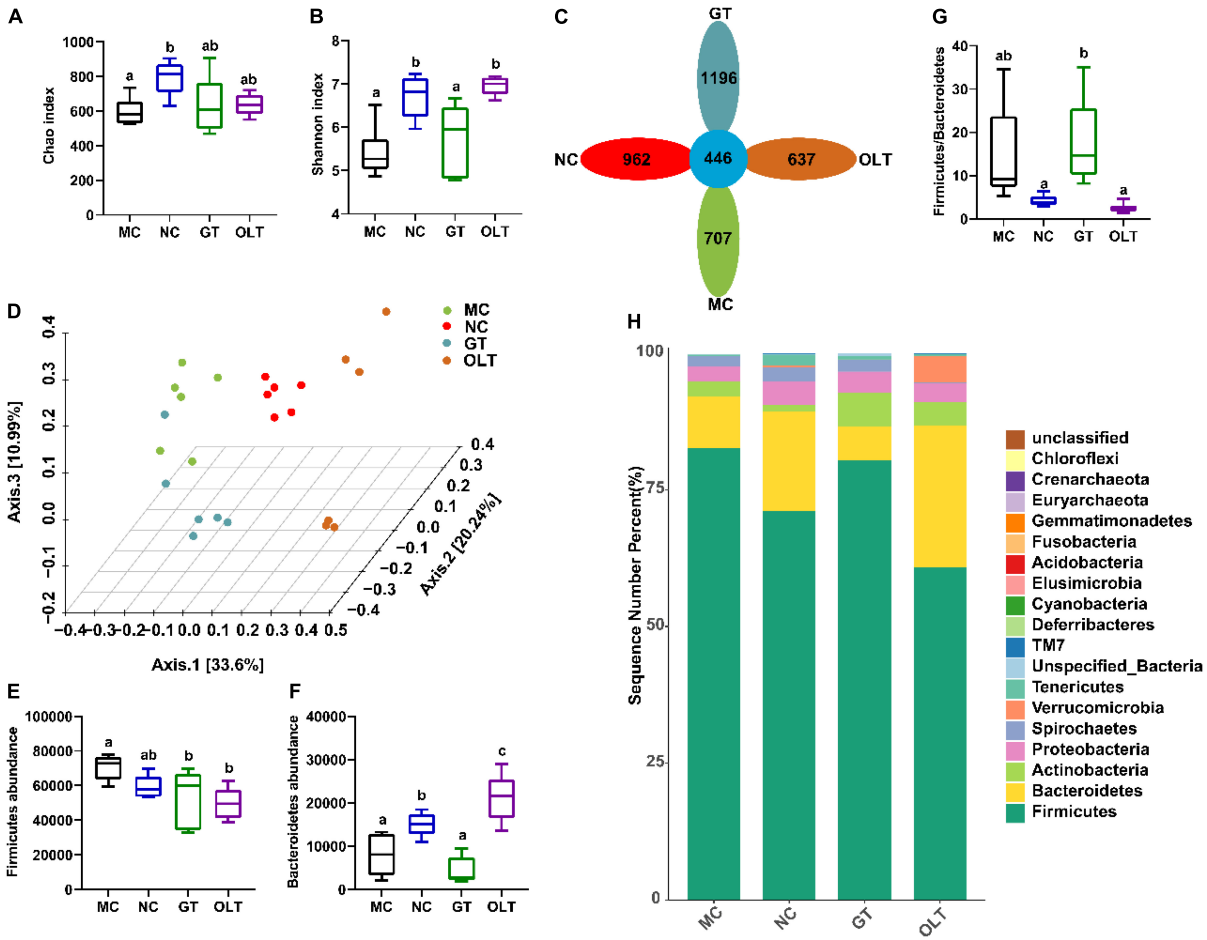


**FIGURE 3 |** GT and OLT altered the gut microbial diversity and composition at the phylum level in rats induced by a high-salt diet. **(A)** Chao index reflects species richness (the number of species). **(B)** Shannon index assessing the species diversity. **(C)** Flower diagram of OTUs, the petals are the number of OTUs unique to the corresponding group, and the center is the number of mutual OTUs of all groups. **(D)** 3D PCoA analysis based on Bray Curtis distance, the percentage represents the contribution value of each principal component to the sample difference. **(E)** Firmicutes abundance at the phylum level. **(F)** Bacteroidetes abundance at the phylum level. **(G)** The ratio of Firmicutes to Bacteroidetes. **(H)** Histogram of the relative distribution of gut microbes at the phylum level. One-way ANOVA analysis followed by a Tukey test was employed to estimate the statistical significance. The different letters represent significant differences between different groups ($p < 0.05$).

As shown in **Figure 5**, the top 15 genera (more than 98% abundance) had mainly consisted of *Lactobacillus*, *Allobaculum*, *Unspecified_S24_7*, *Turicibacter*, *Unspecified_Clostridiales*, *Unspecified_Clostridiaceae*, *Unspecified_Ruminococcaceae*, *Bifidobacterium*, *Treponema*, *Unspecified_Lachnospiraceae*, *Paraprevotella*, *Unspecified_Lachnospiraceae*, *Ralstonia*, *Ruminococcus*, and *Oscillospira*. In high-salt fed rats, several bacteria were significantly decreased compared with a regular diet, including *Unspecified_S24_7*, *Turicibacter*, *Unspecified_Clostridiales*, *Unspecified_Ruminococcaceae*, *Unspecified_Lachnospiraceae*, and *Ralstonia* whereas others were significantly increased, such as *Lactobacillus* and *Unspecified_Clostridiaceae*. Interestingly, the decreased abundance of *Unspecified_S24_7*, *Unspecified_Clostridiales*, *Unspecified_Ruminococcaceae*, and *Unspecified_Lachnospiraceae* observed in the MC group was remarkably reversed by OLT supplementation. Furthermore, OLT intervention significantly reversed the high-salt evoked increase in the abundance of *Unspecified_Clostridiaceae*. Accordingly, GT intervention significantly elevated the relative abundance of *Ralstonia* and notably reduced the relative abundance of *Unspecified_Clostridiaceae*. Of particular interest, GT and OLT supplementation also considerably increased the relative abundance of *Allobaculum* and *Bifidobacterium*. However, surprisingly, the MC group was characterized by a relatively higher abundance of *Lactobacillus* traditionally classified as beneficial microbe.

## Gut Microbial OTU Composition and Its Correlation With Metabolic Parameters

Among the top 50 OTUs (more than 99% abundance), 24 distinct OTUs had undergone noticeable variations through the GT intervention, and 34 distinct OTUs were significantly

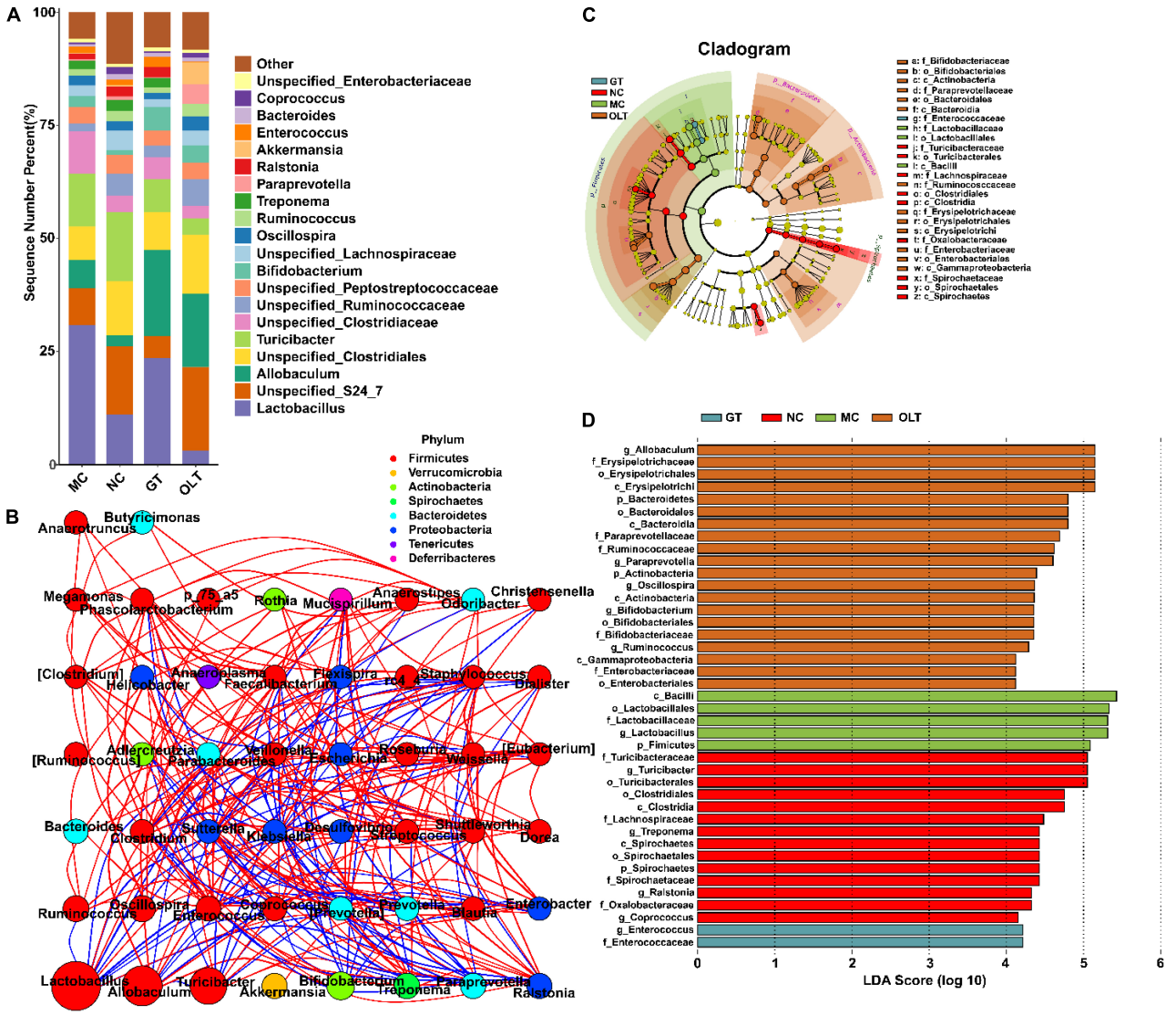


**FIGURE 4 |** GT and OLT changed gut microbiota structure at the genus level in rats in response to a high-salt diet. **(A)** Histogram of the relative distribution of gut microbes at the genus level. **(B)** Microbial interaction network diagram at the genus level based on Spearman correlation analysis. The circle represents a genus, the size represents its relative abundance, different colors represent different phylum classifications, the line between the circles represents a significant correlation between the two bacteria ($p < 0.05$). The red line represents a positive correlation, and the blue line represents a negative correlation. The thicker the line, the greater the absolute value of the correlation coefficient. **(C)** Cladogram based on LEfSe analysis. The cladogram corresponds to different levels of intestinal microbial classification from the inside to the outside, and the connection between the levels represents the belonging relationship. Each circled node represents a classification of bacteria, yellow nodes represent insignificant differences between groups, and non-yellow nodes represent that the bacterium is characteristic microorganisms of the corresponding group (significantly higher abundance in this group). The colored fan-shaped area marks the subordinate classification intervals of the characteristic microorganisms. **(D)** LDA histogram based on LEfSe analysis. Each horizontal column represents a kind of bacterium, and the length of the column corresponds to the LDA value. The higher the LDA value, the greater the difference. The color of the column corresponds to the characteristic microorganisms of the bacterial group (the higher abundance in the corresponding group).

changed by OLT intervention compared with the MC group (**Figure 6A**). Further analysis revealed that 13 and 16 of the OTUs altered by the Control group were reversed in response to GT and OLT interventions, respectively. Likewise, Spearman correlation analysis (**Figure 6B**) was employed to investigate the correlation between the top 50 OTUs and hypertension-associated metabolic parameters. As can be seen, 17 out of 50 OTUs were positively or negatively correlated with at least one parameter associated with hypertension. Therein, *Bacteroides*, *Aggregatibacter*, *Anaerofustis*, *Agrobacterium*, *Elusimicrobium*, *Prevotella*, *Sutterella*, *Paraprevotella*, *Coprococcus*, *Ruminococcus*, and *Akkermansia* were negatively and significantly correlated with hypertension. However, although *Lactobacillus* had a significant correlation with hypertension but showed a controversial effect. Also, *Faecalibacterium*, *Shuttleworthia*, *Clostridium*, *Dorea*, *Bacteroides*, *Aggregatibacter*, *Anaerofustis*,

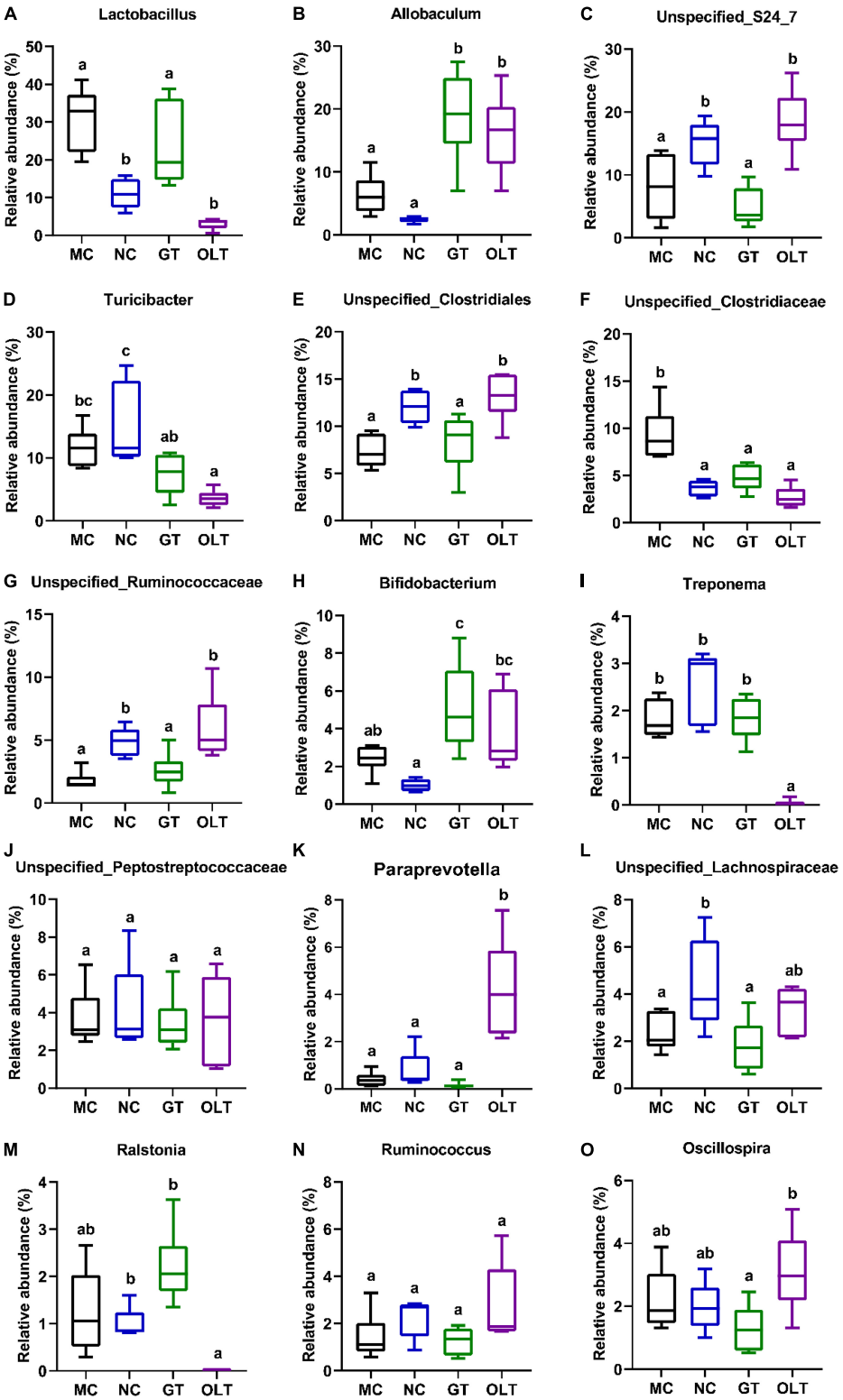


**FIGURE 5 |** The relative abundance of top 15 genera in gut microbial of high-salt fed rats differed after GT and OLT supplementation. **(A)** Lactobacillus, **(B)** Allobaculum, **(C)** Unspecified_S24_7, **(D)** Turicibacter, **(E)** Unspecified_Clostridiales, **(F)** Unspecified_Clostridiaceae, **(G)** Unspecified_Ruminococcaceae, **(H)** Bifidobacterium, **(I)** Treponema, **(J)** Unspecified_Lachnospiraceae, **(K)** Paraprevotella, **(L)** Unspecified_Lachnospiraceae, **(M)** Ralstonia, **(N)** Ruminococcus, **(O)** Oscillospira. One-way ANOVA analysis followed by a Tukey test was employed to estimate the statistical significance. The different letters represent significant differences between different groups ($p < 0.05$).

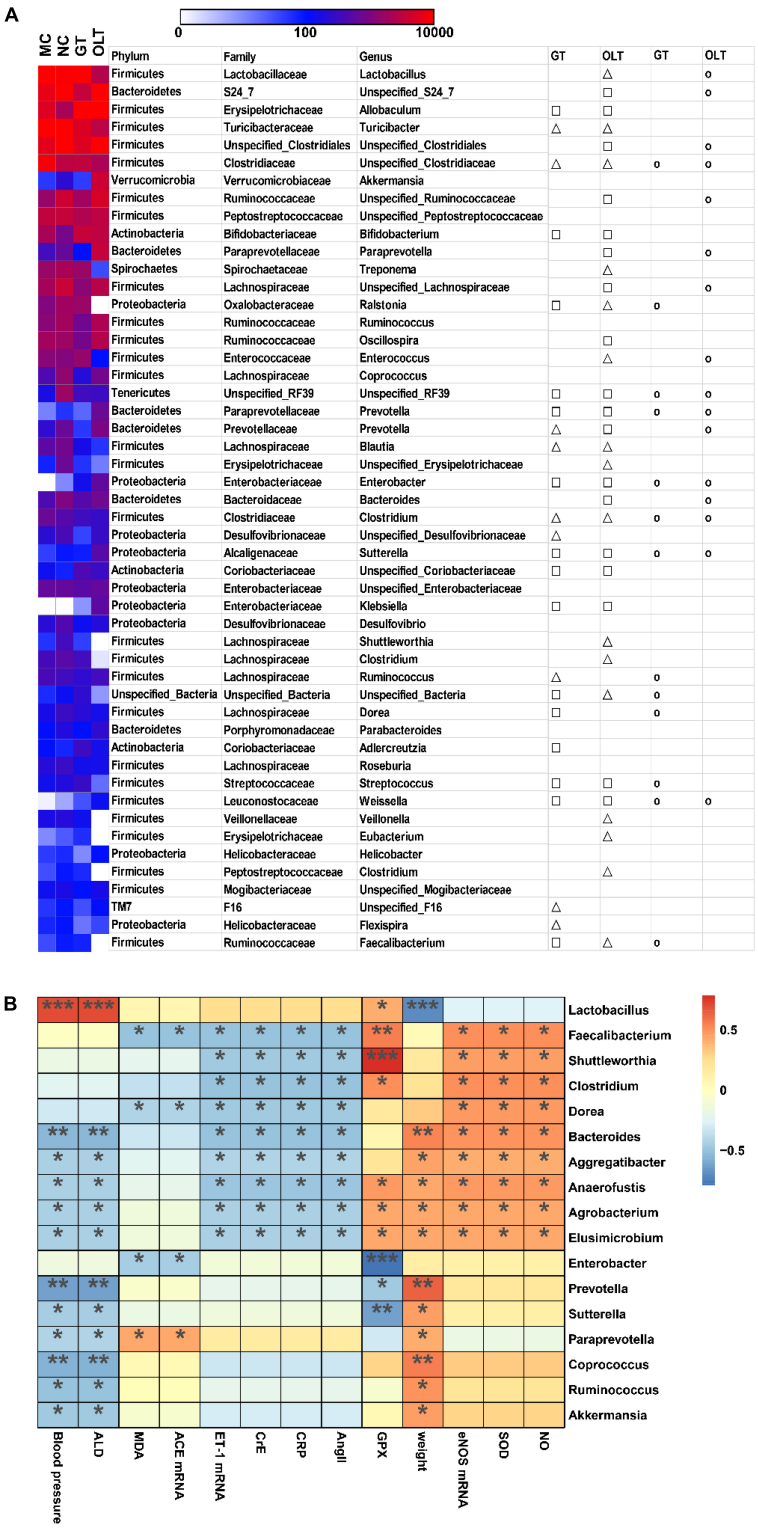


**FIGURE 6 |** GT or OLT treatment reversed the imbalance of intestinal flora abundance of rats caused by a high-salt diet. **(A)** Heatmap in the abundance of the top 50 OTUs of different treatment groups of high-salt fed rats. The triangle and square indicate the less and more relative abundances of OTUs in GT or OLT groups in comparison with the MC group, respectively. The circular indicates that the OTU of the NC group that was changed by hypertension was reversed by GT or OLT treatment. **(B)** Spearman correlation analysis between the microbial genera in the intestinal flora and related parameters of hypertension. The parameters related to blood pressure are on the X-axis, and the bacteria genera are on the Y-axis. The R-value is displayed in different colors, and the * sign indicates that there is a significant correlation between the two. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

*Agrobacterium*, *Elusimicrobium*, *Enterobacter* and *Paraprevotella* were negatively and significantly correlated with hypertension-related disorders. These genera may play a key role in preventing hypertension evoked by a high-salt diet.

## Green Tea and Oolong Tea Regulated the KEGG L3 Pathways Related to Hypertension

Up to date, identified pathways of particular interest regarding BP regulation, including histidine metabolism, tryptophan metabolism, and bile acid metabolism, have been proved to affect host BP through circulating microbial metabolites (11). Based on these researches, the KEGG L3 pathways related to hypertension, including histidine metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, primary bile acid biosynthesis, and secondary bile acid biosynthesis, were investigated to explore the effect of GT or OLT intervention on intestinal microbial function (**Figure 7**). OLT supplementation significantly enhanced histidine metabolism and phenylalanine, tyrosine, and tryptophan biosynthesis compared with the MC group. Moreover, it also significantly decreased the primary and secondary bile acid biosynthesis. However, GT exhibited a limited impact on these pathways.

# DISCUSSION

A western high-salt diet is a risk factor for cardiovascular complications and metabolic syndromes (25). Despite its indispensable involvement in many physiological activities, excessive salt uptake is detrimental to many well-recognized diseases, especially hypertension (25). Changing dietary habits has been proven to regulate BP (25) effectively. In particular, nutritional therapy has exhibited beneficial effects on the prevention and management of hypertension. As mentioned in the literature review, a strong relationship between tea consumption and BP has been reported in animal, population-based cohort, and meta-analysis studies (26, 27). Moreover, prior studies have noted the beneficial effect of various active compounds in tea on anti-hypertension (28). Numerous reports indicate a strong relationship between intestinal flora and host health, and simple diet changes can reshape the host's intestinal flora (29). Excessive salt intake efficiently induces high BP and severely disrupts the intestinal microecology diversity and structure (1, 4). It has been reported that different tea extracts or active compounds could alter the composition and metabolism of the gut microbiota, directly or indirectly regulating the host health through a variety of disease model validations (13). However, the regulation mechanism of tea on hypertension driven by a high-salt diet remains poorly understood. The present study was designed to determine the effect of tea on BP and its potential regulatory mechanism.

In the current study, both GT and OLT supplementation showed a suppressive effect on BP and a protective effect on cardiac and renal tissue injuries but a limited impact on body weight. A similar result also was reported by Szulińska et al., which showed that the anti-hypertensive effects of GT and OLT were not conducted by intervening in body weight (30). Our current findings are consistent with those of Xu and Tanida, who found that GT drinking for 3–16 weeks significantly reduced systolic and diastolic BP in the hypertensive subjects tested and OLT drinking for 14 weeks reduced BP elevation in spontaneously hypertensive rats (26, 31). Besides, studies have shown that a low dose of GT extract (1 mg/kg/day) could significantly improve myocardial stiffness and cardiac compliance of deoxycorticosterone acetate-salt hypertensive rats (32). Although, our results also reveal that the anti-hypertensive activity of OLT was better than that of GT. It seems possible that this result is due to their different chemical compositions (**Table 1**). Owing to the distinctions in the category and source of tea, the distribution of active compounds for lowering BP is significantly different.

In addition to BP, GT or OLT intake also significantly reduced the mRNA expression of ACE and ET-1 and greatly increased the mRNA expression of eNOS. Ang II and ALD levels were significantly decreased for serum BP regulators, and NO level was markedly increased with the administration of OLT. Accordingly, GT supplementation remarkably reduced Ang II level and noticeably elevated NO status but had a limited effect on the ALD level. Renin plays a pivotal role in the development of hypertension. ACE is an integral part of the renin-angiotensin system (RAS) system, which can catalyze angiotensin I (Ang I) into Ang II with high-strength vasoconstriction activity, thereby inducing hypertension (31). Ang II can activate nicotinamide adenine dinucleotide phosphate (NADPH) to increase vascular superoxide anion, and the change of vascular superoxide anion plays a pivotal role in the occurrence of hypertension (12). Furthermore, Ang II can constrict blood vessels and promote the secretion of ALD. The high content of ALD will increase the content of $Na^+$ in the blood, which will overload the blood volume and cause high BP (21). Previous reports revealed that anti-hypertensive candidates could prevent BP elevation, oxidative stress and inflammation (33). As expected, treatment with GT and OLT (no significance between them) significantly enhanced the enzyme activities of SOD and GPX, and significantly reduced the levels of MDA and Cre. Still, no remarkable difference in CRP level was observed. There are similarities between our results and those described by Antonello, who found that GT (6 mg/mL) could inhibit the increase of BP and oxidative stress in male SD rats caused by excessive Ang II (34). Moreover, GT supplementation (4 g/kg diet, 42 d) significantly reduced the concentration of TNF-α (a critical pro-inflammatory cytokine) in the serum of NaCl-induced hypertensive rats and enhanced the body's total antioxidant status (30). ET-1 and eNOS are essential members of the renal endothelial function system. ET-1 is the most effective vasoconstrictor produced by the blood vessel wall and is involved in the pathogenesis of salt-sensitive hypertension in animals and humans (35). eNOS is a specific enzyme that oxidizes L-arginine to produce L-citrulline and NO. Studies have shown that the knockout of the eNOS gene in mice would produce vascular endothelial dysfunction and prone to hypertension. After transfection the eNOS gene, the damaged blood vessels can be recovered (36). Our results indicate that excessive salt

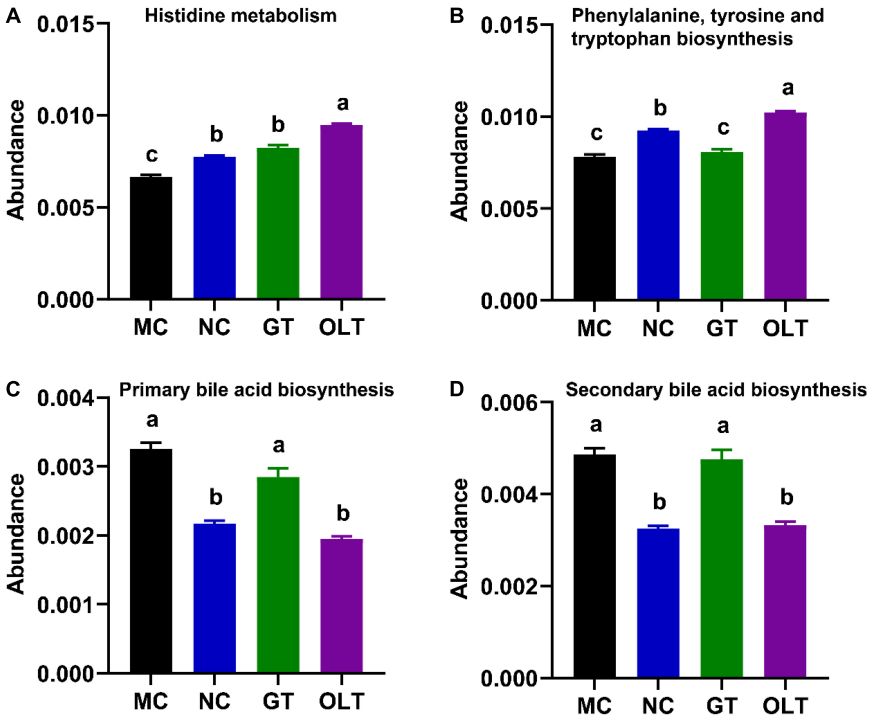


**FIGURE 7 |** GT and OLT regulated the KEGG L3 pathway related to hypertension. **(A)** Histidine metabolism, **(B)** Phenylalanine, tyrosine, and tryptophan biosynthesis, **(C)** Primary bile acid biosynthesis, **(D)** Secondary bile acid biosynthesis. If and only if the *p*-value after ANOVA analysis corrected for "false discovery rate" is less than 0.05, the Duncan test will be further performed. The different letters represent significant differences between different groups ($p < 0.05$).

intake enhanced the oxidative stress state of rats and led to an increase in the expression of ET-1, a decrease in the expression of eNOS, and an increase in the levels of Ang II and ALD. There are several possible explanations for this result. On the one hand, oxidative stress and pro-inflammatory factors can enhance ET-1 expression (12). On the other hand, ET-1 can activate the RAS system, promote the synthesis of Ang II, and release ALD (12). Our results corroborate the findings of a great deal of the previous work on tea and its active compounds in regulating BP. It is reported that GT (6 mg/mL) extracts significantly reduced the systolic and diastolic BP and oxidative stress of SD rats by inhibiting the increase in Ang II levels (34). In addition, black tea extracts exposed to porcine aortic endothelial cells could significantly boost the bioactivity of NO in aortic endothelial cells (37). GT extract regulated vascular homeostasis by its influence on the production of vasoconstrictive substances including Ang II, ET-1 as well as vasodilating substances (38). Interestingly, we found that the regulation effect of OLT was better than GT, which might be explained in part by higher EGCG and theanine contents in OLT. EGCG and theanine are considered to be the main components of tea to lower BP (12). Furthermore, it is speculated that the lower caffeine content might also be the reason for the high activity showed by OLT (39), and the stimulatory implications of caffeine could be decreased by the amount of EGCG in tea (12).

Long-term dietary salt-induced metabolic disorders contribute to aberrant intestinal microbiota *via* poorly understood mechanisms and further lead to high BP, accompanied by symptoms such as inflammation, gastrointestinal diseases, and endocrine disorders (25). The lower richness and diversity of gut microbes are observed in hypertensive individuals induced by a high-salt diet (40). Moreover, a strong relationship between microbial diversity and hypertension-related features has been reported (9). In line with these reports, our results (**Figures 3A–D**) show that the richness and diversity (including α and β) of the intestinal flora of hypertensive rats were significantly reduced. At the same time, GT and OLT supplementation significantly restored microbial richness and diversity, especially OLT. In addition to GT and OLT, many other candidates like yellow, white and dark teas can also increase the richness and diversity of the gut microbiota in dextran sulfate sodium-induced colitis mice (41). All these indicate the effectiveness of tea in regulating the richness and diversity of intestinal microbes, which could be attributed to the rich compounds in tea. At the phylum level, our current study indicates that a high-salt diet elevated gut colonization by bacteria of the phylum Firmicutes, with a resultant increase in the Firmicutes/Bacteroidetes ratio, which also accords with other previous studies (9). Furthermore, it is reported that the Firmicutes/Bacteroidetes ratio is increased in experimental animal and human subjects with obesity and metabolic syndrome (42).

Among members of the Firmicutes phylum, high-salt-enriched bacteria mainly belong to the *Lactobacillus* genus,

belonging to the Lactobacillaceae family. It is well known that the Lactobacillaceae family and *Lactobacillus* genus, recognized intestinal probiotics, are beneficial intestinal flora for improvement of intestinal health and exhibit anti-inflammation, antidiabetic, and anti-obesity (43, 44). Recently and more strikingly, studies have shown that higher levels of *Lactobacillus* were observed in hypertensive rats (11). Moreover, it is reported that the high abundance of *Lactobacillus* is positively correlated with obesity-related features (45). Research shows that the *Lactobacillus* genus contains more than 170 species (46). A recent study showed that some Lactobacillus species like *Lactobacillus reuteri* were related to metabolic disorders with obesity (47). Thus, a high abundance of Lactobacillaceae and *Lactobacillus* may also boost the risks of hypertension induced by a high-salt diet. Early work has shown that *Enterococcus* is a natural inhabitant of the intestinal tract in humans and many animals and is a probiotic because it stimulates immunity, anti-inflammatory activity, and the hypocholesterolemic effect. It can be used as a starter in food fermentation (48). Our results show that GT treatment could significantly enrich the *Enterococcus* genus, thereby helping GT to prevent the increase in BP. However, there are also some reports that *Enterococcus* is an important opportunistic pathogen and can cause many infections (49). This controversial result may be related to different experimental conditions, design, and analytical methods, and it can be explained by more research on microbial regulation by GT. Compared with GT, OLT intake enriched more intestinal microbes, including *Allobaculum*, *Paraprevotella, Oscillospira*, *Bifidobacterium*, and *Ruminococcus*. *Allobaculum* and *Bifidobacterium* (recognized beneficial bacteria) play a valuable role in the body's intestines and play an active role in promoting body health (50). Besides, studies have shown that the abundance of *Paraprevotella* is positively correlated with body strength (51). Chen et al. found that *Oscillospira* was closely related to human health because its abundance was positively correlated with gut microbial diversity and was inversely correlated with BP (52). *Ruminococcus* was reported to produce short-chain fatty acids (SCFAs) and was beneficial to the intestinal environment, whereas SCFAs are generally considered to have a variety of essential roles in maintaining human health, such as lowering BP, reducing inflammation, and protecting the intestinal mucosal barrier (53). In addition, our results reveal that OLT supplementation also significantly increased the abundance of *Unspecified_S24_7*, *Unspecified_Clostridiales*, and *Unspecified_Ruminococcaceae* genera. Thereinto, the Ruminococcaceae family is negatively correlated with arterial stiffness (54). However, there is limited information about *Unspecified_S24_7* and *Unspecified_Clostridiales*, and the relationship between their levels and gut health needs to be further studied. Our results further confirmed the regulatory effect of tea on the intestinal flora, which may be a critical factor in preventing the increase in BP evoked by a high-salt diet.

The abundance analysis of the top 50 OTUs further supports the above analysis, and 24 and 34 different OTUs were markedly altered by GT and OLT administrations, respectively. Therein, *Bacteroides*, *Aggregatibacter*, *Anaerofustis*, *Agrobacterium*, *Elusimicrobium*, *Prevotella*, *Sutterella*, *Paraprevotella*, *Coprococcus*, *Ruminococcus*, and *Akkermansia* were negatively and significantly correlated with hypertension. These bacteria may be involved in intestinal metabolism and microenvironment remodeling, thus exerting their indirect effects on regulating BP. For example, a recent study revealed that *Bacteroides* could play a protective role in hypertension and heart failure in hypertensive rodents (55). In addition, some bacteria, including *Faecalibacterium*, *Shuttleworthia*, *Clostridium*, *Dorea*, and *Enterobacter* genera, may also help lower BP. For instance, studies have shown that fecal *Faecalibacterium* abundance in patients with hypertension was lower than in healthy controls (55). Therefore, our results show that bacterial genera related to BP or its metabolic disorders may be potential therapeutic targets for preventing hypertension.

To further explore the implication of intestinal microbiota on BP, the known pathways related to BP regulation in the KEGG L3 pathway were explored, including histidine metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, primary bile acid biosynthesis, and secondary bile acid biosynthesis. It has been proven that L-histidine can exert anti-hypertensive effects in hypertensive models through central histamine H3 receptors (56). Additionally, the downstream metabolites of tryptophan in the intestine, including serotonin and indole, play an essential role in BP regulation (57). It has been reported that the primary receptor TGR5 was expressed on multiple tissues involved in BP regulation, and TGR5 agonism increased the eNOS activity of endothelial cells, which was beneficial to lower BP (58). Besides, intravenous injection of secondary bile acids could reduce BP in hypertensive rat models (59). As expected, GT and OLT supplementation altered these microbial metabolic pathways, and OLT exhibited a better regulation effect. These results strongly confirm that tea can alleviate high-salt-induced hypertension by regulating the metabolism of intestinal microbes.

In conclusion, both GT and OLT suppressed endothelial dysfunction and alleviated the increase in BP, oxidative stress, inflammation, and tissue damage in mice fed a high-salt diet. In addition, the disturbance of intestinal flora induced by a high-salt diet could be modulated by GT and OLT, which may be related to their differentiated composition. In particular, OLT has shown better anti-hypertension and regulation effects on intestinal flora structure and metabolism.

## DATA AVAILABILITY STATEMENT

The datasets of intestinal flora of rats presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA811498).

## ETHICS STATEMENT

The animal study was reviewed and approved by Experimental Animal Ethics and Use Committee of Shanghai Jiao Tong University.

## AUTHOR CONTRIBUTIONS

XY, XT, and FL: writing—original draft. JZ: data curation and validation. MW: data curation and software. XW: supervision and project administration. YW: supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.892801/full#supplementary-material

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# Effects of Soy Isoflavones and Green Tea Extract on Simvastatin Pharmacokinetics and Influence of the SLCO1B1 521T > C Polymorphism

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**Objectives:** Green tea and soy products are extensively consumed by many people and they may influence the activity of drug metabolizing enzymes and drug transporters to result in drug interactions. This study was performed to evaluate the effect of green tea and soy isoflavone extracts on the pharmacokinetics of simvastatin in healthy subjects and to clarify the role of polymorphisms in the SLCO1B1 drug transporter in this effect.

**Methods:** This was an open-label, three-phase randomized crossover pharmacokinetic study. A single dose of simvastatin 20 mg was taken on three occasions (without herbs, with green tea, and with soy isoflavones) by healthy male Chinese subjects. The green tea and soy isoflavone extracts were given at a dose containing EGCG 800 mg once daily or soy isoflavones about 80 mg once daily for 14 days before simvastatin dosing with at least 4-weeks washout period between phases.

**Results:** All the 18 subjects completed the study. Intake of soy isoflavones was associated with reduced systemic exposure to simvastatin acid [geometric mean (% coefficient of variation) $AUC_{0-24h}$ from 16.1 (44.2) h·μg/L to 12.1 (54.6) h·μg/L, $P < 0.05$) but not the lactone. Further analysis showed that the interaction between simvastatin and the soy isoflavones only resulted in a significant reduction of AUC in subjects with the SLCO1B1 521TT genotype and not in those with the 521C variant allele. There was no overall effect of the green tea extract on simvastatin pharmacokinetics but the group with the SLCO1B1 521TT genotype showed reduced AUC values for simvastatin acid.

**Conclusion:** This study showed repeated administration of soy isoflavones reduced the systemic bioavailability of simvastatin in healthy volunteers that was dependent on the SLCO1B1 genotype which suggested that soy isoflavones-simvastatin interaction is impacted by genotype-related function of this liver uptake transporter.

**Keywords: SLCO1B1, drug interaction, EGCG, green tea, simvastatin, soy isoflavones**

## INTRODUCTION

Cardiovascular diseases (CVDs) are a significant health burden with an increasing prevalence and remain the leading causes of morbidity and mortality worldwide (1). Use of herbal medicines and foods and beverages thought to have beneficial effects in CVD is very common among patients with this condition (2). There may be interactions between herbal medicines or foods with the drugs taken for CVD which may result in toxicity or altered efficacy. The composition of most herbal medicines is complex, with each herb containing a variety of chemical components and each of these components may lead to herb-drug interactions by affecting the activity of drug metabolizing enzymes or drug transporters (3).

Simvastatin is one of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins, which has been used extensively worldwide to reduce low-density lipoprotein cholesterol (LDL-C) and the risk for CVD events. It is administered in the inactive lactone form and is rapidly hydrolyzed to the active open acid form, simvastatin acid (4). This is converted back to simvastatin lactone through a glucuronidation and lactonization pathway (5). Simvastatin acid and lactone are extensively metabolized by cytochrome P450 (CYP) enzymes, mainly CYP3A4 and CYP3A5 with a minor contribution from CYP2C8 (6). Simvastatin acid is a substrate for the liver uptake transporter organic anion–transporting polypeptide 1B1 (OATP1B1) encoded by SLCO1B1 and the adenosine triphosphate (ATP)-binding cassette (ABC) efflux transporters, ABCG2 and ABCB1 (7). The ABCG2 c.421C > A polymorphism contributed toward differences in exposure to simvastatin acid between Caucasian and Asian subjects (8).

The SLCO1B1 c.521T > C single nucleotide polymorphism (SNP) can result in marked inter-individual differences in pharmacokinetics of simvastatin acid (9) and was the only functional genetic variant associated with simvastatin-induced myopathy in a genome wide association study (GWAS) (10) and this variant also influenced the lipid response to simvastatin in a meta-analysis of GWAS (11). A GWAS of the LDL-C response to simvastatin in the Heart Protection Study also identified an ABCC2 variant having a significant effect (12). Drugs and chemicals from herbs and food materials which interact with any of these pathways could therefore influence the systemic exposure and efficacy and safety of simvastatin. A case was reported in which green tea was thought to interact with simvastatin to cause muscle pain (13).

Soybeans contain large amounts of isoflavones, including phytoestrogens, and there is evidence that some isoflavones may modify CYP enzyme expression and activity (14). The average daily intake of soy foods in Asian adults provides an average of 15-45 mg isoflavones/day (15) Rats fed diets containing soy protein isolate showed increased activity and expression of CYP3A1 (16) related to greater binding of the pregnane X receptor (PXR) to a response element on the CYP3A1 promoter (17). Isoflavones also activated human PXR increasing CYP3A4 expression (18). Soy components can activate other nuclear receptors including peroxisome-proliferator activated receptors (PPAR) α and PPARγ and liver X receptor (LXR) resulting in increased expression of CYP3As (19–21). These nuclear receptors can also modulate the expression of drug transporters.

On the other hand, flavonoids can inhibit multiple ABC efflux transporters, including ABCB1, ABCC2 and ABCG2 (22). Many flavonoids can inhibit OATP1B1 in a concentration-dependent manner but rutin had a stimulatory effect (23). In a recent study which investigated the effect of 25 common flavonoids on OATP1B1-mediated uptake of the fluorescent substrate 2′,7′-dichlorofluorescein (DCF) in Chinese hamster ovary (CHO) cells stably expressing human OATP1B1, most of the flavonoids tested had a concentration-dependent inhibitory effect on OATP1B1-mediated DCF uptake, but a low concentration of epicatechin gallate (ECG) showed a stimulating effect of about 160% (24).

This study was conducted to examine the effect of green tea extract and soy isoflavones on the pharmacokinetics of simvastatin in healthy subjects and whether any interactions were influenced by the polymorphisms in SLCO1B1.

## MATERIALS AND METHODS

### Subjects

Eighteen healthy Chinese male subjects who gave written informed consents were recruited for the study. Participants were selected from a group of healthy subjects who had previously been genotyped for the SLCO1B1 521T > C (rs4149056) polymorphism so there would be a reasonable number of subjects in each of the 3 genotype groups. The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki and approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee with reference number CRE-2010.524-T.

Subjects were required to abstain from taking any prescription or non-prescription medications from 2 weeks prior to and during the study. Smoking, alcohol, grapefruit juice, caffeine, soybean milk, tea, dietary supplements, and herbal products were forbidden from 2 weeks before and throughout the entire study. Subjects fasted for 10 h before and 4 h after administration of simvastatin during the blood sampling sessions. Standardized meals were provided to consume at 4 h and 10 h after drug

administration. The meals were provided by the hospital catering service and were the same for all subjects and the same for the 3 phases of the study. Water intake was not allowed from 1 h pre-dose to 1 h post-dose except for the water provided for drug administration. Subjects were asked to report any adverse effects during the pharmacokinetic sampling and at other visits to the study center.

## Simvastatin-Herb Pharmacokinetic Interaction

The study was an open-label, three-phase randomized crossover design. Simvastatin 20 mg (Zocor®, MSD) was given 3 times: 1. simvastatin only; 2. with green tea extract; 3. with soy isoflavones extract. There was a washout period of at least 4 weeks between phases. The extracts of green tea or soy isoflavones were taken as one sachet once daily in the morning before breakfast for 14 days. The herbal extracts were provided as a powder which was taken in 150 ml water at room temperature. Fourteen blood samples were taken at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, and 24 h after the dose to evaluate the pharmacokinetic profile on the simvastatin dosing days. A food diary was used to monitor the food compliance during the study and subjects were asked to record all their daily food intake including the main meals, snacks and beverages. Subjects were requested to follow the requirements on diet carefully.

## Herbal Products

The extracts of green tea and soy isoflavones were manufactured by the Hong Kong Institute of Biotechnology (HKIB) in accordance with Good Manufacturing Practice (GMP). Standard heavy metals, microbial and pesticide testing was performed to ensure the products fulfilled the safety requirements set out by the Department of Health in Hong Kong. Each sachet of green tea extract or soy isoflavones was claimed to contain 800 mg standardized polyphenol (mainly EGCG) or 120 mg total isoflavones, respectively.

## Establishment of Chemical Profiles of Herbal Products

Ultra-Performance Liquid Chromatography (UPLC) methods were used to verify the contents of the green tea extracts and soy extracts in the laboratory of the Institute of Chinese Medicine, the Chinese University of Hong Kong as described previously (25). The green tea extract was compared with a standard mixture containing 7 chemical markers: gallic acid (GA), epigallocatechin (EGC), catechin (C), epigallocatechin gallate (EGCG), caffeine (CAF), epicatechin (EC) and epicatechin gallate (ECG) in methanol. Soy isoflavone extract was compared with a standard mixture containing 7 chemical markers: glycitin, daidzin, genistin, daidzein, glycitein, genistein and acetylgenistin in methanol.

## Quantification of Plasma Concentrations of Simvastatin and Simvastatin Acid

Plasma concentrations of simvastatin and its major active metabolite simvastatin acid were determined by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) employing online sample pre-treatment.

The concentrations of simvastatin acid and lactone in plasma were simultaneously quantified by a method validated according to the U.S. Food and Drug Administration (USFDA) guidance on Bioanalytical Method Validation (26) employing LC-MS/MS using the corresponding isotopically labeled compounds as internal standards. The plasma samples were prepared using liquid–liquid extraction with diethyl ether. Chromatographic separation was accomplished on an XBridge C18 (3.5 μm 2.1 × 30 mm Column; Waters, MA, United States). qqThe mobile phase consisted of a gradient mixture of 0.015 mmol/L ammonium acetate in water (mobile phase A) and methanol (mobile phase B) at a flow rate of 0.4 mL/min. The gradient started at 50% mobile phase B for 0.5 min with a subsequent fast gradient to 98% mobile phase B in 1 min and maintained for another 0.5 min. The gradient was then returned to the initial mobile phase concentration in a chromatographic run of 3 min. Simvastatin acid was detected in a negative ionization mode with a quantification transition of m/z 435.4–115.0 and a qualification transition of m/z 435.4–319.2 while the lactone was quantified in a positive ionization mode with a transition of m/z 441.3–310.2 and monitored by a qualification transition of m/z 441.3–310.2. The lower limits of quantification of simvastatin acid and lactone were 0.1 μg/L by using 300 μL plasma. The linear ranges of the method were from 0.1 to 20.0 μg/L for both simvastatin acid and lactone. The coefficients of variation were lower than 10 and 9% for simvastatin acid and lactone, respectively.

## Effects on Plasma Lipid Profile and Blood Pressure With Green Tea Extract and Soy Isoflavones

Fasting lipid profiles and blood pressure were monitored at baseline and after 14 days of green tea extract and soy isoflavones, respectively. Plasma lipid profile including total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C) was measured on a Roche Modular Analytics system (Roche Diagnostics GmbH, Mannheim, Germany) using standard reagent kits supplied by the manufacturer of the analyzer and LDL-C level was estimated by using the Friedewald formula (27) or directly measured when the triglyceride level was over 4.5 mmol/L. After 5 min of resting seated, clinic blood pressure and heart rate was measured four times at 2-min intervals in the dominant arm with an automatic device (Omron HEM 7080IT, Omron Healthcare). The average of the last three measurements was used in the statistical analyses.

## Genotyping

High Pure PCR Template Preparation Kits (Roche Applied Science) were used to extracted DNA from the blood samples. TaqMan Drug Metabolism Genotyping Assays from Applied Biosystems (Foster City, CA, United States) was used to genotype each subject for the SLCO1B1

388A > G (rs2306283) and 521T > C (rs4149056) polymorphisms.

## Pharmacokinetic Analysis

The pharmacokinetic parameters of simvastatin and its active metabolite simvastatin acid were calculated using non-compartmental methods with the aid of the computer program WinNolin (version 2.1, Pharsight Corporation). The maximum plasma concentration ($C_{max}$) and time to $C_{max}$ ($t_{max}$) were obtained directly from the observed concentration-time data. The terminal elimination rate constant ($\lambda_Z$) was determined by linear regression of the terminal portion of the concentration-time curve and the elimination half-life ($t_{1/2}$) was calculated as $0.693/\lambda_Z$.

Systemic exposure to simvastatin lactone and simvastatin acid was evaluated by calculating the AUC using the linear trapezoidal rule and $AUC_{0-\infty}$ was calculated as $AUC_{0-\infty} = AUC_{0-t} + C_t/K_{el}$ where $C_t$ is the last quantifiable concentration. The oral clearance (CL/F) was calculated as $Dose/AUC_{0-\infty}$.

## Statistical Analysis

The pharmacokinetic parameters of simvastatin with and without herb consumptions calculated by repeated measures ANOVA and the Friedman rank test was used to compare $t_{max}$ values. The geometric mean ratios and 90% confidence intervals (CI) were calculated from the log-transformed values of $C_{max}$ and AUC compared between with and without the herbal extracts. ANOVA analysis was used to compare the pharmacokinetic parameters and interactions among genotype groups if the data was normally distributed, otherwise the Kruskal-Wallis test was used for skewed data. $P < 0.05$ was considered statistically significant.

## Sample Size

Previous studies have shown that significant effects of polymorphisms in drug transporters can be seen for single-dose complete pharmacokinetic studies in small groups of $n = 6$ (28). A herb-drug interaction between baicalin and rosuvastatin was shown to be related to different SLCO1B1 haplotype groups in 18 healthy Chinese subjects, with 6 subjects in each haplotype group. A similar sample size of 18 subjects was used in the current study to explore the potential herb-drug interactions and their relationship with the SLCO1B1 521T > C (rs4149056) polymorphism.

# RESULTS

## Establishment of Chemical Profiles of Herbal Products

The green tea extract contained mainly EGCG and the other 6 chemical markers were present in small amounts. Each sachet continued the amounts of EGCG, ECG, EC, EGC, GA, CAF, and C of 804.6, 45.5, 5.9, 3.7, 1.02, 1.08, and 0.96 mg, respectively.

The soy isoflavone product contained the 7 chemical markers, namely glycitin, daidzin, genistin, daidzein, glycitein, genistein and acetylgenistin in amounts of 58.64, 8.72, 6.48, 2.20, 4.23, 0.42, and 0.90 mg per sachet, respectively. These seven isoflavones were calculated to contribute a total of 81.6 mg per sachet. Some other small unidentified peaks were seen on the chromatogram and these may represent other components of the extract that contribute to the total isoflavones.

## Effect of Green Tea Extract and Soy Isoflavones on the Pharmacokinetics of Simvastatin and Simvastatin Acid

In the 18 healthy Chinese male volunteers (mean ( ± SD) age: 26.6 ± 6.0 years; body weight: 61.7 ± 6.3 kg; body mass index: 21.1 ± 1.7 kg/m$^2$) (**Table 1**), intake of green tea extract 800 mg daily for 14 days had no significant effect on the average pharmacokinetic parameters for simvastatin or simvastatin acid (**Table 2**). However, intake of soy isoflavones significantly reduced the systemic exposure to simvastatin acid with significant reductions in the AUC values but not in $C_{max}$ (**Table 2**). There was no significant effect on any of the pharmacokinetic parameters for simvastatin lactone with the green tea or soy extracts.

The SLCO1B1 521T > C polymorphism was significantly associated with the pharmacokinetics of simvastatin acid, but not the lactone. Compared to the other genotype groups, subjects with the 521CC genotype ($n = 4$) had increased systemic exposure to simvastatin acid, but not simvastatin lactone, at baseline and after intake of green tea and soy isoflavones and the difference remained statistically significant after adjustment for body weight (**Tables 3**, **4**). Further analysis showed that the interaction between simvastatin acid and the soy isoflavones only occurred in subjects with the 521TT genotype but not in those with the TC or CC genotypes (**Table 4** and **Figure 1**). The percentage reduction in $AUC_{0-24h}$ of simvastatin lactone

**TABLE 1 |** Demographics of the 18 study subjects in the simvastatin-herb interaction study.

| Demographics | All subjects ($n = 18$) | SLCO1B1 521TT ($n = 6$) | | | SLCO1B1 521TC ($n = 8$) | | | SLCO1B1 521CC ($n = 4$) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 388AA ($n = 0$) | 388AG ($n = 3$) | 388GG ($n = 3$) | 388AA ($n = 1$) | 388AG ($n = 4$) | 388GG ($n = 3$) | 388AA ($n = 0$) | 388AG ($n = 0$) | 388GG ($n = 4$) |
| Age, years | 26.6 ± 6.0 | | 26.7 ± 6.4 | 26.3 ± 1.5 | 28 | 22.5 ± 1.7 | 36.7 ± 5.1 | | | 25.3 ± 5.1 |
| BW, kg | 61.7 ± 6.3 | | 62.5 ± 6.2 | 60.8 ± 2.4 | 64.2 | 58.0 ± 8.6 | 66.1 ± 8.2 | | | 55.2 ± 5.6* |
| BMI, kg/m$^2$ | 21.1 ± 1.7 | | 20.8 ± 1.9 | 20.5 ± 1.1 | 22.9 | 20.5 ± 2.4 | 22.2 ± 2.4 | | | 19.8 ± 1.7* |

*$P < 0.05$ vs. SLCO1B1 521TT; BW, body weight.*

and simvastatin acid with soy isoflavones in subjects with 521TT genotype were significantly greater than those with CC genotypes (**Figure 2**).

**TABLE 2 |** Effect of green tea extract and soy isoflavones on the pharmacokinetic parametes of simvastatin lactone and simvastatin acid in 18 healthy subjects.

| Variable | Simvastatin | Simvastatin and Green tea | Simvastatinand Soy isoflavones | Overall *P* values |
|---|---|---|---|---|
| **Simvastatin lactone** | | | | |
| $C_{max}$, μg/L | 3.35 (54.9) | 3.68 (48.1) | 3.64 (32.3) | 0.814 |
| GMR (90%CI) | | 0.95 (0.95–1.72) | 0.97 (0.92–1.74) | |
| $AUC_{0-24\ h}$ | 14.4 (49.7) | 16.14 (40.5) | 14.39 (33.7) | 0.319 |
| GMR (90%CI) | | 1.06 (1.00–1.52) | 1.04 (0.90–1.29) | |
| $AUC_{0-\infty}$ | 14.7 (48.6) | 17.17 (39.8) | 15.2 (35.9) | 0.211 |
| $T_{1/2}$, h | 4.47 (27.4) | 4.96 (34.0) | 4.51 (33.6) | 0.772 |
| $T_{max}$, h | 1.0 (0.875, 2.5) | 1.5 (1.0, 2.25) | 2.0 (1.375, 2.25) | 0.138 |
| **Simvastatin acid** | | | | |
| $C_{max}$, μg/L | 2.21 (50.4) | 1.81 (50.0) | 1.78 (53.6) | 0.427 |
| GMR (90%CI) | – | 0.93 (0.67–1.40) | 0.82 (0.73–1.26) | |
| $AUC_{0-24h}$ | 16.09 (44.2) | 14.78 (48.1) | 12.09 (54.6)* | 0.015 |
| GMR (90%CI) | – | 0.85 (0.77–1.27) | 0.73 (0.68–0.95) | |
| $AUC_{0-\infty}$ | 17.76 (45.3) | 16.53 (45.8) | 12.82 (54.6)* | 0.010 |
| $T_{1/2}$, h | 3.94 (31.7) | 5.17 (38.7) | 3.89 (28.3) | 0.262 |
| $T_{max}$, h | 4.0 (3.0, 4.0) | 4.0 (2.88, 4.0) | 4.0 (3.0, 4.0) | 0.976 |

*Data are geometric mean (% CV) except for $T_{max}$ for which median (IQR) are given.*
**P < 0.05 vs. baseline. AUC values in h·μg/L.*
*GMR, geometric mean ratio compared to simvastatin alone value.*

Interestingly, subjects with the 521TT genotype also had a significantly reduced systemic exposure to simvastatin acid with green tea extract, but this was not observed with simvastatin lactone, possibly due to wide variation in the systemic exposure to simvastatin lactone among individuals (**Table 4**). In contrast, subjects with the 521TC genotype but not the two homozygous groups had increased systemic exposure to simvastatin lactone with green tea extract intake. Due to the wide variation in the pharmacokinetics of simvastatin lactone and limited sample size, we cannot be certain if there is an interaction between simvastatin and green tea extract observed in SLCO1B1 genotype subgroup subjects or if this is just a chance finding. There were no statistically significant differences in percentage changes in $AUC_{0-24h}$ of simvastatin lactone and simvastatin acid with green tea extract among the SLCO1B1 genotype groups (**Figure 2**).

## Effect of the Green Tea Extract and Soy Isoflavones on the Plasma Lipid Profiles and Blood Pressure

The consumption of green tea extract for 2 weeks, but not soy isoflavones, was associated with significant reductions in plasma LDL-C (8.1% [95% confidence interval: −2.0, −14.3%], $P < 0.01$) and total cholesterol (4.8 % [0.4 −10.1%], $P < 0.05$) compared to baseline (**Table 5**). Reduction in LDL-C with green tea was observed in 15 out of 18 subjects and the change in LDL-C was not affected by the baseline levels. Neither green tea extract nor soy isoflavones influenced the plasma high-density lipoprotein cholesterol (HDL-C) or triglyceride levels or blood pressure in this normotensive group of subjects (**Table 5**).

**TABLE 3 |** Effects of the SLCO1B1 521 T > C polymorphism on the pharmacokinetics of simvastatin lactone at baseline and after intake of the green tea extract and soy isoflavones.

| Variable | 521TT (*n* = 6) | 521TC (*n* = 8) | 521CC (*n* = 4) | *P* values |
|---|---|---|---|---|
| **Baseline** | | | | |
| $C_{max}$, μg/L | 4.21 (63.5) | 3.4 (55.0) | 2.3 (47.8) | >0.05 |
| $AUC_{0-24h}$, h·μg/L | 24.1 (50.1) | 9.5 (35.9) | 15.0 (42.5) | >0.05 |
| $AUC_{0-\infty}$, h·μg/L | 24.4 (55.8) | 10.3 (35.2) | 16.2 (40.6) | >0.05 |
| $T_{1/2}$, h | 4.1 (16.2) | 4.54 (34.0) | 4.83 (30.2) | >0.05 |
| $T_{max}$, h | 2.0 (1.0, 3.0) | 1.0 (0.625, 1.375) | 2.25 (0.5, 4.0) | >0.05 |
| **After green tea** | | | | |
| $C_{max}$, μg/L | 4.30 (58.5) | 3.13 (53.4) | 4.03 (21.5) | >0.05 |
| $AUC_{0-24h}$, h·μg/L | 19.06 (47.7) | 13.39 (35.6)* | 18.29 (42.7) | >0.05 |
| $AUC_{0-\infty}$, h·μg/L | 20.37 (44.9) | 14.36 (36.6) | 18.98 (42.4) | >0.05 |
| $T_{1/2}$, h | 6.15 (19.4)** | 4.72 (42.7) | 3.98 (31.5) | >0.05 |
| $T_{max}$, h | 1.0 (1.0, 2.25) | 1.25 (1.0, 2.0) | 1.75 (1.5, 3.5) | >0.05 |
| **After soy isoflavones** | | | | |
| $C_{max}$, μg/L | 4.64 (39.1) | 3.42 (29.5) | 2.86 (20.6) | >0.05 |
| $AUC_{0-24h}$, h·μg/L | 15.29 (36.7)* | 12.63 (31.7)* | 17.07 (37.9) | >0.05 |
| $AUC_{0-\infty}$, h·μg/L | 15.61 (36.5)* | 13.23 (33.3)* | 19.30 (44.0) | >0.05 |
| $T_{1/2}$, h | 4.71 (15.5) | 4.71 (39.3) | 3.90 (49.1) | >0.05 |
| $T_{max}$, h | 1.5 (1.0, 1.75) | 2.0 (1.25, 2.5) | 2.0 (1.125, 2.875) | >0.05 |

**P < 0.05 vs. baseline; **P < 0.01 vs. baseline.*

**TABLE 4 |** Effects of the SLCO1B1 521 T > C polymorphism on the pharmacokinetics of simvastatin acid at baseline and after intake of the green tea extract and soy isoflavones.

| Variable | 521TT (*n* = 6) | 521TC (*n* = 8) | 521CC (*n* = 4) | *P* values |
|---|---|---|---|---|
| **Baseline** | | | | |
| $C_{max}$, μg/L | 1.68 (46.3) | 1.78 (38.0) | 5.11 (35.7) | 0.019 |
| $AUC_{0-24h}$, h·μg/L | 12.48 (30.26) | 12.34 (28.03) | 40.06 (26.8) | 0.001 |
| $AUC_{0-\infty}$, h·μg/L | 14.54 (30.7) | 12.87 (29.7) | 43.42 (26.4) | 0.001 |
| $T_{1/2}$, h | 3.63 (25.9) | 3.54 (34.4) | 5.39(30.2) | 0.328 |
| $T_{max}$, h | 3.0 (1.75, 4.0) | 4.0 (4.0, 4.0) | 3.5 (3.0, 4.0) | 0.502 |
| **After green tea** | | | | |
| $C_{max}$, μg/L | 1.02 (31.8) | 1.73 (33.1) | 4.68 (21.9) | <0.0005 |
| $AUC_{0-24h}$, h·μg/L | 8.45 (31.7)* | 14.53 (33.1) | 35.43 (25.4) | 0.001 |
| $AUC_{0-\infty}$, h·μg/L | 10.0 (31.5)* | 16.05 (33.9) | 37.24 (25.8) | 0.002 |
| $T_{1/2}$, h | 5.90 (53.9) | 4.86 (38.2) | 4.82 (16.2) | 0.807 |
| $T_{max}$, h | 4.0 (2.0, 4.0) | 4.0 (3.0, 4.0) | 4.0 (2.875, 4.0) | 0.682 |
| **After soy isoflavones** | | | | |
| $C_{max}$, μg/L | 0.93 (16.0) | 1.76 (26.5) | 4.88 (56.0) | <0.0005 |
| $AUC_{0-24h}$, h·μg/L | 6.44 (17.5)** | 11.0 (27.2) | 37.56 (41.8) | <0.00005 |
| $AUC_{0-\infty}$, h·μg/L | 6.88 (18.1)* | 11.51 (28.3) | 40.45 (37.6) | <0.00005 |
| $T_{1/2}$, h | 3.85 (13.4) | 3.68 (33.0) | 4.41 (40.8) | 0.801 |
| $T_{max}$, h | 4.0 (2.75, 4.0) | 3.5 (2.625, 4.0) | 3.5 (3.0, 4.0) | 0.823 |

**P < 0.05 vs. baseline; **P < 0.01 vs. baseline.*

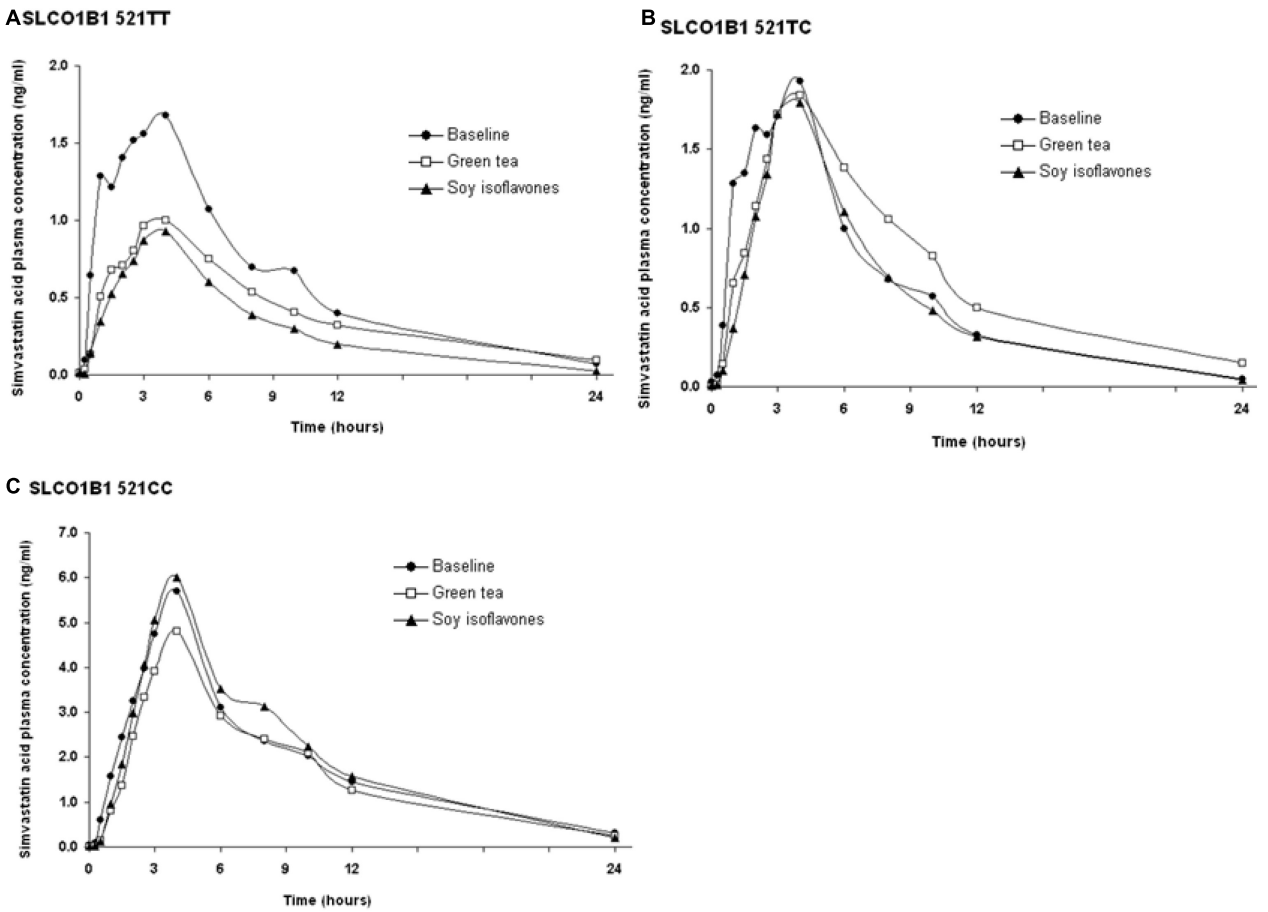


**FIGURE 1 |** Effects of the SLCO1B1 521 T > C polymorphism on the pharmacokinetic interactions between simvastatin acid before and after the green tea extract and soy isoflavones. Data are arithmetic mean.

## Adverse Events

No adverse events were reported during the periods of repeated intake of green tea extract or soy isoflavones or the single doses of simvastatin. The subjects recorded in the food diaries that they complied with the dietary restrictions during the study.

## DISCUSSION

Herbal medicines are often taken concomitantly with therapeutic drugs in many conditions, raising the potential for herb–drug interactions (HDIs). The major pathways leading to HDIs involve the inhibition or induction of CYP-mediated metabolism or drug transporters. The present study showed that administration of ECGC 800 mg per day for 14 days had no significant effect on the pharmacokinetics of simvastatin and simvastatin acid in the overall 18 healthy volunteers, although there was a reduction in systemic exposure to simvastatin acid in the group with SLCO1B1 521TT genotype. The most significant finding of the study is that intake of soy isoflavones with 80 mg daily for 14 days significantly reduced the systemic exposure to simvastatin lactone and simvastatin acid in healthy volunteers with the SLCO1B1 521TT genotype.

This finding is similar to a study which showed that baicalin, derived from the medical plant *Radix scutellariae*, reduced plasma concentrations of rosuvastatin in healthy subjects with haplotypes homozygous for the SLCO1B1 521TT genotype but not in those homozygous for the SLCO1B1 521CC genotype, with intermediate effects in the heterozygotes (28). We previously reported that soy isoflavones did not affect the pharmacokinetics of rosuvastatin (25) but the healthy subjects in that study were not selected for SLCO1B1 genotypes so we cannot exclude the possibility that the number of subjects with the SCLO1B1 521TT genotype was too small to show a significant effect in that previous research.

It has been shown that flavonoids could inhibit multiple ABC efflux transporters, including ABCB1, ABCC2 and ABCG2 (14) as well as the hepatic uptake transporter OATP1B1 (23). However, inhibition of OATP1B1 with soy isoflavones should result in an increased plasma concentration of simvastatin. The previous *in vitro* study examined the effects of individual flavonoids and showed that some of them significantly

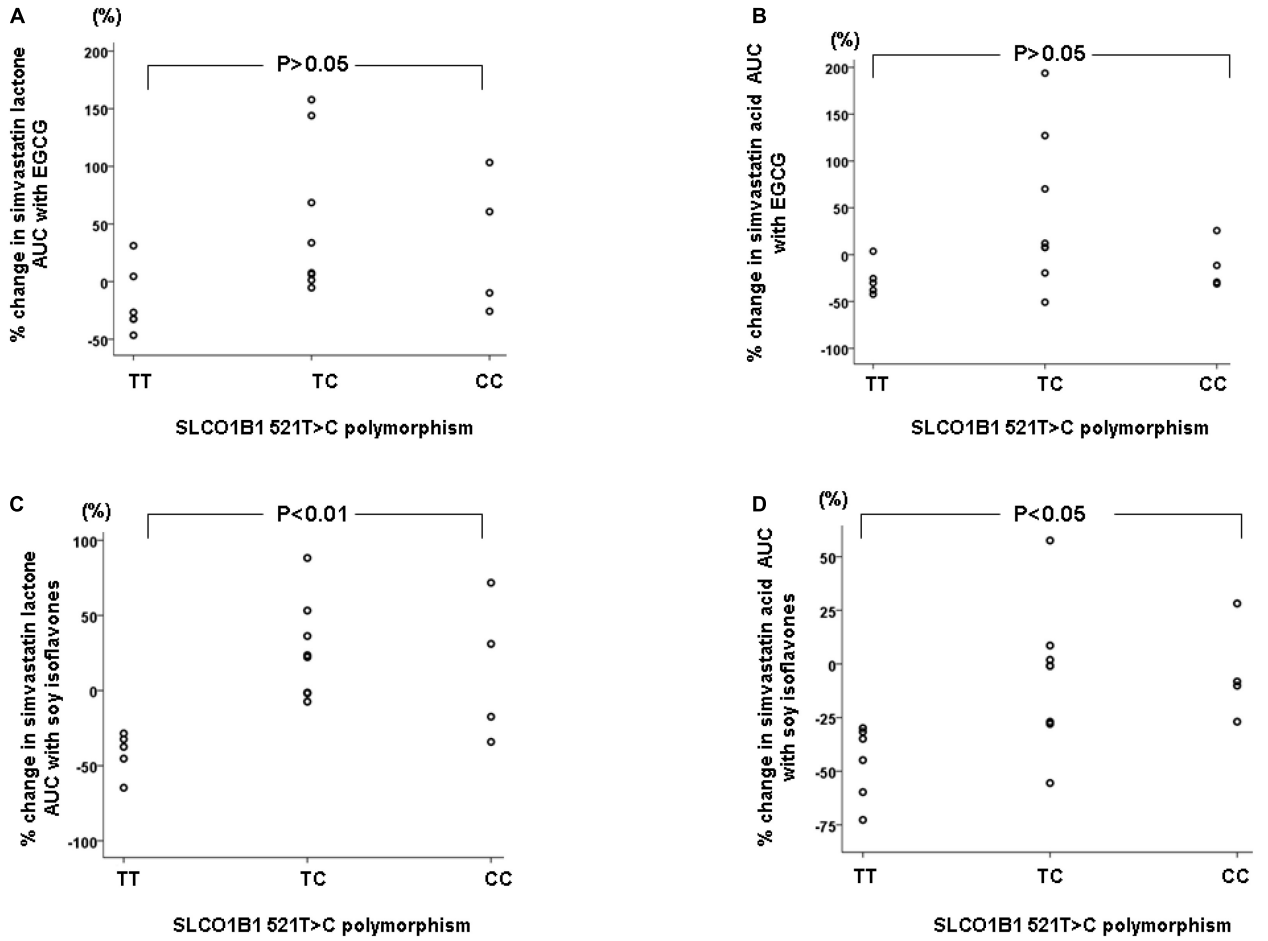


**FIGURE 2 |** Percentage changes in $AUC_{0-24h}$ of simvastatin and simvastatin acid with green tea extract **(A,B)** or soy isoflavones **(C,D)** according to the SLCO1B1 521T > C genotypes.

inhibited [$^3$H] dehydroepiandrosterone sulfate uptake in a concentration-dependent manner in OATP1B1-expressing cells (23). This may not be relevant to the present study which used a combination of flavonoids in the soy extract, which may not reach the same concentrations *in vivo* as those used *in vitro,* and also used a different substrate for OATP1B1.

It was previously demonstrated that feeding rats diets containing soy protein isolate (SPI) results in alterations in expression and inducibility of a number of CYP enzymes, including CYP1A1, CYP1A2, CYP2B1, and CYP2C11 (16, 29). In addition, it was shown that there was a significant elevation in expression and glucocorticoid-inducibility of hepatic CYP3As after feeding SPI-containing diets to rats and mice and after feeding soy infant formula to neonatal piglets (17, 18). The nuclear hormone receptor, PXR regulates multiple drug metabolizing enzymes and transporters including CYP3A4 and activation of PXR by endogenous and exogenous chemicals results in induction of drug metabolism. CYP3A4 is the most important phase I drug metabolizing enzymes that is responsible for the metabolism of approximately 50% of all prescription drugs including simvastatin. Li et al. demonstrated that the soy-associated isoflavones, genistein, daidzein and equol, activated both mouse and human PXR and subsequently upregulated CYP3A enzymes (18). It is therefore possible that the

**TABLE 5 |** Effects of green tea and soy product on plasma lipids and blood pressure.

| Parameters | Baseline | After green tea | After soy isoflavones | *P* |
|---|---|---|---|---|
| Total cholesterol (mmol/L) | 4.24 ± 0.73 | 4.02 ± 0.71* | 4.17 ± 0.86 | 0.111 |
| HDL-C (mmol/L) | 1.42 ± 0.24 | 1.40 ± 0.22 | 1.37 ± 0.17 | 0.332 |
| Triglycerides (mmol/L) | 0.86 ± 0.25 | 0.92 ± 0.34 | 0.90 ± 0.32 | 0.800 |
| LDL-C (mmol/L) | 2.42 ± 0.73 | 2.21 ± 0.70* | 2.39 ± 0.81 | 0.015 |
| Systolic blood pressure, (mmHg) | 113.6 ± 2.0 | 115.6 ± 2.1 | 112.8 ± 2.3 | 0.399 |
| Diastolic blood pressure, (mmHg) | 76.6 ± 9.7 | 77.5 ± 7.8 | 75.9 ± 9.4 | 0.779 |
| Pulse rate, (beats per minute) | 72.2 ± 10.8 | 71.8 ± 10.3 | 68.1 ± 9.4 | 0.413 |

*P values were assessed by repeated measures analyses of variance (ANOVA) for difference among the three groups.*
**P < 0.05 vs. baseline.*

interaction between soy isoflavones and simvastatin is mediated via activation of PXR.

In addition, other studies have shown that soy isoflavones, genistein or daidzein, can activate PPARα and PPARγ and regulate LXR activity indirectly by promoting the phosphorylation of LXRα and LXRβ, leading to differential expression of genes regulated by LXR (20, 30). These nuclear receptors can modulate CYP3A4 and multiple influx and efflux drug transporters suggesting that there may be complex interplays between nuclear receptors, CYP3A4 and drug transporters responsible for the observed interaction between soy isoflavones and simvastatin.

Considering that soy isoflavones significantly reduced the systemic exposure to simvastatin acid, but not the lactone, this may suggest the effect is through a transporter rather than the CYP enzymes as the lactone forms of most statins are metabolized more extensively than the active acid forms (31). Furthermore, the effect was only significant in the SLCO1B1 521TT genotype group with the most active form of the OATP1B1 transporter, suggesting the effect may be mediated by increasing expression or activity of this transporter. There was also a significant reduction in the AUC for simvastatin lactone with soy isoflavones and a significant reduction in the AUC for simvastatin acid with green tea extract in this genotype group. The effect could be mediated via activation of PXR to increase expression of OATP1B1 or a direct effect on the transporter activity by certain flavonoids.

As mentioned above, simvastatin acid is also a substrate for the efflux transporters, ABCG2, ABCB1 and ABCC2 (7, 12), and less active forms of ABCG2 are associated with increased systemic exposure to simvastatin acid (8). It is therefore possible that activation of ABCG2, ABCB1 or ABCC2 would reduce the AUC for simvastatin acid but it seems unlikely that the effect would be influenced by the SLCO1B1 genotype. Likewise, other OATP transporters expressed in hepatocytes and enterocytes such as OATP1B3, OATP2B1 and OATP1A2 may play a minor role in the disposition of simvastatin acid (9), but effects on these are unlikely to be SLCO1B1 genotype-dependent.

A previous case report documented that consumption of green tea might be associated with increased systemic exposure to simvastatin lactone and acid and appeared to trigger statin muscle intolerance in a 61-year-old man with hypercholesterolemia who developed muscle symptoms while receiving low doses of various statins (13). In addition, an *in vitro* study demonstrated that green tea extract (containing EGCG, EGC, EC, and ECG at 43.3, 24.8, 9.7 and 1.7%, respectively, with a total catechin content of 86.5% w/w) weakly to moderately inhibited CYP3A activity in a non-competitive manner as evaluated by midazolam 1′-hydroxylation in rat hepatic microsomes (32). A single oral dose of green tea extract (400 mg/kg) 30 min before simvastatin administration was associated with significantly increased $AUC_{0-6h}$ of simvastatin by 3.4-fold but had no effect on $t_{1/2}$ in rats suggesting that green tea extract did not affect the elimination of simvastatin (32). The $AUC_{0-6h}$ of simvastatin acid was increased by 2.0-fold in this animal study but this was not statistically significant due to large inter-individual variations (32).

The discrepancies between our study and the previous *in vitro* and animal studies may be due to various factors. Firstly, the *in vitro* and animal studies generally use very high doses or concentrations of green tea extract which may not be clinically relevant and may have different effects on drug metabolizing enzymes and transporters. It has been estimated that a freshly brewed cup of green tea may contain 130–180 mg of EGCG (33). The present study used an 800 mg dose of green tea extract, which is equivalent to about 5–6 cups of green tea and may be more relevant to the normal intake, although giving the extract as a single dose will result in higher maximum concentration of catechins than normal consumption of green tea as a beverage throughout the day. Secondly, the previous experiments with green tea extract were performed with a single dose of green tea extract or a single cup of green tea and these may have different effects from the multiple doses used in the present study. This study used an extract which contained predominantly EGCG, which is the most abundant catechin in green tea (up to 80%), whereas other studies may have used other green tea extracts or green tea drinks contain a different mixture of catechins which may again also have different effects on drug metabolizing enzymes and transporters.

In a previous study we showed that the same extract of green tea reduced the systemic exposure to rosuvastatin by about 20% when the extract was given daily for 2 weeks and simultaneously with the dose of rosuvastatin (25). That effect may have been due to activation of liver uptake by OATP1B1 or OATP1B3, or inhibition of intestinal uptake by OATP2B1 or OATP1A2. It has been shown that EGCG can inhibit OATP1A2- and OATP2B1-mediated uptake of estrone-3-sulfate in a concentration-dependent manner in cells expressing these transporters (34). Green tea taken as a drink reduced the systemic exposure to nadolol by 85% in healthy volunteers, which was thought to be due to inhibition of OATP1A2-mediated intestinal uptake of nadolol which was supported *in vitro* studies (35).

Moreover, EGCG was shown to activate OATP1B3 in *in vitro* studies (36). Transporters such as ABCB1 and possibly hepatic OATP1B transporters can be induced by various compounds by activating PXR (37). The effect of the green tea extract used in the present study to reduce the systemic exposure to rosuvastatin in our previous study and possibly reduce the systemic exposure to simvastatin in the SLCO1B1 521TT genotype group in the present study may be due to activation of liver uptake transporters and such effects are known to vary with different substrates (37). Increased liver uptake of these statins may result in increased LDL-C lowering effects and it would be interesting to examine this.

## LIMITATIONS

There are some limitations in this study. It is well known that HDIs may depend on the dosage of herbs used and, in this study, we only investigated one dosage for each herbal product. The dosage was chosen to correspond with a high intake of the natural substances in food or beverages. Secondly, there may be a physical reaction between the herbal extracts and the drug because the

subjects took the herbal extracts and simvastatin simultaneously on the dosing day to try to identify the maximum interaction between the herbs and drug. It was shown that the bioavailability of sunitinib in rats was reduced when taken together with EGCG but not when the EGCG was taken 8 or 4 h before sunitinib, which appeared to be due to a physical reaction between the two compounds when taken together (38). Thirdly, although we instructed the subjects to follow the dietary restrictions, this relied on the subjects' cooperation and honesty and may not be entirely reliable, but it was a practical way to conduct the study.

## CONCLUSION

Repeated green tea catechin administration at a daily dose of about 800 mg EGCG for 2 weeks had no significant overall effect on the pharmacokinetics of simvastatin in healthy volunteers but appeared to reduce the systemic exposure to simvastatin acid in subjects with the SLCO1B1 521TT genotype. Soy isoflavones at a dose of approximately 80 mg daily for 14 days was associated with reduced systemic exposure to simvastatin and simvastatin acid and this interaction appeared to occur in subjects with the SLCO1B1 521TT genotype but not in subjects with the 521C variant allele. Further studies are needed to investigate the underlying mechanisms responsible for these observed interactions and to assess the clinical relevance of these interactions in patients receiving long-term simvastatin.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

WZ and MH analyzed the data and wrote the manuscript. BT designed the research project. MH, BT, HL, EW, CL, CW, and CH performed the experiments. BT and CH revised the manuscript. All authors contributed to the article and approved the submitted version.

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# Tea Ingredients Have Anti-coronavirus Disease 2019 (COVID-19) Targets Based on Bioinformatics Analyses and Pharmacological Effects on LPS-Stimulated Macrophages

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Coronavirus disease 2019 (COVID-19) is a contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that caused millions of deaths and lacks treatment. Although several studies have focused on the major component of green tea, epigallocatechin 3-gallate (EGCG), which is efficient in preventing COVID-19, systemic analyses of the anti-COVID-19 potential of green tea remain insufficient. Here, we co-analyzed the target genes of tea ingredients and COVID-19 signature genes and found that epigallocatechin 3-acetalbehyde was capable of reversing the major molecular processes of COVID-19 (MAPK and NF-κB activation). These findings were further supported by Western blotting (WB), immunofluorescence, and quantitative polymerase chain reaction (qPCR) in LPS-stimulated macrophages. Moreover, using molecular docking analysis, we identified three tea ingredients ((-)-catechin gallate, D-(+)-cellobiose, and EGCG) that may interact with the vital SARS-CoV-2 protein, 5R84, compared with the qualified 5R84 ligand WGS. Thus, our results indicated that tea ingredients have the potential to treat COVID-19 by suppressing the COVID-19 signature genes and interacting with the vital SARS-CoV-2 protein.

**Keywords: COVID-19, molecular docking, network pharmacology, tea ingredients, macrophage, key targets**

## INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a disease with main manifestations involving the lungs and is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). SARS-CoV-2 is rapidly spreading around the world, and the number of confirmed cases and infection-related deaths are increasing every day (2). The severity of COVID-19 is associated with increased inflammatory and chemokine factors; these factors also predict COVID-19 mortality (3). Although the pathogenesis of COVID-19 is not fully understood, the virus and host immune system play key roles in its development (4).

From Delta to Omicron, the new coronavirus is constantly mutating, the global epidemic is at a high level, and the number of infections continues to increase (5). While COVID-19 vaccines can greatly prevent the spread of the virus, they cannot treat patients infected with the virus (6). To treat patients with new coronavirus pneumonia, scientists have made considerable efforts in drug research and development; however, to date, there are still very few drugs that can treat COVID-19 (7). Although some neutralizing antibodies and small molecule inhibitors are being developed, there is uncertainty about their safety and efficacy (8). Therefore, we urgently need to explore new strategies to treat COVID-19.

Tea is popular all over the world as a food drink; in fact, tea has been used as an herbal medicine to prevent and treat various diseases (9). Tea and its characteristic polyphenols—catechins—have been shown to be active in preventing obesity, diabetes, cardiovascular disease, cancer, and other diseases (10–12). Tea ingredients have also been shown to have anti-viral activity as well as protective activity against diseases caused by oxidative stress and inflammation; many of these ingredients may help alleviate and treat COVID-19 (13, 14). Although several studies have focused on epigallocatechin gallate (EGCG), the major component of green tea, which has been shown to be effective in preventing COVID-19 (15), we focused on systematic research of the therapeutic potential of tea components for COVID-19, including inhibition of COVID-19 signature gene transcription and direct interactions with specific COVID-19 proteins. Systematic research about tea and COVID-19 currently remains insufficient. Systematic analyses of the anti-COVID-19 potential of green tea and other teas remains insufficient. In this study, we mainly used bioinformatics and computational network-based pharmacology to explore and determine the efficacy and possible therapeutic mechanisms of tea for the treatment of COVID-19 to reveal the potential uses of tea in the treatment of COVID-19. Using a network pharmacology strategy, we report the pharmacological targets and molecular pathways of tea ingredients. Therefore, in this bioinformatics report, we aimed to reveal the component-target-pathway network and pharmacological mechanisms of tea ingredients in the prevention and treatment of COVID-19.

# MATERIALS AND METHODS

## Identification of the Target Genes of Tea in the Treatment of COVID-19

Using effective tools such as Traditional Chinese Medicine Systems Pharmacology (TCMSP), Swiss Target Prediction, and SuperPred, the target genes of tea were screened from existing databases (16, 17). Other genes related to the occurrence of COVID-19 were obtained using the DisGeNET and GeneCards databases (18). In addition, these putative tea and COVID-19 genes were mapped using the UniProt tool prior to correction. After functional enrichment analysis using FunRich software, all anti-COVID-19 targets of tea were screened and identified.

## Protein-Protein Interaction (PPI) of Candidate Genes

After obtaining the targets of tea and COVID-19, the STRING database was used to further determine and construct a functional protein association network according to a specific algorithm (19, 20). In addition, based on the merged targets of tea and COVID-19, a protein-protein interaction (PPI) network was constructed using Cytoscape software (21, 22). Therefore, the key targets of tea in the treatment of COVID-19 were revealed, visualized, and determined with the topology parameters of the network analyzer tool (23, 24).

## Enrichment Analyses and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway of Intersection Targets

R language packages, such as ClusterProfiler, org.Hs.eg.Db, ReactomePA, and GOplot (3.6.1), have been used for enrichment analysis and visualization of the biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of intersection targets (25). In addition, gene annotation information from org.Hs.eg.Db (26, 27), a $p$-value cutoff = 0.05, and a $q$-value cutoff = 0.05 were used for enrichment before plotting the corresponding bubble chart, histogram, and Circos circle chart.

## Molecular Docking Analysis

To screen and identify key targets for tea-based molecular docking assays, a chemical-protein binding approach was used (28, 29). After searching for a specific protein through the PDB database, the 5R84 protein was selected for docking with the tea compound. The three-dimensional structure of tea was drawn using ChemBio3D Draw in Chem Bio Office 2010 software before docking the molecular structure with AutoDock Vina software (30). The plausibility of the docking parameter settings was assessed by the root-mean-square deviation (RMSD) of the ligand molecules. An RMSD $\leq$4 Å is the threshold for ligand molecular conformation.

## Cell Culture

Murine macrophage RAW 264.7 cells were acquired from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown at 37°C under 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin streptomycin (Gibco, USA) in humidified incubators (Thermo, USA). Lipopolysaccharide (LPS, Escherichia coli 055: B5) and EGCG were purchased from Sigma Chemical Co. (St. Louis, USA). RAW 264.7 cells were treated with LPS, LPS+EGCG, or EGCG (for the concentrations of LPS and EGCG see the figure legend) for 24 h. For viability testing, the cells were starved for 24 h without serum before challenge and seeded at a density of $1 \times 10^5$ cells/mL in 96-well plates with four replications, and cell viability was analyzed with a CCK-8 cell counting kit (Vazyme, China).

## Quantitative Real-Time Polymerase Chain Reaction (QPCR)

The total RNA was isolated from cells using an RNA extraction kit (Vazyme, China). First-strand complementary DNA (cDNA) was synthesized using an iScript cDNA Synthesis Kit (Vazyme, China). Quantitative PCR was performed with SYBR green PCR Master Mix (Vazyme, China) using a ViiA 7 Real-Time PCR System (Applied Biosystems, CA). The primers are detailed in **Table 1**. The following cycle parameters were used: 55°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s and 60°C for 30 s. The relative expressions of the target genes against that of the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were calculated using the $2^{-\Delta\Delta CT}$ method. Cell samples were evaluated in triplicate, and every experiment was performed at least three times. The transcription levels of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, Arg-1, and GAPDH were determined.

## Protein Extraction and Western Blotting (WB)

Total cellular proteins were extracted using radioimmunoprecipitation assay buffer containing 1% sodium dodecyl sulfate (SDS); 40 mg of total lysate was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride membrane, blocked with 5% bovine serum albumin in tris-buffered saline for 90 min, and then incubated with the appropriate primary antibody overnight at 4°C. Membranes were incubated with secondary antibody for 90 min at room temperature after washing and then visualized using ECL Plus Western Blot Detection Reagent (Millipore, USA). The protein expression levels of extracellular signal-regulated kinase (ERK), p-ERK, c-Jun amino-terminal kinase (JNK) and p-JNK, and p38 and p-p38 were determined by Western blotting (WB). GAPDH was used as an internal control.

## Enzyme Linked Immunosorbent Assay (ELISA)

According to the manufacturer's instructions, the cell supernatant concentrations of IL-6, TNF-α, and IL-1β were determined using ELISA kits (ExCell Bio, China).

**TABLE 1** | Primers used for real-time quantitative PCR analysis.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *iNOS* | ACTCAGCCAAGCCCTCACCTAC | TCCAATCTCTGCCTATCCGTCTCG |
| *TNF-α* | CAGGCGGTGCCTATGTCTC | CGATCACCCCGAAGTTCAGTAG |
| *IL-1β* | GCAACTGTTCCTGAACTCAACT | ATCTTTTGGGGTCCGTCAACT |
| *IL-6* | CCAAGAGGTGAGTGCTTCCC | CTGTTGTTCAGACTCTCTCCCT |
| *Arg-1* | CATATCTGCCAAAGACATCGTG | GACATCAAAGCTCAGGTGAATC |
| *GAPDH* | CATCCCAGAGCTGAACG | CTGGTCCTCAGTGTAGCC |

## Immunofluorescence Assay

The expression of phospho-p65 was detected by immunofluorescence assays using a fluorescence microscope. RAW 264.7 cells were cultured directly on glass coverslips in 6-well plates for 24 h. After stimulation with LPS in the presence or absence of EGCG, the cells were fixed with 4% paraformaldehyde in PBS. The membrane was permeabilized by treating the cells for 5 min with 0.1% Triton X-100 in PBS. After a brief washing in PBS, slides were blocked with 5% bovine serum albumin for 1 h and then incubated with rabbit polyclonal anti-human phopho-p65 antibody (dilution, 1:100) overnight at 4°C at room temperature. The next day, the specimens were rinsed with PBS three times. After washing, they were incubated with the secondary antibodies (Alexa Fluor® 594, Thermo Fisher Scientific, CA, USA) for 30 min and counterstained for nuclei with DAPI (Beijing Solarbio Science & Technology, Beijing, China) for 10 min. After a brief washing in PBS, slides were sealed using ProLong® Gold antifade reagent (Molecular Probes® by Life Technologies™, CA, USA). Fluorescence micrographs were acquired with a fluorescence microscope (Nikon ECLIPSE Ti-U, Nikon Co., Japan).

## Data Analysis

Normally distributed data were analyzed using Student's *t*-test (for two-group comparisons) or analysis of variance (for multiple-group comparisons). For non-normally distributed values (as determined by the Kolmogorov–Smirnov test), the Mann–Whitney's rank-sum test was used. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM) and presented using GraphPad Prism 5 software (LaJolla, CA).

# RESULTS

## Identification the Ingredients and Target Genes of Tea

We first downloaded the ingredients and target genes of tea from the Traditional Chinese Medicine Integrated Database (TCMID) database (31). Eleven annotated ingredients and 931 target genes were reported, among which EGCG was the major ingredient and targeted 556 genes (**Figures 1A,B**). According to Gene Ontology (GO) enrichment analysis, the target genes of tea were involved in inflammation and chemokines (positive regulation of cytokine production, positive regulation of leukocyte migration, etc.), coagulation and cell death (neuronal death, extrinsic apoptotic signaling pathways, etc.) (**Figure 1C**), which were previously reported as the molecular characteristics of COVID-19, indicating that the tea has the potential to have anti-COVID-19 activity. The KEGG enrichment analysis also demonstrated that tea has a strong antiviral activity, with target genes that were functionally enriched in COVID-19 and influenza A, and represses inflammation (**Figure 1D**).

In addition to EGCG, there are many additional components such as beta-phenylethyl isothiocyanate,

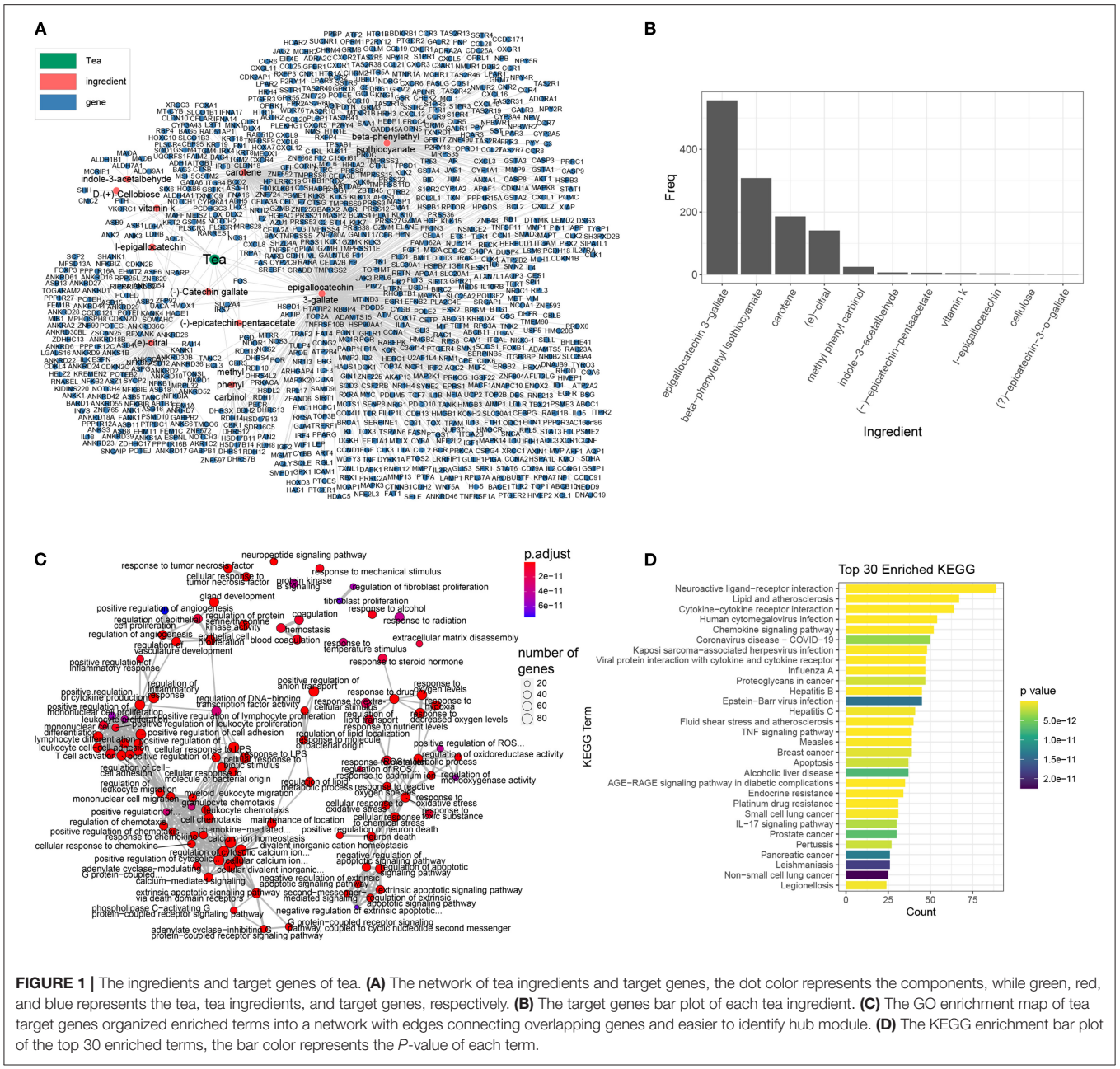


**FIGURE 1 |** The ingredients and target genes of tea. **(A)** The network of tea ingredients and target genes, the dot color represents the components, while green, red, and blue represents the tea, tea ingredients, and target genes, respectively. **(B)** The target genes bar plot of each tea ingredient. **(C)** The GO enrichment map of tea target genes organized enriched terms into a network with edges connecting overlapping genes and easier to identify hub module. **(D)** The KEGG enrichment bar plot of the top 30 enriched terms, the bar color represents the *P*-value of each term.

carotene, and citral, that also have anti-inflammatory and anti-chemotactic effects for COVID-19. We intersected the targets of other tea ingredients with the signature genes of COVID-19 (**Supplementary Figure 1**) and found that the targets of EGCG covered the most signature genes of COVID-19. Furthermore, beta-phenylethyl isothiocyanate and carotene also covered some signature genes. The enrichment analysis of the corresponding intersected genes (**Supplementary Figure 2**) showed that in addition to EGCG, other tea ingredients can repress the corresponding pathological processes involved in COVID-19. For instance, the targets of beta-phenylethyl isothiocyanate are closely related to cell chemotaxis in COVID-19. Citral inhibits inflammation and NK-κB signaling in COVID-19. Finally, cartone is related to coagulation and cytokine secretion. In summary, these results imply that tea can suppress inflammation and prevent coronavirus disease.

## Molecular Characterization of COVID-19 Infection

We next employed the DisGeNET and KEGG databases to characterize the molecular signature of COVID-19 infection. There were 1,288 and 232 COVID-19 signature genes in the DisGeNET and KEGG databases, respectively, among

which 87 genes were shared (**Figure 2A**). These genes were functionally enriched in response to viruses, innate and adaptive immune responses, inflammatory responses, and coagulation (**Figure 2B**). Notably, the signature genes of COVID-19 infection were also enriched in response to LPS, indicating a similar molecular pattern between COVID-19 infection and sepsis (**Figure 2B**). The similarity analysis of the enrichment results of the DisGeNET and KEGG COVID-19 genes revealed that cytokine and chemokine activity, endopeptidase activity, and phosphatidylinositol 3-kinase (PI3K) activity were the major processes of COVID-19 infection (**Figure 2C**). Furthermore, the enrichment of KEGG COVID-19 signature genes showed a MAPK signaling pathway specificity (**Figure 3C**). As for shared genes of the two databases, the PPI analysis implied that they were highly biologically relevant; among these genes, IL-6, TNF, and IL-1B were relevant to the highest degree (**Figure 2D**). In addition to cytokines, the Toll-like receptor (TLR2, TLR3, TLR7, and TLR8) and inflammatory signaling pathways (JAK-STAT, NF-κB, and MAPK signaling pathways) were also important components in the PPI network (**Figure 2D**). The functions of these genes included involvement in the antiviral process (COVID-19, influenza A, etc.) and responses to molecules of bacterial origin and inflammation (**Figures 2E,F**).

## Identification the Candidate Target Genes of Tea and COVID-19

To further verify the anti-COVID-19 activity of tea ingredients, we co-analyzed the target genes of tea with COVID-19 signature genes. There were 249 and 50 shared targets genes of tea with DisGeNET and KEGG COVID-19 gene signatures, respectively, and 33 shared genes in all conditions (**Figure 3A**). The shared GO enrichment items were focused on the response to bacteria and viruses, inflammation (cytokines and chemokines), immune responses, and coagulation; these cover the majority of signature genes of COVID-19 that were enriched (**Figures 2B**, **3B**), suggesting that the ingredients of tea might act as anti-COVID-19 components. The comparison of the GO enrichment results also showed that tea could target the critical pathological processes involved in COVID-19 infection including cytokine and chemokine activity, endopeptidase activity, and the MAPK signaling pathway (**Figure 3C**). The comparison of the KEGG enrichment results also showed a similar pattern that covered the major inflammatory signaling pathways including the JAK-STAT, NF-κB, and MAPK signaling pathways (**Figure 3D**). Furthermore, the shared 33 genes in all three conditions were functionally involved in inflammation and immune responses, which are similar to the major pathological processes of COVID-19, which involve the Toll-like receptor signaling pathway, IL-17 signaling pathway, and cytokine and chemokine activity (**Figure 3E**). Notably, the shared 33 genes were similar to the high degree genes in PPI, such as IL-6, TNF, and IL-1β, revealing that they were centrally involved in the pathological status of COVID-19 infection. Thus, the results demonstrated that the target genes of tea covered the critical processes involved in COVID-19 infection and might serve as anti-COVID-19 components.

## Molecular Docking Analysis of Tea Ingredients With the COVID-19 Protein 5R84

Previous studies have reported that small molecules are able to block the COVID-19 virus through interaction with vital virus proteins, such as 5R84 (32). We next examined the interaction of tea ingredients with the COVID-19 protein 5R84 by molecular docking (33). Six of 11 tea ingredients were capable of interacting with 5R84 and had lower free binding energies than the qualified 5R84 ligand WGS; these were (−)-catechin gallate, carotene, l-epigallocatechin, (−)-epicatechin-pentaacetate, D-(+)-cellobiose, and epigallocatechin 3-gallate (**Figure 4A**). Among these 6 ingredients, (−)-catechin gallate had the lowest binding free energy (−8.8 kcal/mol) and formed 5 hydrogen bonds with the ARG40, TYR54, GLU55, ASN-180, and ARG-188 residues of 5R84 (**Figure 4B**). The other 5 ingredients shared comparable binding free energies (∼-7.26 kcal/mol) and formed 0 to 5 hydrogen bonds with residues (**Figure 4B**). Notably, although carotene had a low binding free energy, it could not form hydrogen bonds with 5R84, implying that the interaction of carotene with 5R84 was not stable. In summary, we identified three tea ingredients ((−)-catechin gallate, D-(+)-cellobiose, and epigallocatechin 3-gallate) that were sufficient to block COVID-19 by interacting with 5R84 protein.

## Epigallocatechin 3-Gallate (EGCG) Reduced the Secretion of Inflammatory Factors by Inhibiting MAPK/NF-$\kappa$B Signaling and Regulating Macrophage Polarization *in vitro*

Based on the abovementioned biometric analysis results, it is reasonable to hypothesize that EGCG is involved in inflammation in COVID-19. To ascertain whether EGCG can protect the body from inflammatory injury, we conducted a CCK8 assay. The results revealed that cell viability began to decline when the concentration of EGCG exceeded 50 nM (**Figure 5A**). Subsequently, we analyzed the effect of EGCG on macrophage polarizations. The LPS (100 ng/mL)-induced mRNA expression of M1 marker genes including *iNOS, TNF-α, Il-1β,* and *IL-6* was significantly reduced by EGCG (**Figures 5B–E**). On the other hand, EGCG showed an increased effect on the level of induction of the M2 marker gene Arg1 stimulated by LPS in RAW264.7 cells (**Figure 5F**). Then, we collected RAW264.7 cell supernatants after LPS stimulation in a culture system with or without EGCG to measure the secretion of inflammatory factors by ELISA. The results showed that EGCG significantly reduced the production of IL-6, TNF-α, and IL-1β compared with the LPS stimulation group (**Figures 5G–I**). Moreover, we also detected the inflammatory factor IL-17A secreted by macrophages and the expression of TLR4 and PI3K, which were previously screened (**Figure 3E**); EGCG significantly suppressed the production of IL-17A and the mRNA levels of TLR4 and PI3K compared with the LPS stimulation group (**Supplementary Figure 3**). These

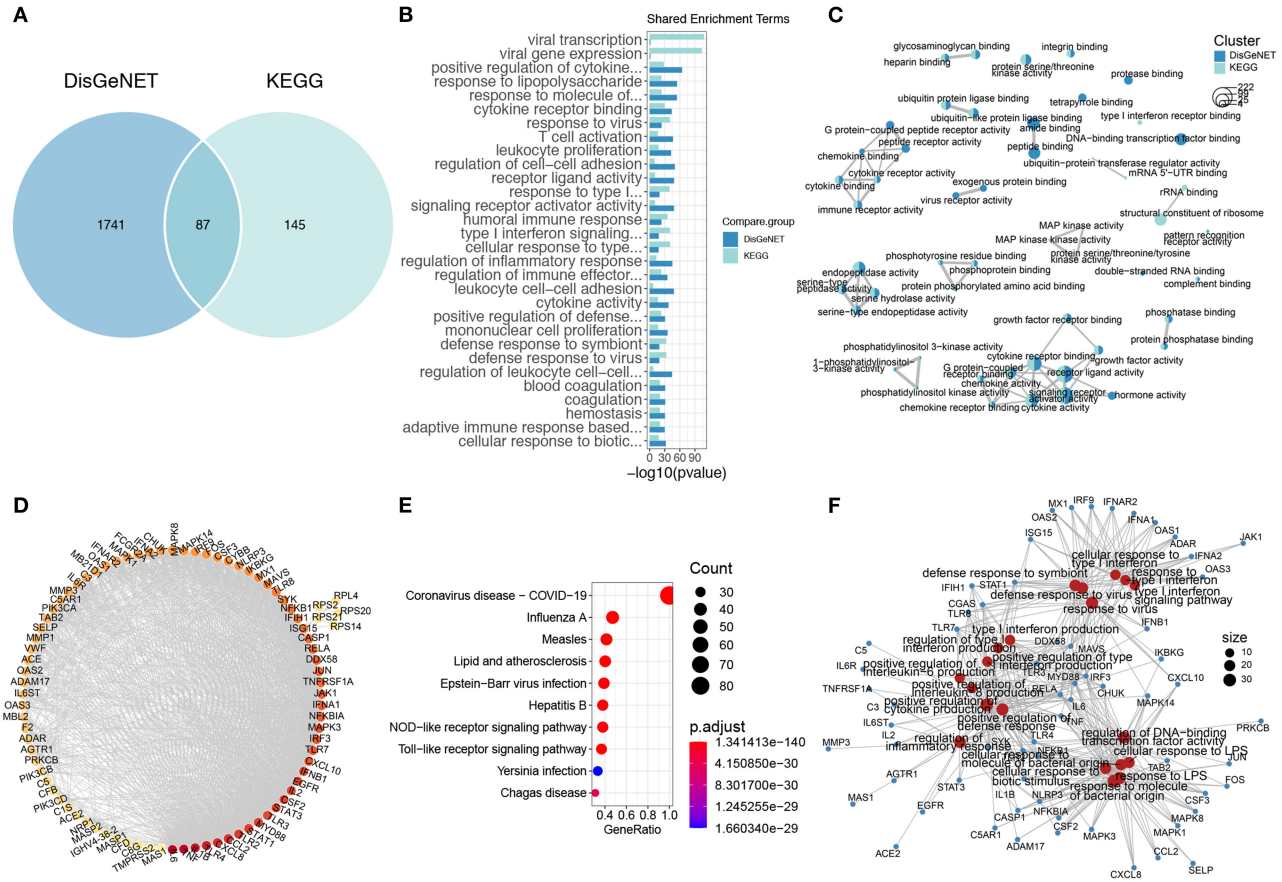


**FIGURE 2 |** The gene signature of COVID-19. **(A)** The Venn plot of COVID-19 gene signature of DisGeNET and KEGG. **(B)** The shared COVID-19 gene signature GO terms of DisGeNET and KEGG. **(C)** The comparison GO enrichment network of DisGeNET and KEGG, the number of circles in the bottom left corner represents the gene number of each enriched term, the proportion of clusters in the pie chart is determined by the number of genes. **(D)** The PPI of shared genes of DisGeNET and KEGG COVID-19 gene signature, the dot color represents the connectivity of each gene, while from yellow to red represents from low to high. **(E)** The top 10 KEGG enriched terms of shared COVID-19 signature genes. **(F)** The genes and GO enriched terms network of shared COVID-19 signature genes, the red dots represent the GO enriched terms while blue dots are the related genes.

results indicate that EGCG reduces the secretion of inflammatory factors *in vitro*.

To further explore the mechanism by which EGCG alleviates inflammatory damage to cells, we investigated the inflammation pathway *in vitro*. We measured the activation of the MAPK pathway. As shown in **Figure 5J**, phosphorylation of p-ERK, p-JNK, and p-p38 in macrophages was significantly increased after LPS challenge; this effect was suppressed by EGCG *in vitro* as determined by WB (**Figure 5J**). This demonstrates that EGCG could effectively inhibit the MAPK pathway. Furthermore, we investigated the suppressive effect of EGCG treatment on the NF-κB signaling cascade in RAW264.7 macrophages. Our investigations indicated that the phosphorylation of p65 was significantly increased after LPS challenge, and this was suppressed by EGCG (**Figure 5K**). This finding confirms that EGCG suppressed inflammation by inhibiting MAPK/NF-κB signaling.

## DISCUSSION

Tea is one of the three most consumed beverages in the world and is known as the beverage of the twenty-first century, not only because of the long history of tea culture but also because of its nutritional value and health care functions (34, 35). Studies have shown that tea contains numerous active ingredients, mainly tea polyphenols, tea pigments, tea polysaccharides, γ-aminobutyric acid, tea saponins, alkaloids, vitamins, pyrroloquinoline quinone, pantothenic acid, minerals, and other ingredients (36, 37). Tea polyphenols are the most abundant soluble components in tea, and they are also the most important substances in tea that exert biological effects (35) that can reduce the incidence of cardiovascular disease, decrease blood lipids, decrease body fat formation, and change the intestinal flora ecology (35, 38). Studies have shown that after drinking a cup of tea for half an hour, the antioxidant capacity (ability to fight oxygen free

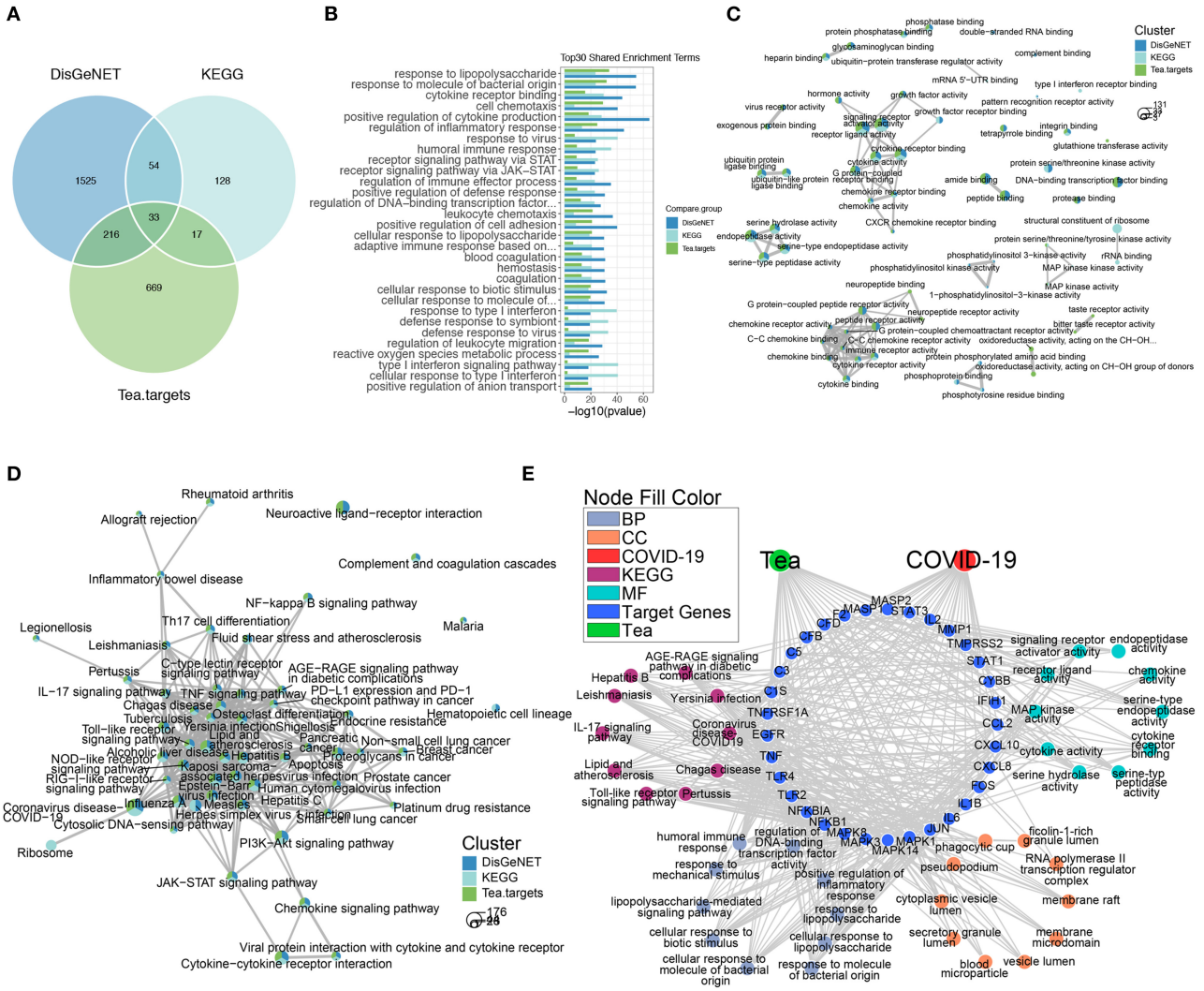


**FIGURE 3 |** The anti-COVID-19 potential of tea. **(A)** The Venn plot of COVID-19 signature genes and tea target genes. **(B)** The shared GO enriched terms of COVID-19 signature genes and tea target genes. The comparison GO **(C)** and KEGG **(D)** enrichment network of COVID-19 signature genes and tea target genes, the bottom left circles stand for the gene number of each enriched term, the proportion of clusters in the pie chart indicates the number of genes. **(E)** The network of shared COVID-19 signatures genes and tea target genes along with the GO and KEGG enrichment terms.

radicals) in the blood increases by 41% to 48% and can last for one and a half hours at a high level (39).

In our work, we first screened the main ingredient of tea, EGCG, in databases, suggesting that EGCG may play an important role in the treatment of COVID-19. EGCG is the main component of green tea polyphenols and is a catechin monomer isolated from tea (40). Studies have shown that EGCG has several functions including significant anti-oxidation, involvement in scavenging free radicals, reduction of inflammation and allergic reactions, anti-mutagenic effects, inhibition of tumor growth, and strong inhibitory effects on dysentery, typhoid fever, *Staphylococcus aureus*, and other bacteria (41–43). EGCG also has the functions of anti-aging, lowering blood lipids, improving low-density lipoprotein, inhibiting the growth of liver fat and cholesterol, preventing atherosclerosis, and enhancing immunity (44–46). In addition, EGCG can inhibit the proliferation of glomerular cell membranes and improve renal function (47). Several studies have reported the potential of EGCG to prevent COVID-19. For instance, EGCG inhibits the angiotensin-converting enzyme 2 (ACE2) receptor (the cellular receptor for SARS-CoV-2) and TMPRSS2, which mediate viral entry into cells, by activating Nrf2 (48, 49). By inhibiting the main protease of SARS-CoV-2, EGCG may inhibit viral reproduction (48). EGCG protects against SARS-CoV-2-induced mitochondrial reactive oxygen species (ROS) (promoting SARS-CoV-2 replication) and ROS bursts caused by neutrophil extracellular traps through its broad antioxidant activity (48, 50). EGCG can potentially

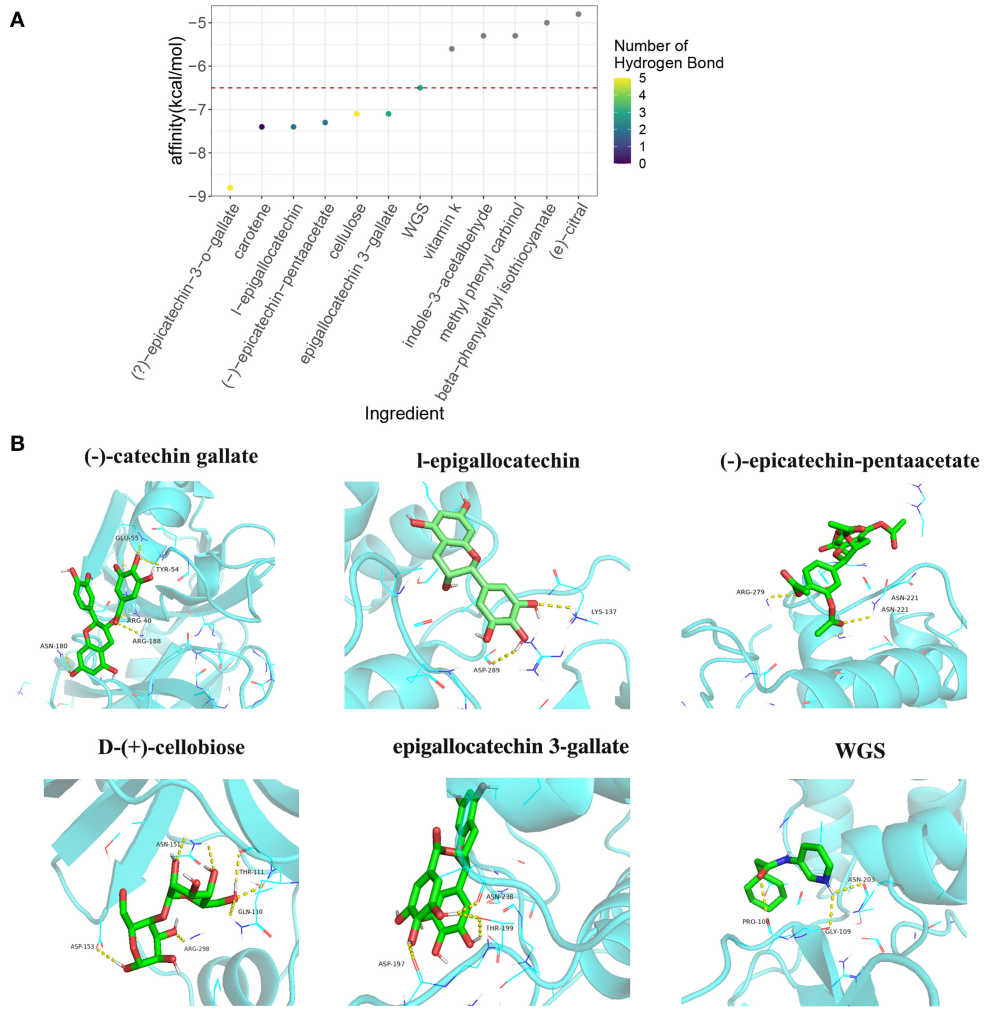


**FIGURE 4 |** The molecular docking analysis of tea ingredients with COVID-19 5R84 protein. **(A)** The binding-free energy and hydrogen bond numbers of tea ingredients with 5R84 protein with qualified 5R84 ligand WGS as a reference, the red dot horizontal line indicates the binding-free energy of WGS with 5R84. **(B)** The representative interaction of tea ingredients and WGS with 5R84, the yellow dot lines indicate the hydrogen bonds of the specific ligand with 5R84.

inhibit the SARS-CoV-2 life cycle by inhibiting ER-resident GRP78 activity and expression (51, 52). EGCG has also been shown to protect against (1) cytokine storm-related acute lung injury/acute respiratory distress syndrome (48, 53), (2) thrombosis through inhibition of tissue factor and activation of platelets (54), (3) inactivation of redox-sensitive HMGB1-induced sepsis (55), and (4) pulmonary fibrosis by increasing Nrf2 and inhibiting NF-κB (13). However, these activities remain to be further confirmed in animals and humans.

Studies have shown that macrophages play an important role in COVID-19 (56). Cytokine storm syndrome (CSS) refers to the continuous activation and expansion of lymphocytes and macrophages caused by the infection of microorganisms, and a variety of cytokines such as TNF-α, IL-1, IL-6, IL-12, interferon (IFN)-α, IFN-β, IFN-γ, monocyte chemoattractant protein (MCP)-1, and IL-8 are rapidly produced in large quantities (57). CSS is an excessive immune phenomenon of the body to external stimuli and is an important cause of acute respiratory distress syndrome and multiple organ failure (58). Studies have shown that cytokine storms play a key role in the transition to severe and critical illness in most coronavirus-infected patients (59). In addition, one study found that there is a highly pro-inflammatory macrophage microenvironment in the lungs of severely ill patients with the new strain, which the researchers said may help to elucidate the underlying mechanism behind the immune response triggered by the new coronavirus (60). Therefore, we focused on the role of EGCG in regulating changes in macrophage function to improve COVID-19. The inflammatory response in COVID-19 is much more complex than that in LPS-induced RAW264.7 cells, and it is extremely important to distinguish the inflammatory subtypes of different diseases. However, the inflammatory response in COVID-19 still shares some common signatures with the inflammatory

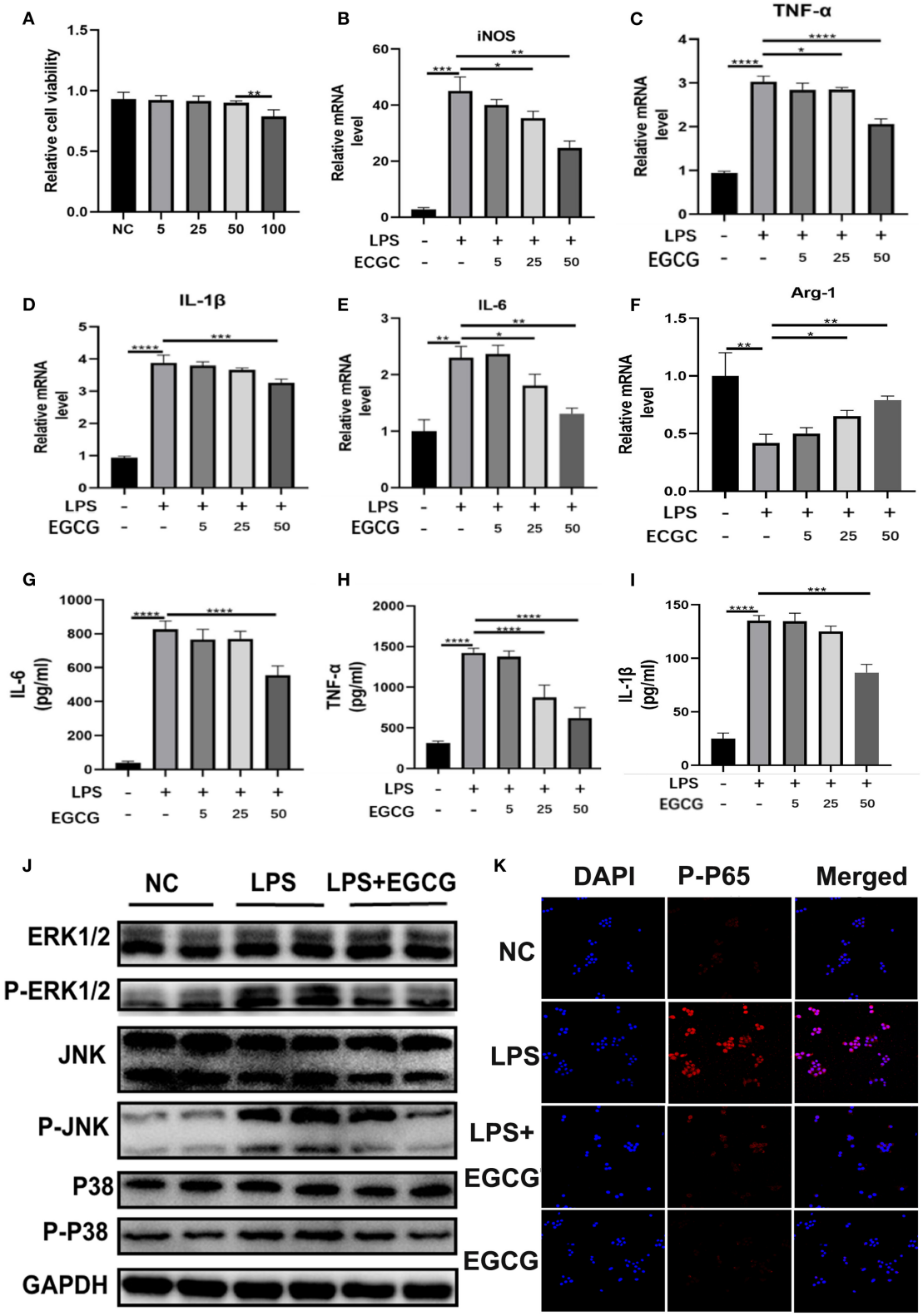


**FIGURE 5 |** EGCG suppressed secretion of inflammatory factors, macrophage polarization, and MAPK/NF-κB signaling *in vitro*. **(A)** RAW 264.7 cells were incubated with EGCG (50 mM) for 24 h. Cell viability was determined by CCK8 assay ($n = 5$). **(B–F)** The mRNA levels of *iNOS, TNF-α, Il-1β, IL-6*, and *Arg1* in the RAW 264.7

*(Continued)*

**FIGURE 5 |** cells with LPS (100 ng/ml) and EGCG (50 nM) for 24 h were detected by q-PCR ($n = 3$). **(G–I)** The concentrations of IL-6, TNF-α, and IL-1β in RAW 264.7 cell supernatant after LPS and EGCG treatment for 24 h were determined by ELISA kits ($n = 4$). **(J)** The protein levels of ERK1/2, P-ERK1/2, JNK, P-JNK, P38, and p-p38 in the RAW 264.7 cells treated with LPS (100 ng/ml) and EGCG (50 nM) for 24 h were detected by Western blotting. **(K)** The expressions of p-p65 (red) and DAPI (blue) in RAW 264.7 cells were detected by using an immunofluorescence staining assay (scale bar: 50 μm). $^{*}P < 0.1$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

response in LPS-induced RAW264.7 cells, among which the most typical are TLR4, NF-κB, and other signaling pathways and their corresponding cytokines (including IL-6, TNF, IL-1β, etc.) (**Figure 2D**). Through the LPS-stimulated macrophage model, we attempted to demonstrate the possibility of EGCG molecules indirectly inhibiting COVID-19 inflammation.

EGCG has been reported to alleviate acute lung injury, regulate the polarization of macrophages to M2 (61), and inhibit secretion of inflammatory factors, and its protective mechanism may be related to the inhibition of the MAPK and NF-κB signaling pathway (62–64). In addition, EGCG derivatives have anti-inflammatory activity in LPS-stimulated mouse macrophages (65). Furthermore, EGCG-modified collagen membranes have been shown to downregulate the expression of inflammatory factors and promote M2 (CD163 and CD206) macrophages (66). EGCG also stimulates LC3-II production and autophagosome formation and inhibits LPS-induced upregulation and extracellular release of HMGB1 (67). Our results are consistent with those described above; however, the origins of the abovementioned research and our study are different. There is some heterogeneity in the inflammatory responses of different diseases and different states of certain diseases. Starting from the gene signature of COVID-19, we co-analyzed the target genes of each component of tea in an attempt to identify the potential of specific components of tea for the treatment of COVID-19. The results showed that the intersection of COVID-19 signature genes and tea target genes was highly focused on the response to LPS stimulation (**Figure 3B**). This phenomenon itself is an important discovery. Among the different components of tea, EGCG is obviously an important molecule regulating this process in COVID-19; furthermore, it has the most target genes and is the major active ingredient in tea. We then indirectly verified our findings in LPS-stimulated macrophages *in vitro* to examine the suppression effects of EGCG on the LPS-like responses in COVID-19. Finally, our study is slightly different from the abovementioned literature (61–67) in terms of molecular signaling pathways. Based on the results of the bioinformatics analysis, we focused on the most credible MAPK (ERK1/2-JNK-P38) signaling pathway. In addition, EGCG reduced the secretion of inflammatory factors and regulated macrophage polarization (from M1 to M2) *in vitro*. These cell experiments verified the results of our bioinformatics analysis; namely, the active ingredient of tea, ECGC, can directly act on macrophages in the cytokine storm environment of COVID-19, and inhibit the secretion of inflammatory factors and the activation of the MAPK and NF-κB signaling pathways, improving the prognosis of COVID-19.

Moreover, Douangamath et al. (68) performed a large-scale electrophilic and non-covalent fragment screening of the major proteases of SARS-CoV-2 by combined mass spectrometry and X-ray and found that 5R84 is one of two cysteine viral proteases essential for viral replication. We therefore examined the interaction of tea components with the COVID-19 protein 5R84. Through molecular docking analysis, we identified three tea ingredients ((−)-epicatechin-3-o-gallate, D-(+)-cellobiose, and EGCG) that likely interact with the vital SARS-CoV-2 protein, 5R84, compared with the qualified 5R84 ligand WGS. According to the description in PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/24802025#section=Household-Products), D-(+)-cellobiose is indeed insoluble in water and cannot be absorbed by the human body; thus, it is nearly impossible to inhibit SARS-CoV-2 through absorption from the gastrointestinal tract and into circulation. However, considering the droplet transmission and fecal-oral transmission of SARS-CoV-2, namely, that SARS-CoV-2 exists on the surfaces of the respiratory tract and digestive tract, D-(+)-cellobiose may directly interact with SARS-CoV-2 on the corresponding surfaces. However, the roles of (−)-epicatechin-3-o-gallate and D-(+)-cellobiose in COVID-19 should be studied further in cell and animal experiments.

In summary, our research systematically analyzed the active ingredients of tea, namely, (−)-epicatechin-3-o-gallate, D-(+)-cellobiose and EGCG, which have the potential to treat COVID-19 by suppressing the target genes and signaling pathways of COVID-19 and interacting with the vital SARS-CoV-2 protein. In addition, we validated the above results in macrophages. Our study analyzed the anti-COVID-19 effects of the active ingredients of tea and provided new ideas for the prevention and treatment of COVID-19.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

LW and QT: conception and design, collection and/or assembly of data, data analysis and interpretation, visualization, and manuscript writing and final approval of the manuscript. ZW and JS: collection and/or assembly of data. WY and LZ: collection and/or assembly of data. XY, LW, and YS: financial support, administrative support, provision of study material, supervision, data analysis and interpretation, visualization, manuscript writing, and final approval of the manuscript. All authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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# The Influence of EGCG on the Pharmacokinetics and Pharmacodynamics of Bisoprolol and a New Method for Simultaneous Determination of EGCG and Bisoprolol in Rat Plasma

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**Background and Aim:** Research has shown that green tea catechins may influence the activity of drug metabolizing enzymes and drug transporters. We examined whether epigallocatechin-3-gallate (EGCG) affected the pharmacokinetics and pharmacodynamics of bisoprolol in rats.

**Methods:** A sensitive, specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was established for the quantitative determination of EGCG and bisoprolol. The pharmacokinetic parameters of EGCG and bisoprolol in Sprague-Dawley (SD) rats were analyzed using non-compartmental methods with the aid of the computer program WinNolin. Blood pressure (BP) of spontaneously hypertensive rats (SHRs) was monitored by the tail-cuff method. Bisoprolol was given as single doses of 10 mg/kg with or without EGCG 100 mg/kg by gavage or by intravenous injection.

**Results:** Intake of EGCG with bisoprolol by gavage significantly reduced the $C_{max}$ (mean $C_{max}$ from 2012.31 to 942.26 ng/mL, $P < 0.05$) and increased the $T_{max}$ (mean $T_{max}$ from 0.5 to 0.83 h, $P < 0.01$) for bisoprolol. After intravenous injection, EGCG significantly increased the apparent volume of distribution of bisoprolol (mean Vz/F from 1629.62 to 2473.27 mL/Kg, $P < 0.05$) and tended to increase the clearance. The absolute bioavailability of bisoprolol was reduced from 92.04 to 66.05% in rats when bisoprolol was administered with EGCG. Heart rate reduction was less in SHRs when EGCG was given by gavage with bisoprolol whereas BP reduction occurred more rapidly.

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**Conclusion:** This study showed that the simultaneous administration of EGCG by gavage at a dose of 100 mg/kg was associated with decreased $C_{max}$ and increased $T_{max}$ of bisoprolol, and the Vz/F of bisoprolol was increased when administered with EGCG by intravenous injection in SD rats. Moreover, the early heart rate reduction with bisoprolol was attenuated and BP reduction occurred earlier when EGCG was given with bisoprolol by gavage in SHRs.

**Keywords: bisoprolol, epigallocatechin-3-gallate (EGCG), green tea, pharmacokinetics, pharmacodynamics, hypertension**

## INTRODUCTION

The behavior of drinking tea has a history of more than 5,000 years and tea has become the second most consumed beverage in the world after water with total annual sales exceeding $43 billion globally, of which more than $11 billion is accounted for by green tea (1). In Asian countries, the average green tea consumption is about three cups per day, providing 240–320 mg of polyphenols (2). Epigallocatechin-3-gallate (EGCG) is the main abundant catechin in green tea, accounting for 50–80% of the total catechins (3). EGCG is a potent antioxidant and is considered to have the potential to treat various human diseases such as cancer, inflammation, endometriosis, diabetes, cardiovascular disease and even has antiviral activity against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection (4–7).

Because of these benefits, green tea is often taken concomitantly with therapeutic drugs in many conditions, which leads to the potential for herb–drug interactions (HDIs). The major pathways responsible for HDIs involve the inhibition or induction of cytochrome P450 (CYP) enzyme activity and the expression of drug transporters such as P-glycoprotein (P-gp) and organic anion transporting polypeptide 1A2 (OATP1A2).

Bisoprolol is a β1-blocker with high oral bioavailability and is one of the first choices for the treatment of hypertension and angina. The highly β1-selective property of β-blockers has the advantage of reducing side effects and improving efficacy in the treatment of hypertension and other cardiovascular diseases (8). Bisoprolol is moderately lipophilic with an oral bioavailability of more than 90% and its half-life is 10–12 h (9). It is used once a day for the treatment of hypertension. Bisoprolol is rapidly absorbed after oral administration, and reaches peak plasma concentration after about 3 h and is eliminated with 50% renal excretion as unchanged drug and 50% *via* hepatic metabolism to pharmacologically inactive metabolites which are then excreted by the kidneys (10). Bisoprolol is mainly metabolized by the drug metabolizing enzymes CYP3A4 (95%) and CYP2D6 (5%) both of which are isoenzymes of cytochrome P450 (CYP). Therefore any drugs or herbs that induce the activity of CYP3A4 and CYP2D6 can accelerate the metabolic clearance of bisoprolol and result in drug interactions. Furthermore, the absorption and excretion of bisoprolol is closely related to the activity of OATP1A2 and P-gP (11). Therefore, any concomitant drug that affects the activity of CYP3A4, CYP2D6, P-gP or OATP1A2 may lead to changes in the plasma concentration of bisoprolol, which may be the molecular basis for the pharmacokinetic interactions between other drugs or herbs and bisoprolol. In recent years, many studies have reported the influence of green tea extract and its major ingredient, EGCG, on drug interactions, which ultimately affect the blood concentration and efficacy of the drug. Therefore, the present study was performed to investigate the effect of EGCG on the pharmacokinetics and pharmacodynamics of bisoprolol in rats.

## MATERIALS AND METHODS

### Chemicals and Reagents

EGCG (purity > 99%) and bisoprolol fumarate (purity > 99%) were purchased from Selleckchem, USA. The Bisoprolol-D7 isotope (internal standard [IS], HPLC grade) and loratadine

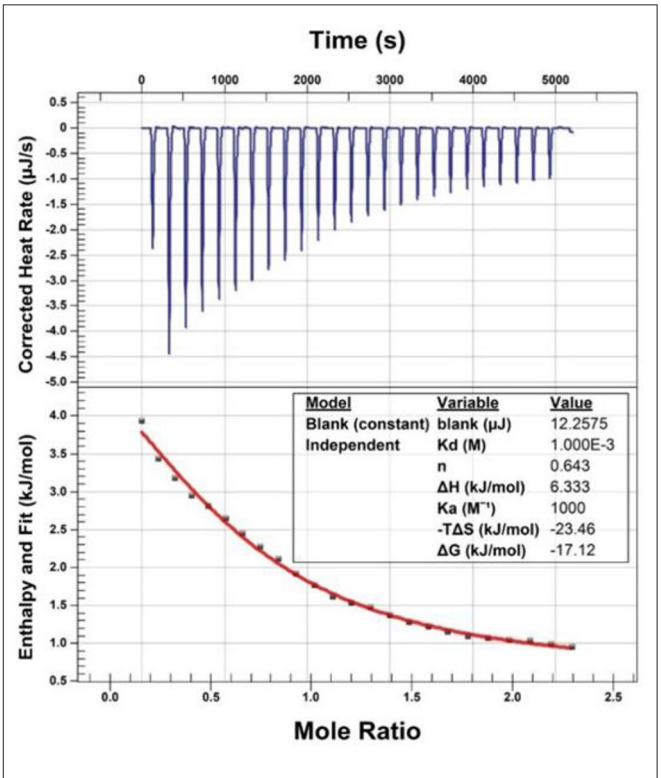


**FIGURE 1 |** Isothermal titration calorimetry (ITC) assay.

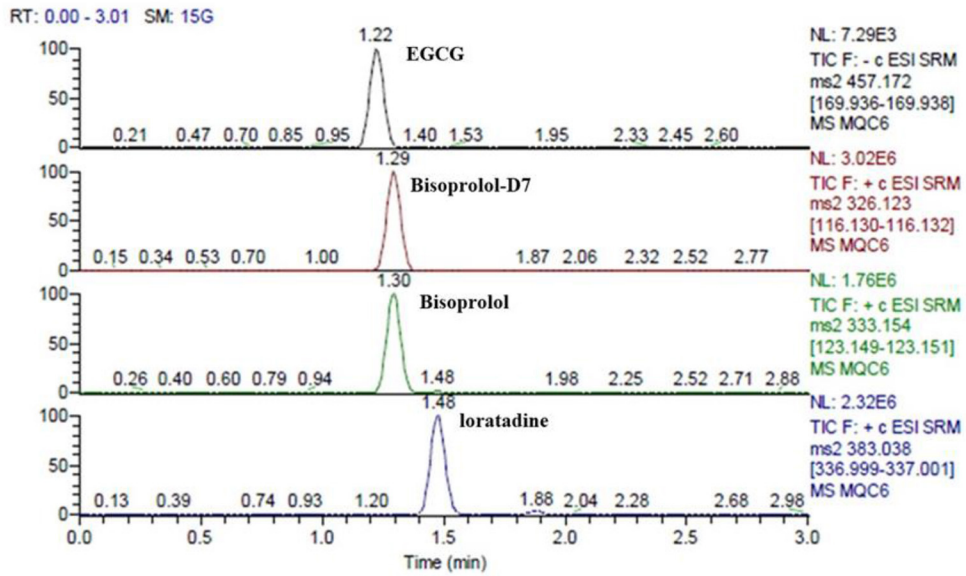


**FIGURE 2 |** The retention times of EGCG, bisoprolol, deuterated bisoprolol and loratadine in plasma sample at the same time.

(IS of EGCG) were obtained from CDN isotopes, Canada. LC–MS grade acetonitrile and methanol were purchased from TEDIA, USA. Ethylenediaminetetraacetic acid (EDTA) and Vitamin C were obtained from Source Leaf Organisms, China. Other chemicals employed throughout the experiment were of analytical grade and available commercially. Deionized and double-distilled water used in all assays was produced by a Milli-Q purification System (Millipore, Bedford, MA).

## Apparatus and Analytical Conditions

Samples were analyzed through Dionex Ultra-Liquid Chromatography (Thermo ScientificTM, USA) and TSQ QuantumTM Access MAX (Thermo ScientificTM, USA) systems on a Hypurity C18 column (50 × 2.1 mm,3 μm) using an injection volume of 10 μL. The mobile phases consisted of 0.1% formic acid water (A) and acetonitrile (B). The gradient elution system was optimized as follows: 0–0.5 min 10% B, 0.5–0.8 min 10–95% B, 2.0–2.2 min 95–10% B, 2.2–2.5 min 10% B. The flow rate was 0.8 mL/min. The autosampler was maintained at 4°C, and 100 μL was automatically injected into the system. MS/MS data acquisition was performed under negative/ positive electrospray ionization (ESI) mode. The Multiple Reaction Monitoring (MRM) mode was employed to monitor EGCG, bisoprolol, loratadine (IS) and D7-Bisoprolol (IS) with the precursor-to-product ion transition of m/z 457.13 → 169.98, m/z 325.96 → 115.96, m/z 383.01 → 336.94 and m/z 325.96 → 115.96, respectively. The parameters were optimized as follows: a capillary voltage of −3500 V, a gas flow rate of 12 L/min, and the dry gas temperature of 350 °C. For EGCG, bisoprolol, loratadine (IS) and D7-Bisoprolol (IS), the collision energies were 20 eV, 17 eV, 18 eV, and 24 eV, respectively. The other parameters, including cone voltage (CV), collision energy (CE), and the dwell time, were also achieved for the maximum abundance of the ions of interest by the automatic tune procedure of the instrument. Thermo Xcalibur (2.2 SP1.48, Thermo Scientific, USA).

**TABLE 1 |** Regression equation of EGCG and bisoprolol in rats plasma determined by HPLC-MS (weight $W=1/X^2$).

| Analyte | Calibration curves | $R^2$ | Weight | %RE |
|---|---|---|---|---|
| EGCG | $Y = 3.028e^{-5}X - 1.009e^{-4}$ | 0.9982 | $1/X^2$ | −7.37 ~ 4.19 |
| | $Y = 2.098e^{-5}X - 7.199e^{-5}$ | 0.9936 | $1/X^2$ | −13.15 ~ 9.91 |
| | $Y = 2.144e^{-5}X - 7.308\ e^{-5}$ | 0.9918 | $1/X^2$ | −11.19 ~ 10.60 |
| Bisoprolol | $Y = 1.193e^{-2}X + 3.506e^{-3}$ | 0.9996 | $1/X^2$ | −3.16 ~ 1.91 |
| | $Y = 1.117e^{-2}X + 1.742e^{-5}$ | 0.9991 | $1/X^2$ | −5.00 ~ 4.25 |
| | $Y = 1.119e^{-2}X + 2.248e^{-3}$ | 0.9992 | $1/X^2$ | −4.60 ~ 3.51 |

## Preparation of Calibrators and Quality Control (QC) Samples

An 8-level series of calibrators was prepared using pooled plasma. Briefly, pooled plasma was spiked with working solution to give 50,100,500,1,000,2,000,5,000,10,000 and 20,000 ng/mL calibrators. Pooled plasma was used as blank. Calibrators were aliquotted and stored at −80°C. A 4-level QC was prepared using pooled plasma. Briefly, pooled plasma was spiked with working solution to give 15,000,1500,150 and 50 ng/mL QC. All QC samples were aliquotted and stored at −80°C.

## Extraction Procedure

50 μL of Plasma,d7-Bisoprolol (1 μg/mL),Loratadine (1 μg/mL) and Antioxidant Mixture Were Added Into a 1.5 mL Centrifuge Tube, After 1 min of Vortex Mixing, 200 μL of Acetonitrile Was Added and Vortex Mixed for 3 min, Then Mixed Evenly and let to Stand at Room Temperature for 5 min. After 5 min of

**TABLE 2 |** Intra-day and inter-day precisions and accuracies for the determination of EGCG and Bisoprolol from the assay samples (mean ± SD, $n = 6$).

| | Concentration | Intra batch | | | Inter batch | | |
|---|---|---|---|---|---|---|---|
| **Analyte** | **(ng/ml)** | **Mean ± SD** | **RE (%)** | **RSD (%)** | **Mean ± SD** | **RE (%)** | **RSD (%)** |
| EGCG | 5 | 5.79 ± 1.06 | 15.77 | 18.35 | 5.99 ± 0.76 | 19.70 | 12.63 |
| | 15 | 15.48 ± 1.13 | 3.17 | 7.32 | 14.61 ± 1.10 | −2.60 | 7.50 |
| | 150 | 137.34 ± 6.48 | −8.44 | 4.72 | 136.75 ± 1.31 | −8.84 | 0.96 |
| | 1500 | 1,547.44 ± 32.52 | 3.16 | 2.10 | 156.87 ± 48.11 | 4.19 | 3.08 |
| Bisoprolol | 5 | 4.23 ± 0.21 | −15.42 | 4.89 | 4.30 ± 0.10 | −13.95 | 2.37 |
| | 15 | 14.04 ± 0.31 | −6.40 | 2.21 | 14.15 ± 0.51 | −5.66 | 3.57 |
| | 150 | 150.30 ± 1.91 | 0.20 | 1.27 | 152.75 ± 2.99 | 1.83 | 1.96 |
| | 1500 | 1,502.17 ± 18.52 | 0.14 | 1.23 | 1,508.16 ± 10.97 | 0.54 | 0.73 |

**TABLE 3 |** Extraction recovery and matrix effect for the EGCG and Bisoprolol in plasma (mean ± SD, $n = 6$).

| | Concentration | Matrix effect | | Recovery | |
|---|---|---|---|---|---|
| **Analyte** | **(ng/ml)** | **Mean ± SD** | **RSD (%)** | **Mean ± SD** | **RSD (%)** |
| EGCG | 1500 | 90.12 ± 4.13 | 4.58 | 79.09 ± 2.97 | 3.76 |
| | 150 | 91.00 ± 4.59 | 5.04 | 60.96 ± 4.20 | 6.89 |
| | 15 | 97.58 ± 4.05 | 4.15 | 72.69 ± 10.22 | 14.06 |
| Bisoprolol | 1500 | 99.91 ± 2.14 | 2.14 | 98.82 ± 5.07 | 5.15 |
| | 150 | 98.53 ± 1.42 | 1.45 | 99.71 ± 3.49 | 3.50 |
| | 15 | 100.14 ± 3.34 | 3.34 | 96.88 ± 7.72 | 7.96 |

Centrifugation at 1400 rpm, 180 μL of Supernatant Was Added Into Another Clean EP Tube and Centrifuged Again at 14,00 rpm for 5 min, Then 10 μL of the Supernatant Was Taken and Subjected to LC-MS/MS Analysis.

## Bisoprolol-EGCG Pharmacokinetic Interaction

A total of 30 Sprague-Dawley (SD) rats including 15 male and 15 female, weighing 180–220 g, were acquired from the Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, China, license no. SCXK 2016-0029). The SD rats were housed in clean cages under an optimal temperature range of 24–26°C and 12 h light/dark cycle with free access to food and water. All the animal procedures complied with the institutional animal ethics guidelines set by the Animal Care and Use Committee of Sun Yat-Sen University. The rats were randomly allocated to two phases of this pharmacokinetic interaction study. The dosing of 18 rats was given by intragastric (i.g.) gavage and another 12 rats were dosed by tail vein injection. The 18 rats were randomly allocated to three groups: bisoprolol (10 mg/kg) group, EGCG (100 mg/kg) group and the bisoprolol (10 mg/kg) + EGCG (100 mg/kg) combination group. The 12 rats were randomly allocated to two groups: bisoprolol (10 mg/kg body weight, i.g.) group, bisoprolol (10 mg/kg body weight, i.g.) + EGCG (100 mg/kg body weight, i.g.) group. The dose of the 12 rats was calculated from bisoprolol (10 mg/kg) and EGCG (100 mg/kg) dissolved in a 0.7% saline solution. The rats were fasted for 12 h prior to drug administration without restriction of drinking water, and feeding restarted at 4 h after dosing. The blood samples (approximately 0.3 mL) were collected into heparinized centrifuge tubes *via* orbital venous plexus sampling before dosing (denoted as 0 min), and at 5 min,10 min,15 min,30 min,1 h,1.5 h,2 h,4 h,8 h, and 24 h after dosing. The supernatant was collected by centrifugation of the blood samples immediately at 4000 rpm for 10 min at 4°C and stored at −20°C until further analysis.

## Blood Pressure Measurements of SHRs

Twelve male spontaneously hypertensive rats (SHRs) of body weight about 320 g and average blood pressure more than 180 mm Hg were used. The SHRs were divided into two groups and 6 in each group (including three female and three male). The rats were housed in clean cages under an optimal temperature range of 24–26°C and 12 h light/dark cycle with free access to food and water. The rats were randomly allocated to the bisoprolol (10 mg/kg body weight, i.g.) group and the bisoprolol (10 mg/kg body weight, i.g.) + EGCG (100 mg/kg body weight, i.g.) group. The dose selection mainly was refered to the previous study in SHRs (12). Fasting was carried out for 12 h prior to drug administration without the restriction of drinking water, and feeding restarted at 4 h after dosing. The dosing was given by gavage. A real-time blood pressure monitor (Intelligent non-invasive blood pressure monitor mouse BP-2010A, China) with non-invasive manometry was used to measure blood pressure at 0 min,15 min,30 min,45 min,1 h,75 min,1.5 h,105 min,2 h,4 h,6 h, 8 h,12 h, and 24 h after dosing. The average value of three recordings of blood pressure from the tail artery in the awake state of the rats was used for analysis.

## Isothermal Titration Calorimetry Assay

Isothermal titration calorimetry (ITC) assay was performed to investigate the binding of EGCG to bisoprolol on a NanoITC LV-190 μL (Waters GmbH, TA Instruments, Eschborn, Germany). Titration calorimetry was performed at 25°C in the assay buffer (PBS, PH 6.5). Briefly, the sample and syringe cell were filled with bisoprolol (2.1 mM) and EGCG (13 mM), respectively, which were degassed prior to use. The titrations consisted of 25 consecutive injections of 1.96 μL each with a 200 s interval

**TABLE 4 |** Stability of EGCG and Bisoprolol in plasma (mean ± SD, $n = 6$).

| Storage condition | Analyte | Concentration (ng/ml) | Mean ± SD (ng/ml) | RSD (%) | RE (%) |
|---|---|---|---|---|---|
| Room temperature 4 h | EGCG | 15 | 14.13 ± 1.76 | 12.44 | −5.80 |
| | | 150 | 131.46 ± 15.90 | 12.09 | −12.36 |
| | | 1500 | 1,446.46 ± 24.61 | 1.70 | −3.57 |
| | Bisoprolol | 15 | 14.06 ± 0.41 | 2.90 | −6.36 |
| | | 150 | 151.73 ± 7.25 | 4.78 | 1.15 |
| | | 1500 | 1,500.19 ± 24.77 | 1.65 | 0.01 |
| Autosampler for 12 h (4°C) | EGCG | 15 | 14.49 ± 1.60 | 11.02 | −3.41 |
| | | 150 | 135.76 ± 5.73 | 4.22 | −3.41 |
| | | 1500 | 1,506.53 ± 49.58 | 3.29 | 0.44 |
| | Bisoprolol | 15 | 13.68 ± 0.33 | 2.41 | −8.82 |
| | | 150 | 151.72 ± 2.33 | 1.54 | 1.15 |
| | | 1500 | 1,519.58 ± 23.01 | 1.51 | 1.31 |
| Three Freeze/thaw cycle at −80°C | EGCG | 15 | 13.48 ± 1.13 | 8.41 | −10.11 |
| | | 150 | 129.87 ± 6.29 | 4.48 | −13.42 |
| | | 1500 | 157.56 ± 63.32 | 4.02 | 5.04 |
| | Bisoprolol | 15 | 14.89 ± 0.51 | 3.43 | −0.72 |
| | | 150 | 150.54 ± 1.10 | 0.73 | 0.36 |
| | | 1500 | 144.94 ± 22.43 | 1.50 | 0.01 |
| Long term for 30 d at −80 °C | EGCG | 15 | 12.93 ± 1.03 | 7.99 | −13.80 |
| | | 1500 | 1,391.63 ± 87.32 | 6.27 | −7.22 |
| | Bisoprolol | 15 | 14.44 ± 0.33 | 2.26 | −3.70 |
| | | 1500 | 1,432.24 ± 44.86 | 3.13 | −4.52 |

between injections. The data were analyzed using the instrumental internal software package and fitted with an independent model.

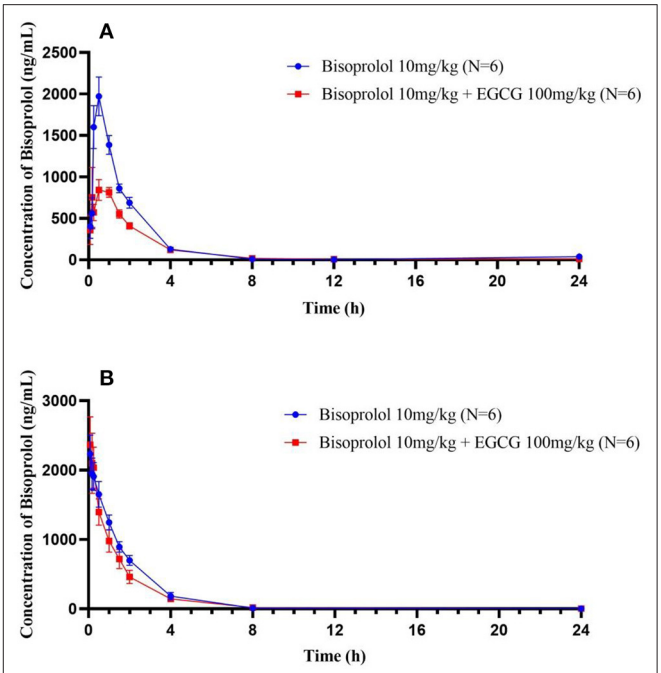


**FIGURE 3 |** The plasma concentration time curves of bisoprolol after oral **(A)** and intravenous **(B)** administration.

## Ethics Statement

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Laboratory Animal Center of Sun Yat-Sen University. The protocol was approved by the Institutional Review Board of Baoan Women's and Children's Hospital, Shenzhen, China with IRB No LLSC2020-03-05, and the Animal Care and Use Committee of Sun Yat-Sen University.

## Pharmacokinetic Analysis

The pharmacokinetic parameters of bisoprolol were calculated using non-compartmental methods with the aid of the computer program WinNolin (version 8.1, Pharsight Corporation). $C_{max}$ and $T_{max}$ were obtained directly from the observed plasma concentration-time data. The terminal elimination rate constant ($\lambda_Z$) was determined by linear regression of the terminal portion of the plasma concentration-time curve and the elimination half-life ($t_{1/2}$) was calculated as $0.693/\lambda_Z$. Systemic exposure to bisoprolol was evaluated by calculating the AUC using the linear trapezoidal rule and $AUC_{0-\infty}$ was calculated as $AUC_{0-\infty} = AUC_{0-t} + C_t/K_{el}$ where $C_t$ is the last quantifiable concentration. Apparent volume of distribution (Vz/F) = dose * bioavailability / plasma drug concentration. The oral clearance (CL/F) was calculated as $Dose/AUC_{0-\infty}$.

## Statistical Analysis

All data were verified for normal distribution. Data are presented as mean ± SD. A probability value $<0.05$ was considered statistically significant. The pharmacokinetic parameters of bisoprolol with and without EGCG were compared by repeated

**TABLE 5 |** Effect of EGCG on the pharmacokinetic parameters of bisoprolol in SD rats.

| Parameters | Intragastric administration | | Intravenous administration | |
|---|---|---|---|---|
| | Bisoprolol ($n = 6$) | Bisoprolol+EGCG($n = 6$) | Bisoprolol ($n = 6$) | Bisoprolol+EGCG($n = 6$) |
| $t_{1/2}$, h | 3.89 ± 2.03 | 4.49 ± 1.45 | 2.47 ± 0.32 | 2.88 ± 0.94 |
| $T_{max}$, h | 0.50 ± 0.10 | 0.83 ± 0.26** | 0.25 ± 0.37 | 0.12 ± 0.07 |
| $C_{max}$, mg/mL | 2,012.31 ± 510.67 | 942.26 ± 230.23* | 2,259.56 ± 607.55 | 2,438.08 ± 962.08 |
| $AUC_{0-t}$, h·ng/mL | 3,709.61 ± 827.46 | 2,260.37 ± 579.78 | 4,030.01 ± 1,091.01 | 3,421.82 ± 1,124.34 |
| $AUC_{0-\infty}$, h·ng/mL | 3,914.09 ± 1081.55 | 2,347.37 ± 586.96 | 4,050.09 ± 1,117.69 | 3,436.69 ± 1,115.84 |
| $V_z$/F, mL/Kg | 3,687.71 ± 2021.28 | 7,693.73 ± 5,114.65 | 1,629.62 ± 279.05 | 2,473.27 ± 1,814.65* |
| Cl/F, mL/h/Kg | 661.27 ± 225.56 | 1,028.96 ± 374.99 | 466.34 ± 111.29 | 546.72 ± 206.45 |

*$P < 0.05$, ** $P < 0.01$.

**TABLE 6 |** Pharmacokinetic parameters of EGCG after intragastric administration in SD rats.

| Parameters | EGCG($n = 6$) | EGCG+Bisoprolol ($n = 6$) | *P*-value |
|---|---|---|---|
| $t_{1/2}$, h | 5.06 ± 2.99 | 2.81 ± 0.96 | 0.130 |
| $T_{max}$, h | 0.36 ± 0.35 | 0.18 ± 0.06 | 0.270 |
| $C_{max}$, mg/mL | 6,007.28 ± 8,136.92 | 4,768.19 ± 4,636.59 | 0.753 |
| $AUC_{0-t}$, h·ng/mL | 3,499.28 ± 2,489.96 | 3,219.20 ± 2,614.72 | 0.853 |
| $AUC_{0-\infty}$, h·ng/mL | 3,634.08 ± 2,400.23 | 3,274.21 ± 2,625.90 | 0.809 |
| $V_z$/F, mL/Kg | 96,970.06 ± 109,217.92 | 120,821.98 ± 205,173.74 | 0.807 |
| Cl/F, mL/h/Kg | 11,109.16 ± 10,466.19 | 25,615.88 ± 39,010.30 | 0.400 |

measures ANOVA and the Friedman rank test was used to compare $T_{max}$ values. SPSS 25.0 for Windows (SPSS, Chicago, IL) was used.

# RESULTS

## The Direct Binding Effect of EGCG and Bisoprolol *in vitro*

In this assay, bisoprolol was titrated with EGCG at room temperature. The thermodynamics parameters of interaction between EGCG and bisoprolol can be calculated by fitting the raw ITC data. The parameter values were as follows: $\Delta Go = -17.12$ kJ/mol, $\Delta Ho = 6.33$ kJ/mol, $-T\Delta So = -23.46$ kJ/mol. The equilibrium dissociation constant (KD) was determined after analysis of the normalized ITC curve by the NanoAnalyze Software. The data indicated that EGCG can bind to bisoprolol (see **Figure 1**).

## Assay Validation

The retention times of EGCG, bisoprolol, deuterated bisoprolol and loratadine were 1.22, 1.29, 1.30, and 1.48 min, respectively. There were no impurity peaks in the blank plasma samples at or near the peak time (**Figure 2**). The linear relationship between EGCG and bisoprolol was present in the range of 5–2,000 ng/mL, and the correlation coefficient ($R^2$) was more than 0.99. The deviation of each concentration in the standard curve was within the acceptable range of ±15%. The standard curve results are shown in **Table 1**. Accuracy and precision all met acceptable requirements, precision (RSD, %) were <15% and accuracy (RE, %) were within ±15%. The accuracy and precision of LLOQ did not exceed 20% (**Table 2**). The recoveries and matrix effects of EGCG and bisoprolol are shown in **Table 3**. The recoveries for all analytes ranged from 61.0 to 99.7%. No significant signal was observed in the mass spectrometry affecting rat plasma. Under current analytical conditions, matrix effects were negligible. The accuracy of bisoprolol and EGCG measurement was not significantly affected under different storage condition including 12 h in the autosampler, 4 h on a laboratory table at room temperature, 30 days in a low temperature freezer at −80°C or 3 freeze-thaw cycles (**Table 4**). No significant differences were observed in test results compared to freshly prepared samples.

## Effect of EGCG on the Pharmacokinetics of Bisoprolol

After intragastric administration, intake of EGCG (100 mg/kg body weight) with bisoprolol significantly increased the $T_{max}$ (mean $T_{max}$ from 0.5 to 0.83 h, $P < 0.01$) and reduced the $C_{max}$ (mean $C_{max}$ from 2012.31 to 942.26 ng/mL, $P < 0.05$) of bisoprolol (**Figure 3** and **Table 5**). Moreover, intake of EGCG tended to reduce the systemic exposure to bisoprolol (mean $AUC_{0-\infty}$ from 3914.09 to 2347.37 h.ng/mL, $P = 0.17$) but this was not significant and there was no significant effect on the elimination half-life ($t_{1/2}$; **Figure 3** and **Table 5**). After intravenous administration, intake of EGCG with bisoprolol significantly increased the apparent volume of distribution (mean $V_z$/F from 1629.62 to 2473.27 mL/Kg, $P < 0.05$) but there was no significant effect on other pharmacokinetic parameters (**Figure 3** and **Table 5**). In the male group, the trend of increasing Cl/F after co-administration was stronger than in the female group. This means that the inhibitory effect of EGCG on the elimination of bisoprolol was stronger in the male rats. $AUC_{0-t}$ and $AUC_{0-\infty}$ also went down in the male group more than in the female group, which means that EGCG had more of an impact on bisoprolol's

absorption than it did in the female rats. So, it can be said that there is a gender difference in the way EGCG affects the way bisoprolol is absorbed, used, and excreted (Data didn't shown).

## The Pharmacokinetics of EGCG

The pharmacokinetic parameters of EGCG after intragastric administration in SD rats were shown in **Table 6**. There were no significant differences in a single dose of EGCG and combined with bisoprolol.

## Effect of EGCG on the Bioavailability of Bisoprolol

Based on the pharmacokinetic study results of intragastric administration and tail vein administration, the absolute bioavailability of bisoprolol in the single-dose bisoprolol group and the combined EGCG-administered group was calculated. The results showed that the absolute bioavailability of bisoprolol when administered alone ($F_{abs.Bisoprolol} = 92.04\%$) was greater than when it was administered in combination with EGCG ($F_{abs.Bisoprolo+EGCG} = 66.05\%$).

## Effect of EGCG on the Pharmacodynamics of Bisoprolol

After gavage administration, intake of EGCG (100 mg/kg body weight) with bisoprolol lowered SBP at the first measurement at 0.5 h post dose of EGCG and bisoprolol, while the SBP only started to decrease at 4 h after administration of bisoprolol alone. The largest reduction of SBP was 37.37 ± 10.91 mmHg (change of −20.8%) at 8 h post dose with EGCG and bisoprolol combined and was similar with bisoprolol alone. MBP and DBP reached the maximum reduction of 28.38 ± 9.30 mmHg (change of −20%) and 31.68 ± 12.96 mmHg(change of −24.6%), respectively, at 1 h post dose of EGCG and bisoprolol. However, the reduction of MBP and DBP were relatively slow, and reached the maximum reduction at 8 h after the single dose of bisoprolol. The largest reduction of heart rate (HR) was 140.72 ± 26.28 beats/min (change of −30.0%) at 1 h after the dose of bisoprolol alone and it was greater than the effect of the combination of EGCG and bisoprolol with a reduction of 60.18 ± 80.37 beats/min (change of −13.2%; **Table 7** and **Figure 4**).

# DISCUSSION

Green tea is taken as a common drink worldwide and it can interact with various medications and may alter their pharmacokinetic and pharmacodynamic properties. In this study, we found for the first time that the green tea component EGCG (100 mg/kg) can reduce the $C_{max}$ of bisoprolol and delay the $T_{max}$ and tend to reduce the AUC when given simultaneously by gavage in rats, while the Vz/F and clearance of bisoprolol tended to increase. The $T_{max}$, $C_{max}$ and the AUC are all related to the absorption process of bisoprolol. The results suggest that EGCG can inhibit the absorption of bisoprolol when given together and this can lead to a lower early reduction in HR. The attenuation in reduction of HR with EGCG combined with bisoprolol was associated with a greater early reduction in SBP, which may reflect the various mechanisms involved in the reduction of BP with a beta-blocker. In order to detect the plasma concentrations of bisoprolol and EGCG simultaneously, a new HPLC-MS/MS method was developed and validated. The method meets the guidelines for the validation of methods for the definitive analysis of biological samples (Version 2020) and was applied for the detection of bisoprolol and EGCG in this study. We also employed isothermal titration calorimetry (ITC), a thermodynamic method with high sensitivity and reproducibility *in vitro*, to study biomolecular interactions. We tested the direct binding effect of EGCG and bisoprolol *in vitro* and found that EGCG and bisoprolol showed a significant binding phenomenon, similar to that of EGCG and atenolol.

EGCG is the main abundant catechin in green tea, accounting for 50–80% of the total catechins and has been reported to improve many cardiovascular risk factors including blood pressure (13). Intake of catechins 400–500 mg daily can significantly reduce systolic and diastolic blood pressure (14). However, several studies have reported that green tea polyphenols may affect the expression or activities of drug-metabolizing enzymes such as CYP1A1, CYP2D6, CYP3A4 and drug transporters which leads to changes in the absorption and metabolism of certain drugs (15, 16). In addition, it was reported that green tea catechins can inhibit the activities of CYP1A1, CYP2A6, CYP2C9 and CYP3A4 (17). Some other studies have also reported the inhibition of the ABCB1 and ABCG2 transporter activity by EGCG (18). Roth et al. showed that EGCG inhibited OATP1A2— and OATP2B1-mediated uptake of estrone-3-sulfate in an *in vivo* study (19). Another study has shown that the consumption of green tea extract can significantly reduce the bioavailability of nadolol through the inhibition of OATP1A2-mediated uptake (20). Therefore, EGCG may inhibit the efflux of bisoprolol by inhibiting the action of P-gp, resulting in increased bioavailability.

A study in a Korean population found that co-administration of rosuvastatin with EGCG resulted in a 19% decrease in the plasma concentrations of the drug apparently due to inhibition of drug absorption by the transporters OATP1A2 and OATP2B1(21). In a study in Hong Kong, green tea extract was reported to affect the pharmacokinetics of rosuvastatin and reduced the peak plasma drug concentration by 30% (22). It has also been reported that green tea was able to inhibit the drug metabolizing enzyme CYP3A4 and the efflux transporter P-gP for simvastatin, resulting in increased simvastatin plasma concentrations (23). Moreover, in a study in Japan there was a significant effect of green tea on the pharmacokinetics and therapeutic efficacy of nadolol after 14 days of simultaneous administration of nadolol and green tea. In 10 healthy volunteers, the peak plasma concentration ($C_{max}$) and area under the plasma concentration time curve up to 48 h ($AUC_{0-48}$) decreased by 85.3 and 85.0%, respectively, while the time to $C_{max}$ ($T_{max}$) was significantly shortened, but no effect on the clearance was found. Further study identified the mechanism involved in the inhibition of the absorption of nadolol by EGCG through the transporter OATP1A2 which resulted in a decrease in the plasma concentration of nadolol (24). In addition, EGCG

**TABLE 7 |** The reduction of blood pressure and heart rate after treatment in the two groups in SHRs.

| Time post dose | SBP, mmHg | | DBP, mmHg | | MBP, mmHg | | HR,beats/min | |
|---|---|---|---|---|---|---|---|---|
| | Bisoprolol | Bisoprolol + EGCG | Bisoprolol | Bisoprolol + EGCG | Bisoprolol | Bisoprolol + EGCG | Bisoprolol | Bisoprolol + EGCG |
| 0.5 h | −13.62 ± 15.41 | 11.72 ± 8.37** | 3 ± 16.77 | 1.93 ± 11.02 | −2.5 ± 14.97 | 4.85 ± 10.26 | 139.48 ± 31.73 | 45.28 ± 71.11** |
| 1 h | −9.18 ± 16.61 | 23.23 ± 13.29** | 3.68 ± 15.03 | 31.68 ± 12.96** | −0.73 ± 15.43 | 28.38 ± 9.30** | 140.72 ± 26.28 | 60.18 ± 80.37** |
| 2 h | 0.17 ± 13.95 | 20.5 ± 12.07** | 9.45 ± 15.97 | 6.17 ± 11.73 | 6.28 ± 14.67 | 10.62 ± 10.67 | 118.88 ± 36.24 | 47 ± 74.06 |
| 4 h | 10.92 ± 11.59 | 21.05 ± 21.14 | 14.22 ± 11.12 | 23.07 ± 16.13 | 13.05 ± 10.23 | 20.43 ± 15.31 | 120.78 ± 21.76 | 53.77 ± 79.17 |
| 8 h | 36.13 ± 17.46 | 37.37 ± 10.91 | 33.78 ± 18.89 | 24.83 ± 9.03 | 34.5 ± 17.88 | 28.88 ± 8.27 | 82.78 ± 34.10 | 23.98 ± 80.04 |
| 24 h | 16.08 ± 13.65 | 26.83 ± 18.43 | 18.48 ± 14.05 | 18.6 ± 26.53 | 17.7 ± 13.32 | 21 ± 22.24 | 20.08 ± 54.12 | −21.43 ± 71.55 |

*Negative values indicate increases.*
*$^{*}P < 0.05$, $^{**}P < 0.01$.*

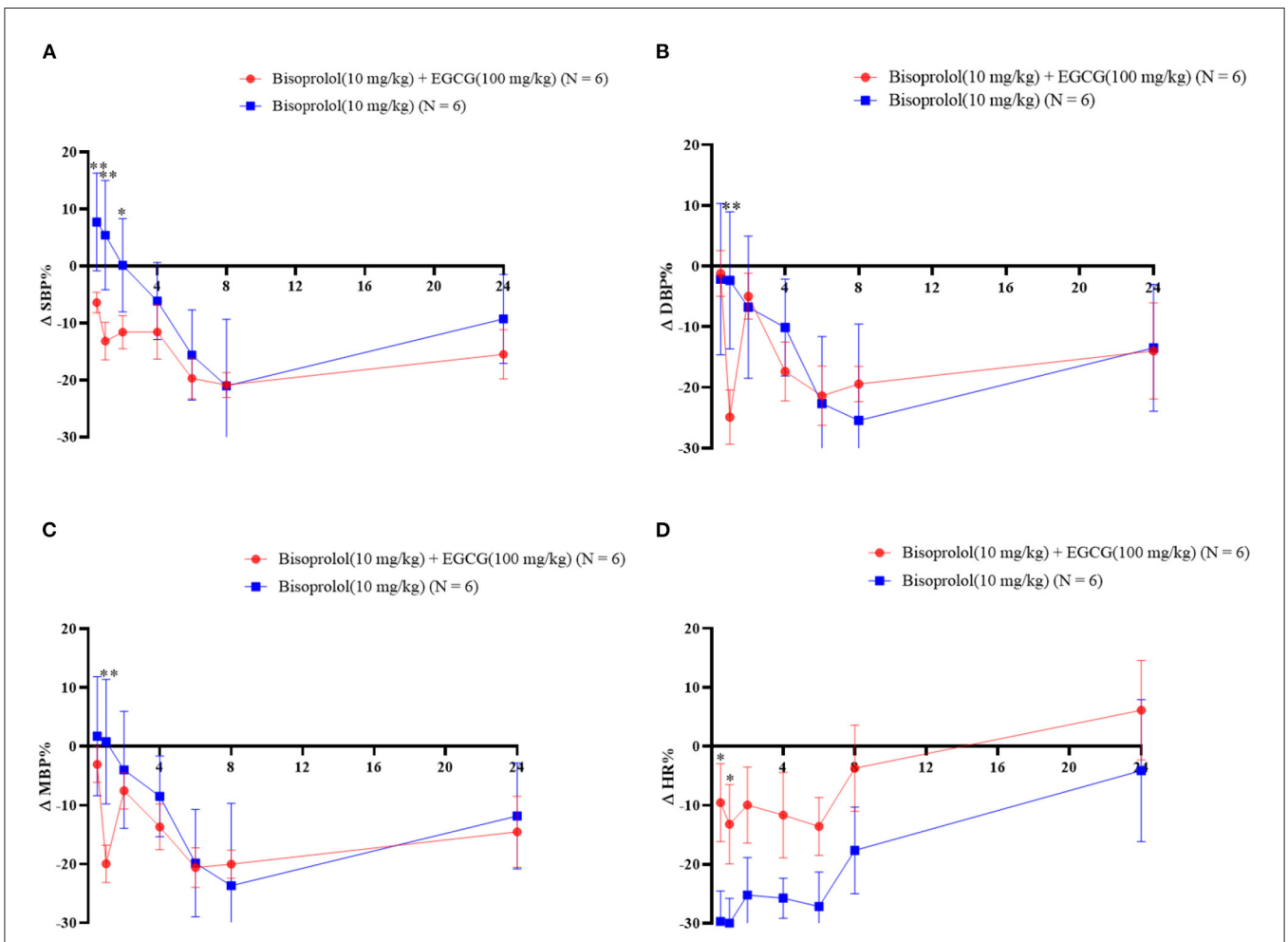


**FIGURE 4 |** The blood pressure and heart rate changes as percentages over time after gavage administration of bisoprolol with or without EGCG. *$P < 0.05$, **$P < 0.01$. **(A)** Percentage change of SBP; **(B)** Percentage change of DBP; **(C)** Percentage change of mean BP; **(D)** Percentage change of heart rate.

can interact with atenolol and form a precipitate under acidic conditions *in vitro*, resulting in the inhibition of the absorption of atenolol (25).

EGCG has been reported to increase the AUC of tamoxifen and diltiazem when combined with these drugs by inhibiting the activity of CYP3A4 and P-gp (18). Tamoxifen and diltiazem are known to be metabolized by the same pathway as bisoprolol. However, the AUC and $C_{max}$ of bisoprolol were reduced in this study. That is mainly because of the differences in the first-pass effect during the absorption of drugs. The first-pass effect of bisoprolol is much smaller than that of tamoxifen or diltiazem. Therefore, we will further investigate the absorption pathway of bisoprolol and the factors affecting this.

The metabolism of bisoprolol is mainly dependent on the CYP3A4 enzyme, and it has been shown that EGCG can inhibit the CYP3A4 enzyme (26), so it may be predicted that EGCG could inhibit part of the metabolism of bisoprolol by inhibiting the activity of CYP3A4, resulting in a reduction in the metabolism of bisoprolol and causing accumulation. In addition, when the metabolic pathway of bisoprolol *via* CYP3A4 enzymes is inhibited, renal clearance becomes the main route of elimination of bisoprolol, so renal clearance increases and the overall elimination half-life is prolonged although no significant effect was observed in the present study.

The absorption of bisoprolol in the intestine may be dependent on organic cation transporters (OCTs) for completion (27). However, it has been shown that EGCG inhibits the activity some of the OCTs (28), so EGCG may affect the absorption of bisoprolol by inhibiting the action of OCTs, resulting in a reduction in the absorption and a delay in absorption time, leading to a delay in the $T_{max}$ and a reduction of $C_{max}$ and AUC. The increase in the apparent volume of distribution of bisoprolol after intravenous administration with EGCG may also be related to effects on transporters resulting in increased distribution of bisoprolol to body tissues or more rapid clearance of bisoprolol by metabolism or renal excretion.

## LIMITATION

This study has some limitations that need to be considered. Firstly, this study only assessed one dosage for EGCG (100 mg/kg). It is known that interactions between herbs and drugs may be dose-dependent. Evaluating a higher dose or a lower dose may help to provide a better understanding of the interaction between bisoprolol and green tea. Secondly, it has been shown that taking EGCG 8 or 4 h before sunitinib administration had no effect on the pharmacokinetics of sunitinib in rats, whereas taking the two together reduced the bioavailability of sunitinib, probably because of a physical reaction between the two compounds (50), suggesting separation of dosing of green tea and drugs may reduce any herb-drug interaction. EGCG was given simultaneously with bisoprolol in the present study and we did demonstrate by isothermal titration calorimetry that a physico-chemical reaction does occur between EGCG and bisoprolol and this may be responsible for some of the interaction. It would be useful to assess whether the separation of dosing or repeated dosing of EGCG and bisoprolol have different effects.

## CONCLUSION

This study showed that administration of EGCG at a single dose of 100 mg/kg with a single dose of bisoprolol of 10 mg/kg was associated with decreased $C_{max}$ and $T_{max}$ and a tendency for decreased AUC and increased Vz/F and clearance for bisoprolol in SD rats when bisoprolol was taken simultaneously with EGCG. Moreover, administration of EGCG significantly attenuated the early HR reduction with bisoprolol and resulted in an earlier reduction in BP compared to when bisoprolol was given alone in SHRs.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Review Board of Baoan Women's and Children's Hospital with IRB No LLSC2020-03-05 and the Animal Care and Use Committee of Sun Yat-sen University.

## AUTHOR CONTRIBUTIONS

WZ and SL analyzed the data and wrote this manuscript. WZ and GZ designed the research project. SL, YG, and YW performed the experiments. MH, BT, GZ, and WZ revised this manuscript. All authors contributed to the article and approved the submitted version.

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# Effects of Different Green Teas on Obesity and Non-Alcoholic Fatty Liver Disease Induced by a High-Fat Diet in Mice

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Non-alcoholic fatty liver disease (NAFLD) and obesity are serious public health problems. Green tea is widely consumed in the world and different green teas could possess different bioactivities. In this study, the effects of 10 selected green teas on obesity and NAFLD were evaluated and compared. The mice fed with a high-fat diet were intervened with green tea extract (200 mg/kg body weight) for 15 weeks. Most of these teas were first evaluated for their effects on obesity and NAFLD. The results showed that Selenium-Enriched Chaoqing Green Tea and Jieyang Chaoqing Tea showed the most prominent inhibition of obesity and body weight gains of mice in these two tea intervention groups and model groups were 5.3, 5.5, and 13.7 g, respectively. In addition, Jieyang Chaoqing Tea, Taiping Houkui Tea, and Selenium-Enriched Chaoqing Green Tea exerted the most notable effect on NAFLD, which was attributed to decreasing body weight, and lipid content and ameliorating oxidative stress. Furthermore, 13 phytochemicals were determined in these teas by high-performance liquid chromatography and the correlation analysis found that epigallocatechin gallate, gallocatechin, and epigallocatechin might contribute to the decrease of hepatic weight, while epicatechin might reduce oxidative stress. In general, several green teas could prevent the development of obesity and NAFLD and could be developed into functional foods. This study was also helpful for the public to select appropriate tea to prevent obesity and NAFLD.

**Keywords: green tea, obesity, non-alcoholic fatty liver disease, prevention, antioxidant activity**

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a threatening chronic non-communicable disease with continuously increased incidence worldwide. The prevalence of NAFLD is estimated at 25% among adults, 3–10% in the pediatric population, and could rise to 70% among children with obesity (1–3). Various factors contribute to the development of NAFLD. Increasing evidence revealed that obesity is a strong contributor to the occurrence of NAFLD and obesity itself is also a serious public health problem. In the condition of similar body weight, people with more visceral fat are more likely to

develop NAFLD (4–6). The lipid metabolism relied on the action of the liver, such as the *de-novo* lipogenesis, lipid oxidation, and uptake/secretion of lipoproteins. When excessive intake of dietary fat and energy exceeds the capacity of the liver to metabolize lipids, the synthesis and deposition of a lipid would increase in the liver and body, leading to lipid metabolic disturbance, which is of great importance in the pathogenesis of NAFLD. The increase in the level of free fatty acids is accompanied by the overload of lipids, which could promote the over generation of reactive oxygen species (ROS) and the consumption of antioxidants. Oxidative stress could further impair the function of hepatocytes and even destroy the hepatic structure, which would accelerate the process of NAFLD (7, 8). NAFLD could develop into more severe liver diseases, such as fibrosis, cirrhosis, and hepatocellular carcinoma (9, 10). Therefore, prevention and treatment of NAFLD are vital. On the other hand, some natural products have shown strong antiobesity and/or antioxidant activity, which could be a good alternative for the prevention of NAFLD and obesity because of the limited efficacy and potential side effects of chemically synthetic drugs.

Tea (*Camellia sinensis*) is a popular drink with a long history. It has shown various bioactivities, such as antioxidant, anti-inflammation, antidiabetic, antiobesity, anticancer, and cardiovascular protection (11–15). Tea could be divided into green, white, yellow, oolong, black, and dark teas based on different fermentation degrees. Green tea extract has been used to treat antiobesity (11). On the other hand, growing studies showed the effect of green tea on the prevention and management of NAFLD (16–18). The hepatoprotective and antiobesity effects of green tea are mainly attributed to its rich bioactive compounds, such as polyphenols. However, different kinds of green teas could have very different compositions and contents of bioactive compounds. Hence, the effects of different kinds of green teas on NAFLD and obesity could be different. In this study, the effects of 10 selected green teas on obesity and NAFLD induced by a high-fat diet (HFD) were evaluated and compared in mice at a dose of 200 mg/kg body weight (bw). Most of these green teas were first evaluated for their effects on obesity and NAFLD. The findings could serve the public to choose tea for the prevention of obesity and NAFLD. In addition, several teas could be developed into functional food for the prevention and management of obesity and NAFLD.

# MATERIALS AND METHODS

## Chemicals and Materials

Methanol, formic acid, and isopropanol were obtained from Macklin Chemical Factory (Shanghai, China). The standard chemicals were offered by Derick Biotechnology Corporation Ltd. (Chengdu, China), namely, gallic acid, quercitrin, kaempferol, astragalin, quercetin, ellagic acid, theaflavin, myricetin, chlorogenic acid (CA), caffeine, catechin, epicatechin (EC), gallocatechin (GC), catechin gallate (CG), epigallocatechin (EGC), epicatechin gallate (ECG), gallocatechin gallate (GCG), and epigallocatechin gallate (EGCG). The determination kits of hepatic triglyceride (TG) and malondialdehyde (MDA) were provided by Apply-gen Technologies Corporation Ltd. (Beijing, China) and kits for superoxide dismutase (SOD) and glutathione (GSH) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Moreover, the contents of serum TG, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), aspartate transaminase (AST), and alanine transaminase (ALT) were determined by kits from Roche Diagnostics (Shanghai, China).

**TABLE 1 |** The details of 10 selected green teas from China.

| No. | Name | Production place |
|---|---|---|
| GT1 | Dianqing Tea | Kunming city, Yunnan province |
| GT2 | Jieyang Chaoqing Tea | Jieyang city, Guangdong province |
| GT3 | Fenggang Zinc-Selenium-Enriched Tea | Guiyang city, Guizhou province |
| GT4 | Liping Xiang Tea | Liping city, Guizhou province |
| GT5 | Taiping Houkui Tea | Huangshan city, Anhui province |
| GT6 | Xihu Longjing Tea | Hangzhou city, Zhejiang province |
| GT7 | Chaoqing Green Tea | Yichang city, Hubei province |
| GT8 | Selenium-Enriched Chaoqing Green Tea | Enshi city, Hubei province |
| GT9 | Selenium-Enriched Matcha | Enshi city, Hubei province |
| GT10 | Seven Star Matcha | Shaoxing city, Zhejiang province |

The details of 10 selected green teas are shown in **Table 1**.

## Preparation of Green Tea Extracts

The preparation of 10 kinds of green tea extracts was carried out according to the literature (19). The 10 g green tea sample and 100 ml boiling deionized water were mixed in a conical flask and extracted in a 98°C water bath for 10 min. The mixture was filtered to collect the infusion. The tea sample was extracted 3 times and the infusions were collected and merged. Then, the collected infusion was concentrated using a vacuum rotary evaporator at 60°C and about 15 ml of concentrated infusion was obtained. Finally, the concentrated infusion was freeze-dried into powder by a lyophilizer and kept at −80°C. The powders were dissolved in deionized water to obtain the tea extracts with a concentration of 20 g/l (w/v) before the administration to the mice.

## Animal Study

The C57BL/6J male mice (18–20 g) used in this study were purchased from the Experimental Animal Center of Guangdong Province (Guangzhou, China). All the mice were housed in a specific pathogen-free (SPF) animal laboratory, where the humidity was set at 40–60%, the room temperature was 22 ± 0.5°C, and the light/dark cycle was 12 h. After acclimatization for 1 week, 8-week-old mice were randomly divided into the normal diet (ND) control group, the high-fat diet (HFD) model group, and the green tea (GT) treatment groups (including 10 different green tea treatment groups), totally 12 groups ($n$ = 10/group). The ND control group was fed with a normal diet with an energy of 3.6 kcal/g (12% calories from fat), which was provided by Jiangsu Xietong Pharmaceutical Bioengineering Corporation Ltd. (Nanjing, China). Meanwhile, the HFD model group and the treatment group were given a high-fat diet with the energy of 5.0 kcal/g (60% calories from fat), which

was purchased from Trophic Animal Feed High-technology Corporation Ltd. (Nantong, China). Mice in the treatment groups were intragastrically administrated with tea extracts at a dose of 200 mg/kg bw daily for 15 weeks according to the literature (20, 21). In addition, mice in the control and model groups were received 10 ml/kg bw of deionized water by gavage because the concentration of the intervention solution was 20 g/l, which was equal to 10 ml/kg bw when a dose of 200 mg/kg bw was used. The daily food consumption and weekly body weight of mice were recorded. At the end of the 15-week intervention, all the mice were fasted for 12 h and then weighed, anesthetized, and sacrificed to collect blood and liver samples, and perirenal and epididymal adipose tissues. All the experimental procedures involving animals in this study have received approval from the Ethics Committee in the School of Public Health, Sun Yat-Sen University (No. 2019–002; 28 February 2019).

## Measurement of Serum Alanine Transaminase, Aspartate Transaminase, Triglyceride, Total Cholesterol, and Low-Density Lipoprotein Cholesterol Levels

After being collected and placed at room temperature (25°C) for 1 h, the blood samples were centrifuged at 3,000 × *g* (4°C) for 15 min to acquire serum. The levels of serum ALT, AST, TG, TC, and LDL-C were determined with the instruction of detection kits. In brief, enzymatic tests were used to measure the contents of TG, TC, and LDL-C and the velocity tests were carried out to determine the levels of AST and ALT.

## Biochemical Analysis of Hepatic Tissue

The determination of hepatic TG content was performed according to the instruction from the detection kit. The 25 mg hepatic tissue was completely homogenized with 500 μl lysis buffer to obtain the liver homogenate. After placing for 10 min, the upper layer of liver homogenate was separated and heated at 70°C for 10 min and then centrifuged (2,000 × *g*, 25°C) for 5 min to obtain the supernatant to measure the TG content.

The status of hepatic oxidative stress in mice was assessed by the determination of GSH, MDA, and SOD levels. The 200 mg hepatic tissue was mixed and homogenized with 1.8 ml of 0.9% NaCl to acquire hepatic homogenate. The homogenate was centrifuged (2,500 ×*g*, 4°C) for 10 min to get the supernatant for the determination of GSH and SOD. In addition, 10 mg hepatic tissue was mixed and homogenized with 500 μl lysis buffer to obtain liver homogenate, which was then centrifuged (10,000 × *g*, 4°C) for 10 min to obtain the supernatant to measure MDA content.

## Observation of Histopathological Changes in Liver and Adipose Tissues

The histopathological changes in liver and epididymal adipose tissues were examined using H&E staining. The hepatic and epididymal adipose tissues were soaked in 4% paraformaldehyde for a few days and then embedded in paraffin. The embedded samples were sliced into 5-μm-thick sections and then deparaffinized, rehydrated, and stained with H&E. The image of the liver and epididymal adipose tissue was captured and observed with a microscope.

## Measurement of Bioactive Compounds in Green Tea Extracts

The bioactive components in green tea extracts were qualitative and quantitative analyzed using high-performance liquid chromatography (HPLC) in comparison with the standard compounds, which is according to our previous study (19).

## Statistical Analysis

The analysis of experimental data was performed with the software SPSS version 25.0 (IBM Incorporation, Armonk, NY, USA) and the results were expressed as mean ± SD. A one-way ANOVA combined with the least significant difference (LSD) test was conducted to compare the difference between the experimental groups. Statistical significance was set at $p < 0.05$. In addition, the software GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) was applied to draw figures.

# RESULTS AND DISCUSSION

## Effects of Different Green Teas on Body Weight

The effects of 10 kinds of green teas on body weight gain and energy intake are shown in **Figure 1**. As shown in **Figure 1A**, mice fed with HFD had a significantly larger weight gain than those of the control group ($p < 0.01$). The average body weight gain of mice in the model group was 13.7 g, which was 2.14-fold that in the control group (6.4 g). In addition, the body weight gain in all the green tea groups was markedly smaller than that in the model group ($p < 0.01$), but the effect greatly varied in different green teas. The Selenium-Enriched Chaoqing Green Tea (GT8) and Jieyang Chaoqing Tea (GT2) showed the strongest inhibition on obesity with 5.3 and 5.5 g body weight gain, respectively. Moreover, the energy intake in the model group was significantly bigger than that in the control group ($p < 0.05$). The energy intake in mice treated with 8 green teas was reduced compared with the model group ($p < 0.01$) (**Figure 1B**). The results of energy intake were generally coincident with those of body weight gain, which manifested that green tea played a role in the inhibition of obesity partly because of its effect on the reduction of energy intake.

A large amount of research has proved that excessive and frequent consumption of a high-calorie diet led to the prevalence of obesity and related NAFLD. In this study, HFD induced a dramatic increase in body weight gain in C57BL/6 mice, while green tea could effectively prevent obesity at a dose of 200 mg/kg bw. The results were consistent with previous studies (16, 20, 22). For example, a study found that the oral gavage with 500 mg/kg bw of green tea extracts markedly attenuated body weight gain in C57BL/6 mice compared with the HFD group (16). Weight loss is usually accompanied by a decrease in energy intake. In this study, most of the 10 green teas significantly inhibited the energy intake, except Chaoqing Green Tea (GT7) and Seven Star Matcha (GT10). Correspondingly, the body weight gain in mice treated

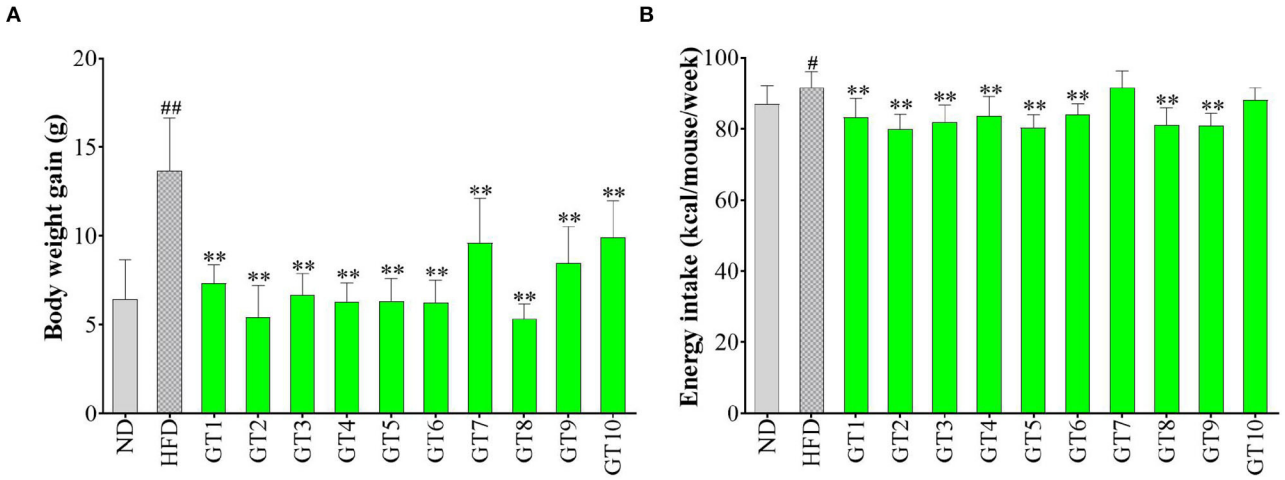


**FIGURE 1 |** Effects of green teas on body weight (BW) gain **(A)** and energy intake **(B)**. # $p < 0.05$, ## $p < 0.01$, the model group vs. the control group; ** $p < 0.01$, the green tea groups vs. the model group. ND, normal diet; HFD, high-fat diet. GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium -Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

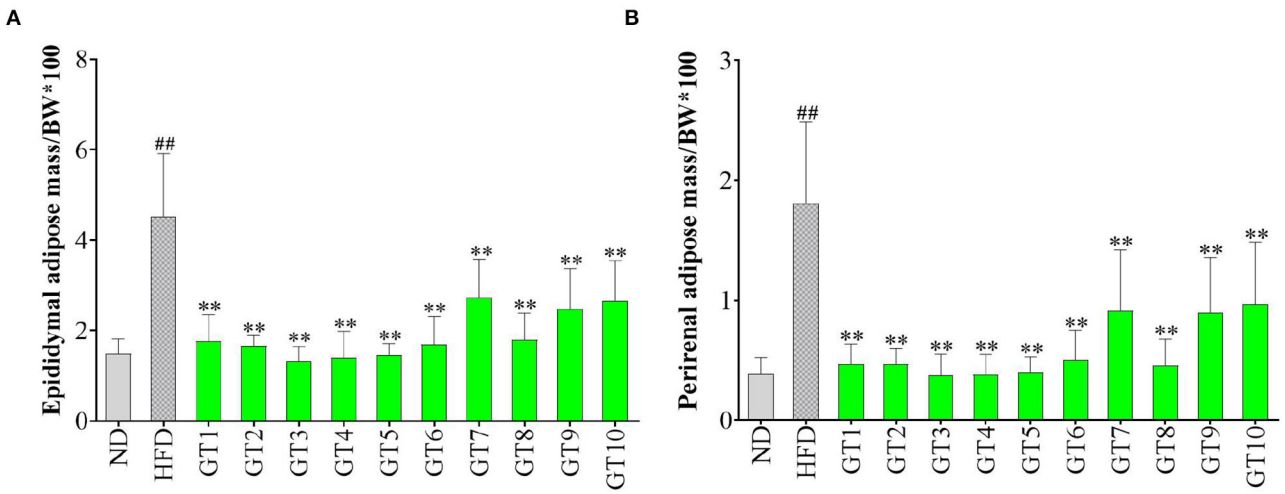


**FIGURE 2 |** Effects of green teas on visceral adipose tissue. **(A)** Epididymal adipose mass/BW × 100 and **(B)** Perirenal adipose mass/BW × 100. ## $p < 0.01$, the model group vs. the control group; ** $p < 0.01$, the green tea groups vs. the model group. BW, body weight; ND, normal diet; HFD, high-fat diet; GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

with Chaoqing Green Tea (GT7) and Seven Star Matcha (GT10) was more obvious than in other teas. Hence, the antiobesity effects of 10 different green teas varied greatly, which was partly due to the inhibitory difference in energy intake.

## Effects of Green Teas on Visceral Adipose Tissue

Epididymal and perirenal adipose is the important visceral fat in the body. The ratio of their mass to body weight was used to evaluate the effect of green teas on visceral adipose in this study and the results are shown in **Figure 2**. The percentages of both the epididymal and perirenal adipose masses in the body weight of mice in the model group significantly increased compared with those of the control group ($p <$ 0.01), while the treatment of different green teas could reduce the accumulation of visceral fat in mice induced by HFD at varying degree. Among 10 kinds of green teas, Fenggang Zinc-Selenium-Enriched Tea (GT3), Liping Xiang Tea (GT4), and Taiping Houkui Tea (GT5) showed the most remarkable effectiveness in suppressing the increase of visceral adipose tissue. Furthermore, the histopathological changes in epididymal adipose tissue have been studied and the results are shown in

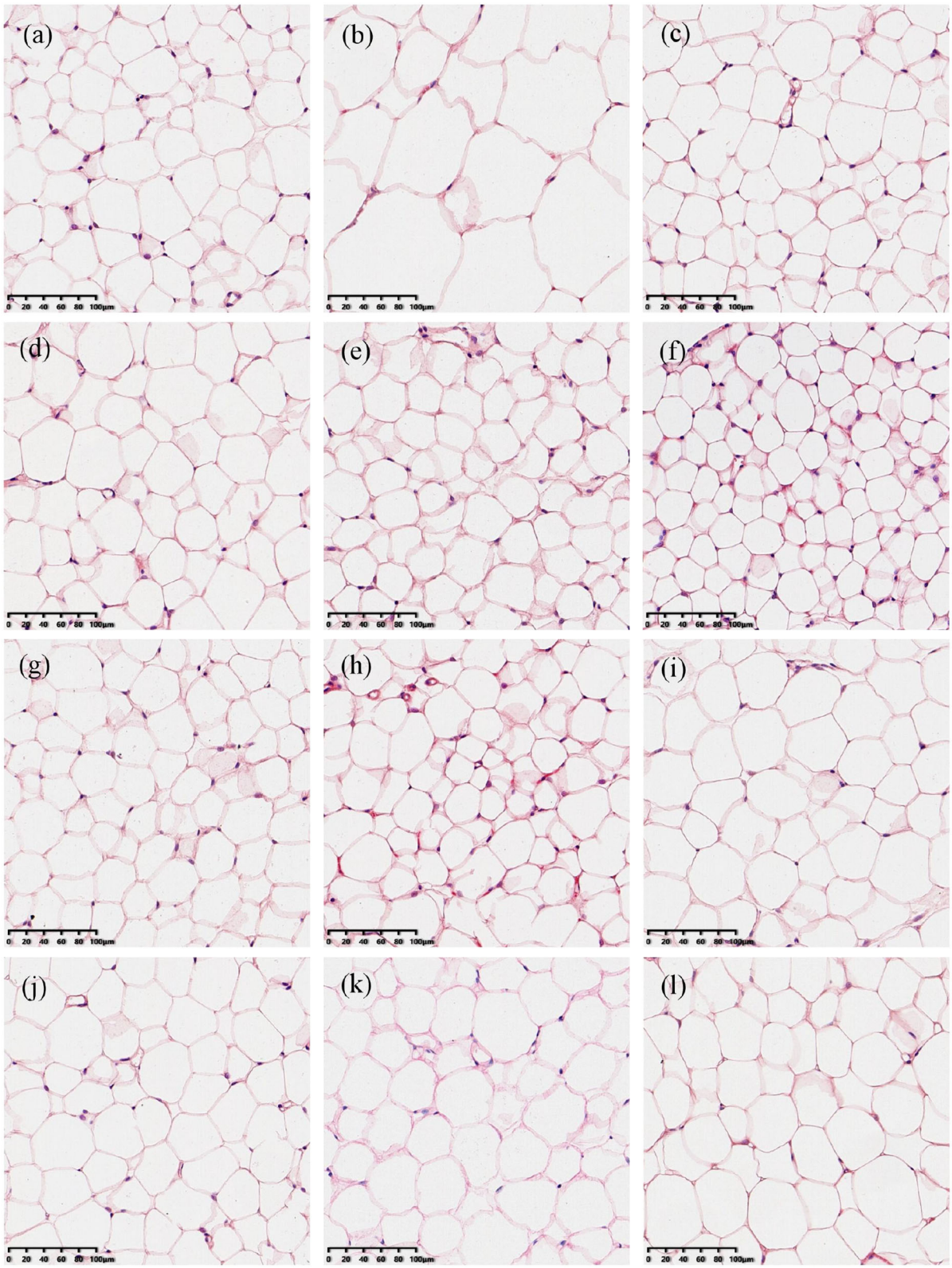


**FIGURE 3 |** Histopathological changes of epididymal adipose tissues (200X magnification). **(a)** The control group; **(b)** the model group; **(c)** the GT1 group; **(d)** the GT2 group; **(e)** the GT3 group; **(f)** the GT4 group; **(g)** the GT5 group; **(h)** the GT6 group; **(i)** the GT7 group; **(j)** the GT8 group; **(k)** the GT9 group; and **(l)** the GT10 group. ND, normal diet; HFD, high-fat diet; GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

**Figure 3**. It could be seen that the sizes of epididymal adipocytes in the model group had an incredible expansion and the shapes of adipocytes showed an abnormal irregularity. On the other hand, epididymal adipocytes in the different green tea groups were significantly smaller and had more regular shapes compared with the model group.

Increasing evidence revealed that the accumulation of visceral adipose tissue was a strong predictor of the occurrence of NAFLD. In the case of similar body weight, people with a higher proportion of visceral fat were more prone to develop NAFLD (23, 24). In this study, the visceral adipose tissues in the model group markedly increased and the morphology of epididymal adipose tissue was abnormal mainly characterized by the expansive sizes and irregular shapes of adipocytes. These adverse changes were ameliorated by the intervention of green tea. Some studies also revealed the effect of green tea on inhibiting the accumulation of visceral fat. In a previous study, the visceral adipose mass of obese mice and lean littermates fed with diets containing 1 or 2% green tea extract was significantly lower than their respective controls fed with a green tea extract-free diet (25). In another study, an HFD significantly increased the weight of epididymal and perirenal adipose tissues, and the size of adipocytes in mice, while the dietary supplement Matcha prevented these unfavorable changes (26). Likewise,

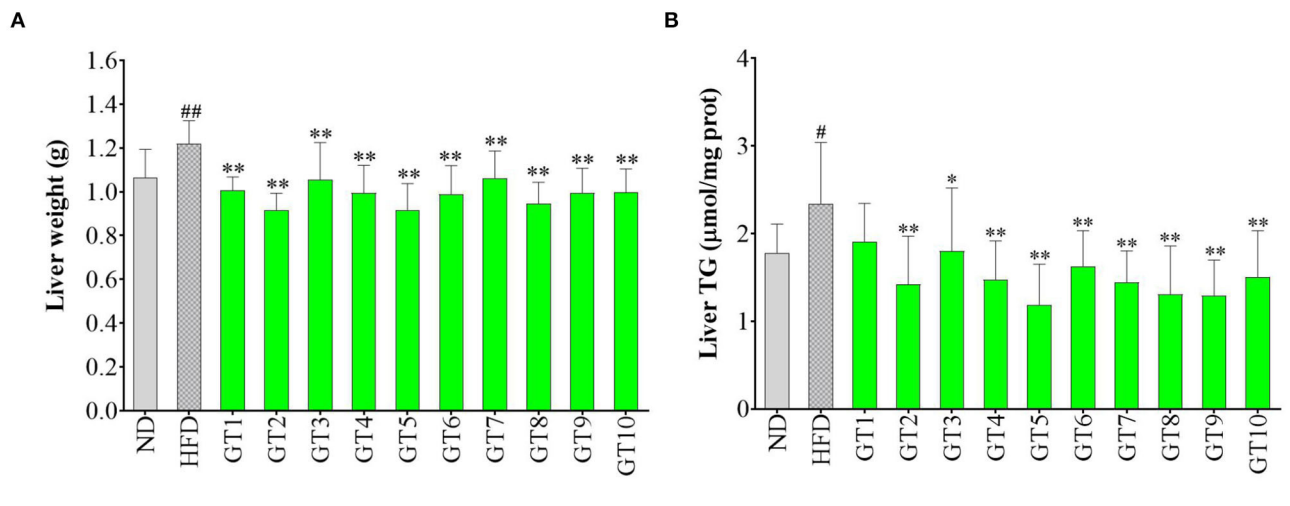


**FIGURE 4 |** Effects of green teas on weight and TG content of liver. **(A)** Liver weight and **(B)** Liver triglyceride (TG) content. # $p < 0.05$, ## $p < 0.01$, the model group vs. the control group; * $p < 0.05$, ** $p < 0.01$, the green tea groups vs. the model group. ND, normal diet; HFD, high-fat diet; GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

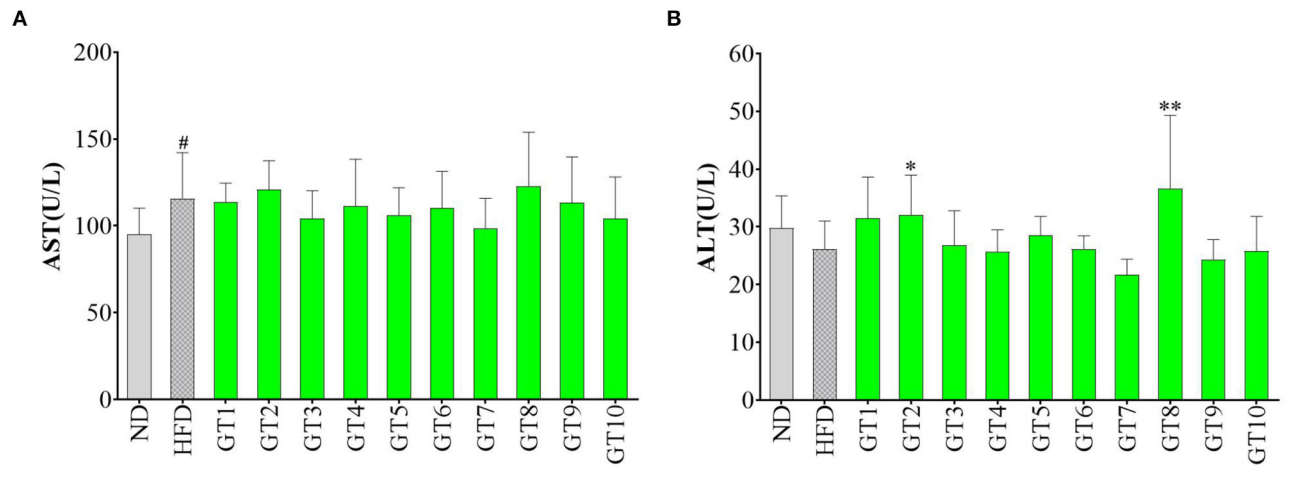


**FIGURE 5 |** Effects of green teas on liver injury. **(A)** Serum AST level and **(B)** Serum ALT level. # $p < 0.05$, the model group vs. the control group; * $p < 0.05$, ** $p < 0.01$, the green tea groups vs. the model group. AST, aspartate transaminase; ALT, alanine aminotransferase; ND, normal diet; HFD, high-fat diet; GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

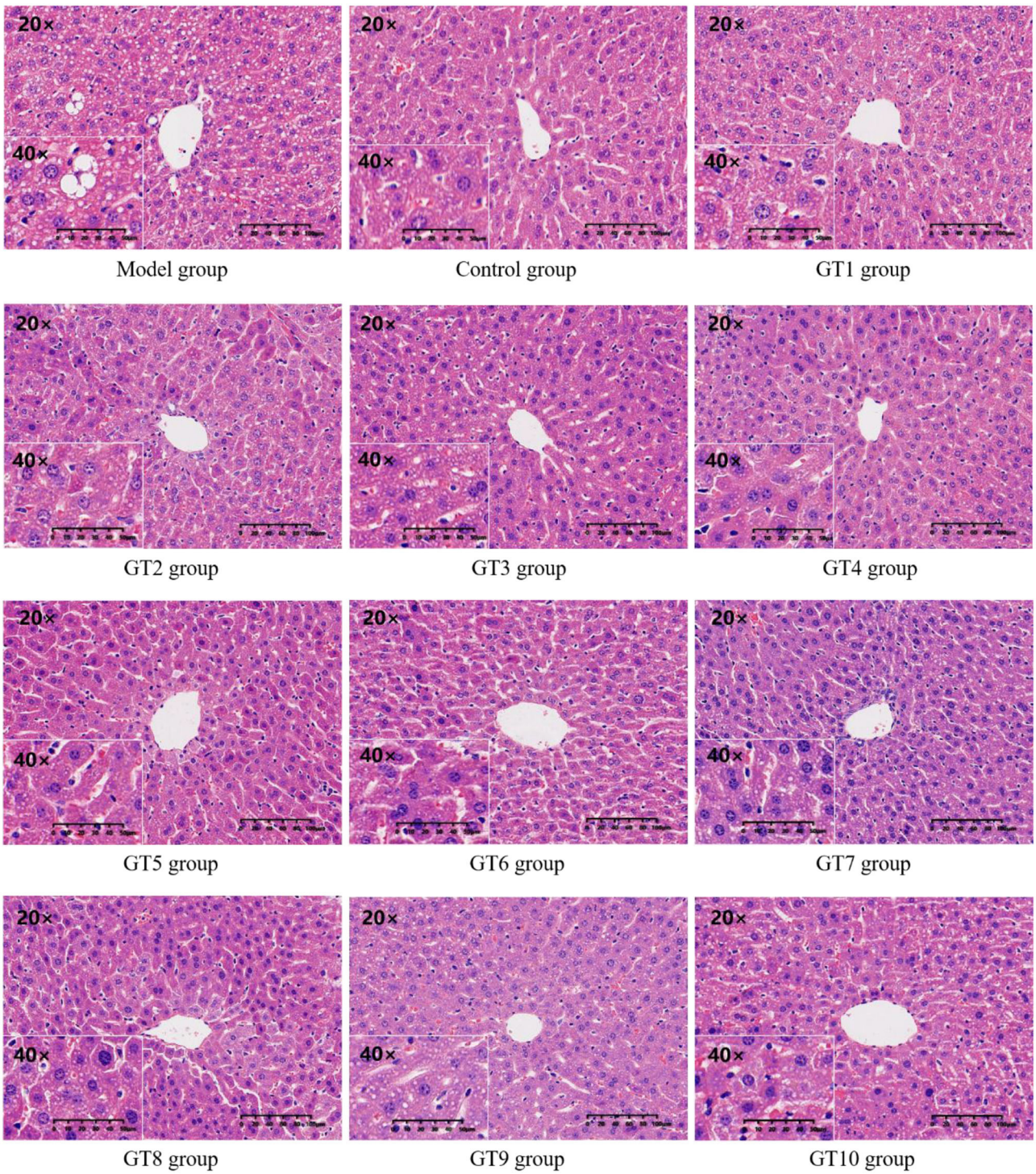


**FIGURE 6 |** The histopathological images of hepatic tissue (200 and 400X magnification). GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

Selenium-Enriched Matcha (GT9) and Seven Star Matcha (GT10) in this study could reduce the accumulation of adipose and the hypertrophy of adipocytes. In addition, we found that several other green teas had better effects on visceral adipose tissues than the Matcha teas, such as Fenggang Zinc-Selenium-Enriched Tea and Liping Xiang Tea.

## Effects of Green Teas on Hepatic Weight and Triglyceride Content of Liver

In this study, the hepatic weight and TG content of the liver in mice fed with HFD markedly increased compared with mice fed with a normal diet ($p < 0.05$) (**Figure 4**). All the 10 green teas could significantly decrease the hepatic weight ($p < 0.01$), while the effect varied in different teas (**Figure 4A**). Jieyang Chaoqing Tea (GT2), Taiping Houkui Tea (GT5), and Selenium-Enriched Chaoqing Green Tea (GT8) exerted a relatively notable effect on decreasing the hepatic weight. Accordingly, the majority of 10 green teas, except Dianqing Tea (GT1), could reduce the content of hepatic TG ($p < 0.05$) (**Figure 4B**). The most effective teas of decreasing TG content were Taiping Houkui Tea (GT5), followed by Selenium-Enriched Matcha (GT9) and Selenium-Enriched Chaoqing Green Tea (GT8).

The hepatic weight and TG content of liver were important predictors of hepatic lipid accumulation and the hepatic steatosis (25). The effects of green teas on hepatic weight and TG content in this study were in line with previous studies (16, 22, 27). For example, a study pointed out that the dietary supplement of 1% green tea extract decreased hepatic weight and TG content in obese mice (27).

## Effects of Green Tea on Aspartate Transaminase and Alanine Transaminase Levels

The liver injury was accessed by the detection of serum AST and ALT levels and results are shown in **Figure 5**. In comparison with the control group, although no significant increment of ALT level was observed in the model group ($p > 0.05$) (**Figure 5B**), AST level in serum was remarkably elevated ($p < 0.05$) (**Figure 5A**),

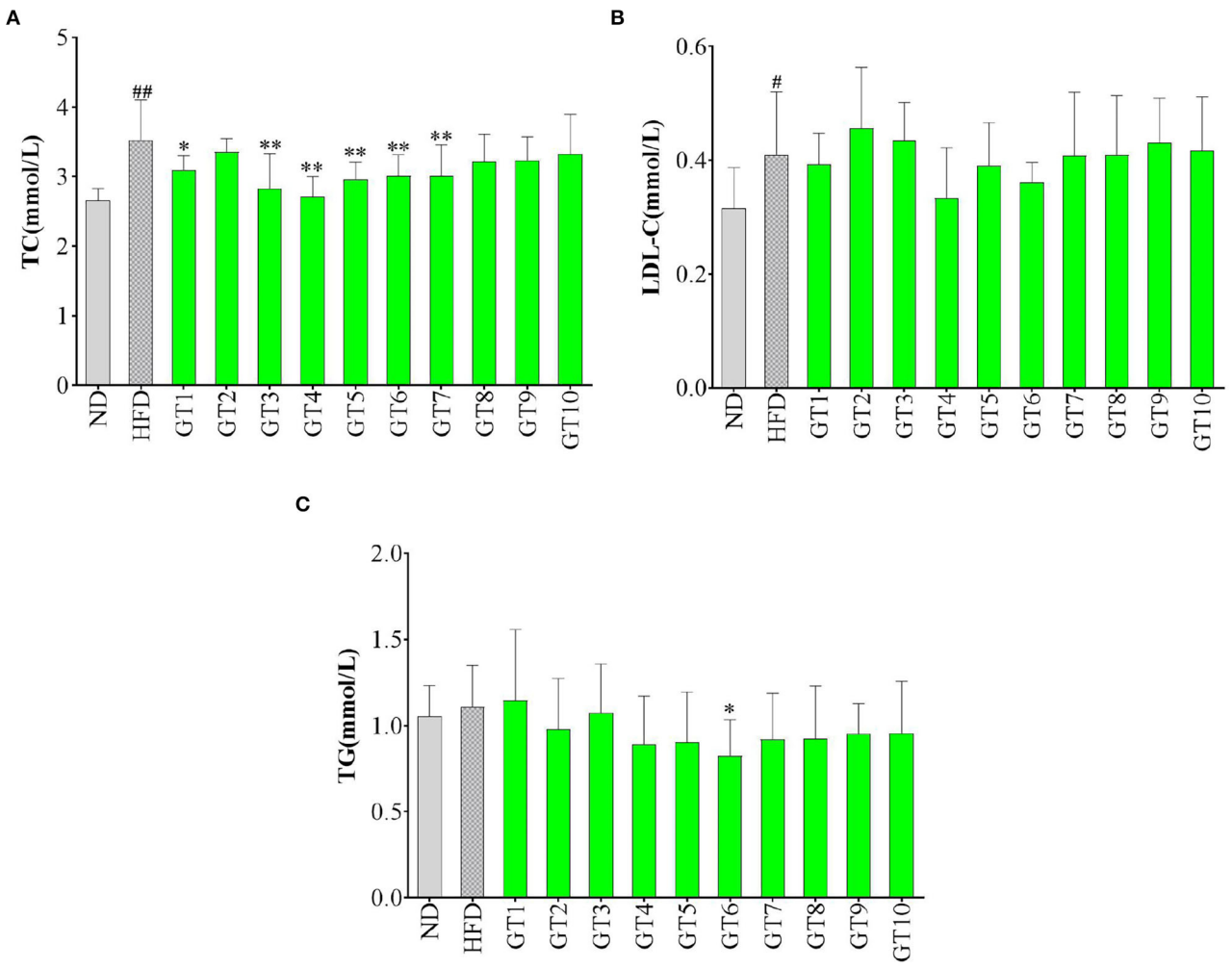


**FIGURE 7 |** Effects of green teas on serum lipid levels. **(A)** serum total cholesterol (TC) level; **(B)** serum low-density lipoprotein cholesterol (LDL-C) level; and **(C)** serum TG level. # $p < 0.05$, ## $p < 0.01$, the model group vs. the control group; * $p < 0.05$, ** $p < 0.01$, the green tea group vs. the model group. GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

hinting the existence of liver injury induced by HFD. No significant difference in AST and ALT levels was observed between most of the 10 green teas groups and model group, but a decreasing trend of AST and ALT levels was found in the Fenggang Zinc-Selenium-Enriched Tea (GT3) and Chaoqing Green Tea (GT7) groups ($p > 0.05$). On the other hand, Jieyang Chaoqing Tea (GT2) and Selenium-Enriched Chaoqing Green Tea (GT8) could further increase the ALT activity ($p < 0.05$), which hinted their possible damage to the hepatocytes in mice.

The hepatocytes were gradually destroyed by various factors induced by HFD, leading to the liver injury and NAFLD. The increase of serum ALT and AST activities was sensitive indicator in response to the liver injury. A previous study found that the oral gavage with green tea extract (500 mg/kg bw) for 12 weeks could prevent the increase of serum ALT and AST activities in male C57Bl/6 mice fed with HFD and protect against liver injury (16). Another study showed that green tea intervention reversed the increase of serum ALT activity induced by HFD (28). In this study, 10 green teas did not remarkably decrease the activities of these two transaminases, which could be because the dosage of intervention was smaller (200 mg/kg bw) than that in the literature (500 mg/kg bw) (16).

## Histopathological Evaluation of Liver

The H&E staining was performed to observe the histopathological changes of liver induced by a HFD and to further verify the effect of green teas on NAFLD. The results are given in **Figure 6**. Compared with the control group, many unequal size lipid droplets presented in the hepatocytes of the model group. Of note, lipid droplets in hepatocytes were significantly reduced by the intervention of green teas, particularly in Fenggang Zinc-Selenium-Enriched Tea (GT3), Liping Xiang Tea (GT4), Chaoqing Green Tea (GT7), and

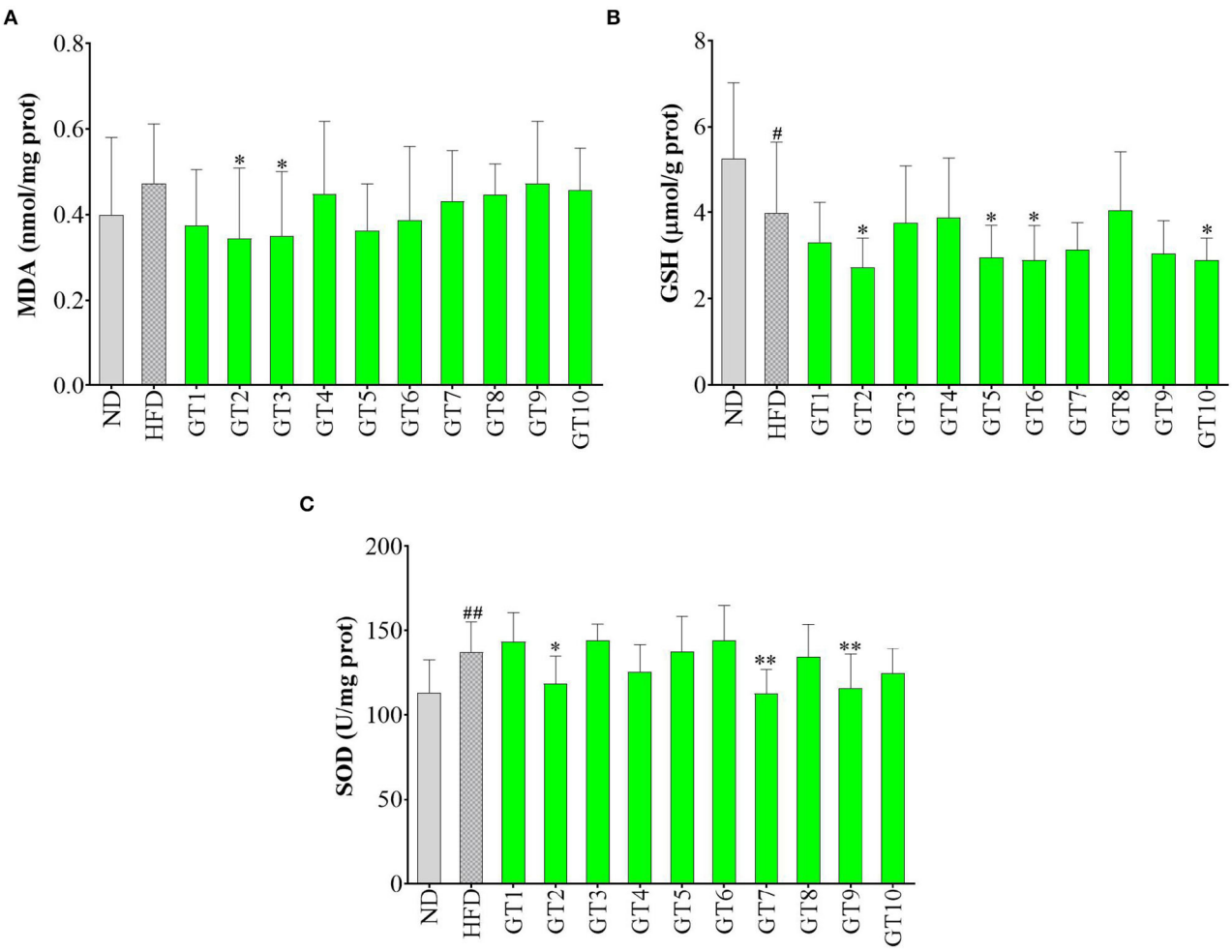


**FIGURE 8 |** Effects of green teas on hepatic oxidative stress. **(A)** Glutathione (GSH) (μmol/g protein); **(B)** superoxide dismutase (SOD) (U/mg protein); and **(C)** malondialdehyde (MDA) (nmol/mg protein). # $p < 0.05$, ## $p < 0.01$, the model group vs. the control group; * $p < 0.05$, ** $p < 0.01$, the green tea groups vs. the model group. GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha.

Selenium-Enriched Chaoqing Green Tea (GT8). The results were consistent with previous studies (16, 17, 20).

## Effects of Green Teas on Serum Lipid Levels

The serum TC, TG, and LDL-C levels have been used to evaluate the lipid levels in this study and the results are shown in **Figure 7**. Compared with the control group, mice fed with HFD had markedly higher levels of serum TC and LDL-C ($p < 0.05$), which reflected the presence of hyperlipidemia. The effects of 10 green teas on lipid levels varied greatly and some green teas showed prominent effectiveness in decreasing lipid levels. The 6 out of 10 green teas showed significant effect in lowering TC levels, namely, Liping Xiang Tea (GT4), Fenggang Zinc-Selenium-Enriched Tea (GT3), Taiping Houkui Tea (GT5), Xihu Longjing Tea (GT6), Chaoqing Green Tea (GT7), and Dianqing Tea (GT1) (**Figure 7A**). Although there was no significant difference existed in LDL-C level between the model group and the 10 green tea groups ($p > 0.05$), a decreasing tendency was observed in the Liping Xiang Tea (GT4) and Xihu Longjing Tea (GT6) groups (**Figure 7B**). On the other hand, TG content in mice fed with a HFD was not evidently elevated compared with that in mice fed with normal diet ($p > 0.05$). In addition, there was no significant difference in TG level between the model group and the majority of the 10 green tea groups ($p > 0.05$), except the Xihu Longjing Tea (GT6) group obviously decreased the level of TG ($p < 0.05$) (**Figure 7C**).

Lipid metabolism disorder is one of the most prominent manifestations of NAFLD, which could be presented as hyperlipidemia, hypertriglyceridemia, and hypercholesterolemia (29, 30). The TC, TG, and LDL-C contents in serum were the most commonly used indicators to reflect the lipid profiles (21, 31). Several studies showed that green tea could ameliorating the development of NFLDA *via* decreasing the contents of serum lipids and improving the lipid profile (20, 25, 27, 32). In this study, different green teas exerted different effects on lipid indicators. From a comprehensive perspective, Xihu Longjing Tea (GT6) and Liping Xiang Tea (GT4) could be the most efficient green teas to improve lipid profile.

## Effects of Green Teas on Hepatic Oxidative Stress

In this study, the levels of hepatic MDA, GSH, and SOD were detected to reflect the redox state in the liver and the results

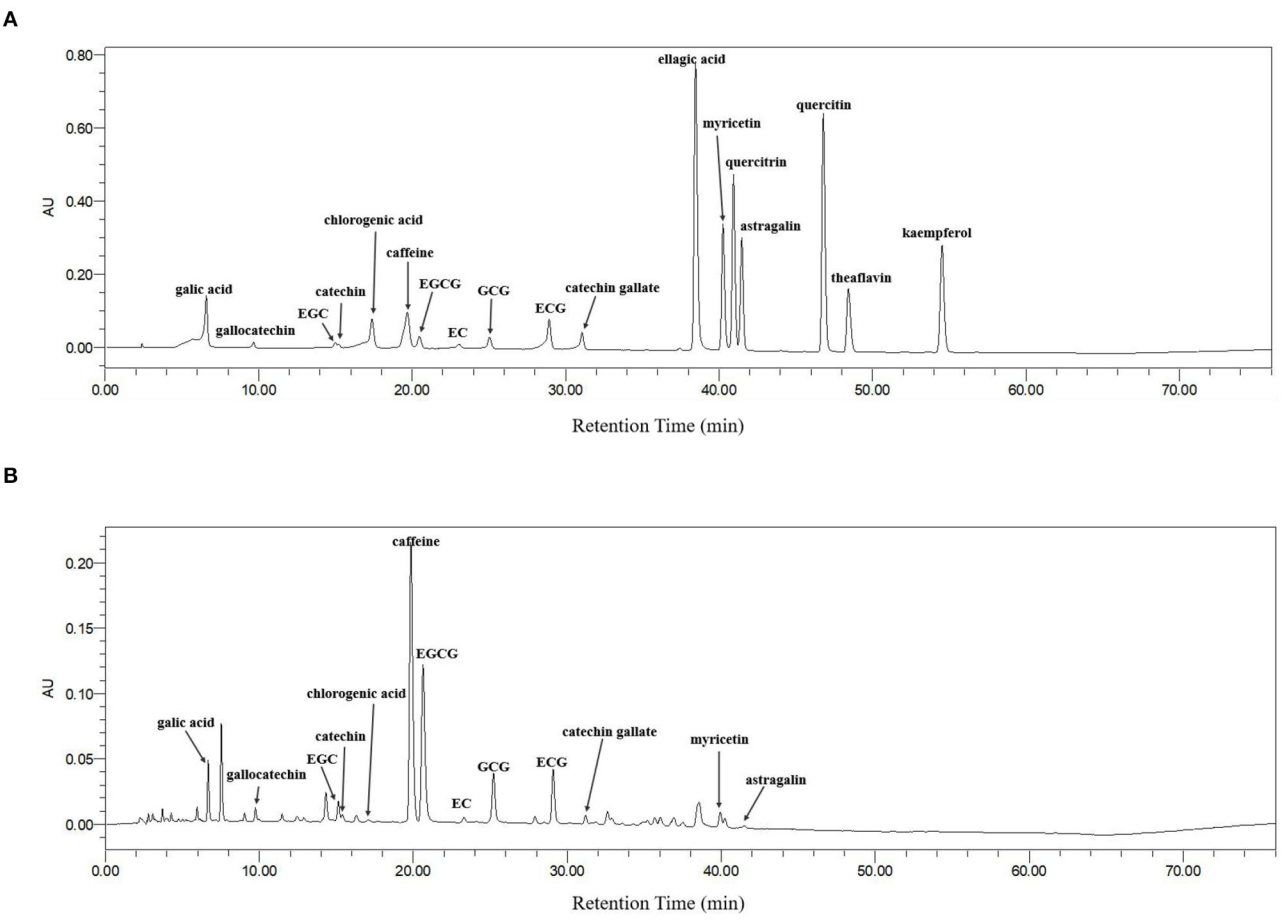


**FIGURE 9 |** The high-performance liquid chromatography (HPLC) chromatograms of the standard components **(A)** and Liping Xiang Tea **(B)** at 254 nm.

are shown in **Figure 8**. As shown in **Figure 8**, the model group showed a lower content of GSH and an increasing tendency of MDA content in contrast with the control group, indicating that oxidative stress occurred in the model group. In addition, the Jieyang Chaoqing Tea (GT2) and Fenggang Zinc-Selenium-Enriched Tea (GT3) groups showed a significant decrease of the MDA content compared with the model group ($p < 0.05$), suggesting their antioxidant activity (**Figure 8A**).

Oxidative stress is regarded as a crucial contributor to the occurrence and development of NAFLD. With the accumulation and oxidation of fatty acids in the liver, the antioxidants are consumed and excessive reactive oxygen species are produced, gradually destroying the structure and function of hepatocytes and eventually accelerating the process of NAFLD (33, 34). MDA is a representative product of lipid peroxidation (35). This study found that HFD led to an escalating trend of MDA level in mice, while some green teas, namely, Jieyang Chaoqing Tea (GT2) and Fenggang Zinc-Selenium-Enriched Tea (GT3), could significantly lower the MDA level, hence inhibiting the lipid peroxidation and oxidative stress. The results were consistent with some previous report. For example, in an ob/ob mice Non-alcoholic steatohepatitis model, diet supplementation with 0.5 and 1% green tea extract for 6 weeks inhibited the generation of ROS, along with the reduction of lipid peroxidation (36). SOD is an important antioxidative enzyme and GSH is a non-enzymatic antioxidants (10). The overgeneration of ROS caused the consumption of antioxidant substances, subsequently might led to the decrease of GSH content and SOD activity. However, in this study, several green teas could lower the levels of GSH and SOD, which was contrary to the expectation. This could be because the concentration of antioxidants in these teas was too high and they would show pro-oxidant activity in the body, just as vitamin C, which is a strong antioxidant *in vitro* and will be pro-oxidant under high concentration *in vivo* (37, 38).

## Bioactive Components in Green Tea Extracts

Bioactive components in green tea extracts were identified by HPLC *via* comparison with standard compounds. The chromatograms of the standard substances and Liping Xiang Tea

**TABLE 2 |** The contents of major phytochemicals in 10 green tea extracts (mg/g DW).

| Phytochemicals | GT1 | GT2 | GT3 | GT4 | GT5 |
|---|---|---|---|---|---|
| EGCG | 237.64 ± 2.77 | 262.32 ± 5.81 | 157.91 ± 5.00 | 248.37 ± 4.34 | 266.45 ± 7.40 |
| GCG | 50.24 ± 0.86 | 103.66 ± 1.52 | 40.15 ± 1.81 | 54.66 ± 0.96 | 77.75 ± 1.70 |
| ECG | 40.77 ± 0.43 | 16.83 ± 0.23 | 28.85 ± 0.70 | 24.02 ± 0.35 | 26.02 ± 0.29 |
| EGC | 31.22 ± 0.28 | 107.48 ± 3.49 | 37.87 ± 0.91 | 26.77 ± 0.43 | 40.99 ± 3.58 |
| Catechin | 34.68 ± 0.37 | - | 38.60 ± 2.15 | 17.31 ± 0.51 | 36.38 ± 0.52 |
| Epicatechin | 20.11 ± 1.03 | 30.07 ± 2.09 | 25.93 ± 0.96 | 14.48 ± 0.29 | 17.08 ± 1.05 |
| GC | 19.47 ± 1.40 | 95.36 ± 1.20 | 28.81 ± 0.59 | 18.93 ± 0.34 | 44.27 ± 3.59 |
| CG | 8.04 ± 0.27 | 5.21 ± 0.74 | 6.46 ± 0.60 | 6.13 ± 0.28 | 7.59 ± 0.44 |
| Caffeine | 78.11 ± 0.43 | 154.22 ± 3.00 | 106.46 ± 0.54 | 75.29 ± 1.23 | 107.33 ± 1.11 |
| Gallic acid | 8.40 ± 0.09 | 16.17 ± 0.77 | 11.52 ± 0.33 | 8.83 ± 0.40 | 12.27 ± 0.31 |
| CA | 5.18 ± 0.06 | - | 4.51 ± 0.04 | 1.69 ± 0.02 | - |
| Astragalin | - | 2.05 ± 0.21 | 5.22 ± 0.31 | 5.74 ± 0.18 | 3.16 ± 0.02 |
| Myricetin | 1.83 ± 0.06 | - | - | 4.17 ± 0.14 | - |
| **Phytochemicals** | **GT6** | **GT7** | **GT8** | **GT9** | **GT10** |
| EGCG | 232.93 ± 6.39 | 162.03 ± 3.75 | 196.69 ± 1.94 | 234.29 ± 13.30 | 201.48 ± 5.72 |
| GCG | 63.22 ± 1.34 | 38.56 ± 1.10 | 30.95 ± 1.19 | - | - |
| ECG | 15.19 ± 0.33 | 14.68 ± 0.30 | 9.20 ± 0.10 | 22.75 ± 0.91 | - |
| EGC | 43.65 ± 1.87 | 18.40 ± 0.26 | 86.84 ± 1.85 | 66.37 ± 1.60 | 46.08 ± 1.61 |
| Catechin | - | - | 16.52 ± 0.30 | - | 53.15 ± 4.53 |
| Epicatechin | 12.95 ± 0.41 | 11.03 ± 0.49 | 12.82 ± 0.20 | 19.24 ± 0.68 | 10.68 ± 0.65 |
| GC | 33.69 ± 0.39 | 16.44 ± 0.24 | 51.34 ± 0.23 | 41.31 ± 1.77 | - |
| CG | 3.96 ± 0.09 | 3.55 ± 0.17 | 2.08 ± 0.09 | - | - |
| Caffeine | 88.18 ± 0.17 | 56.98 ± 0.59 | 54.40 ± 0.28 | 110.22 ± 1.25 | 131.10 ± 0.92 |
| Gallic acid | 7.16 ± 0.24 | 9.49 ± 0.25 | 2.60 ± 0.11 | 19.57 ± 0.25 | 1.91 ± 0.18 |
| CA | 2.11 ± 0.02 | 1.66 ± 0.04 | - | 2.93 ± 0.01 | - |
| Astragalin | 2.65 ± 0.11 | - | 4.47 ± 0.14 | 9.62 ± 0.15 | - |
| Myricetin | 2.20 ± 0.15 | - | - | - | - |

*Abbreviations: –, not detected; DW, dry weight; GT1, Dianqing Tea; GT2, Jieyang Chaoqing Tea; GT3, Fenggang Zinc-Selenium-Enriched Tea; GT4, Liping Xiang Tea; GT5, Taiping Houkui Tea; GT6, Xihu Longjing Tea; GT7, Chaoqing Green Tea; GT8, Selenium-Enriched Chaoqing Green Tea; GT9, Selenium-Enriched Matcha; GT10, Seven Star Matcha; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; GC, gallocatechin; EGC, epigallocatechin; CA, chlorogenic acid.*

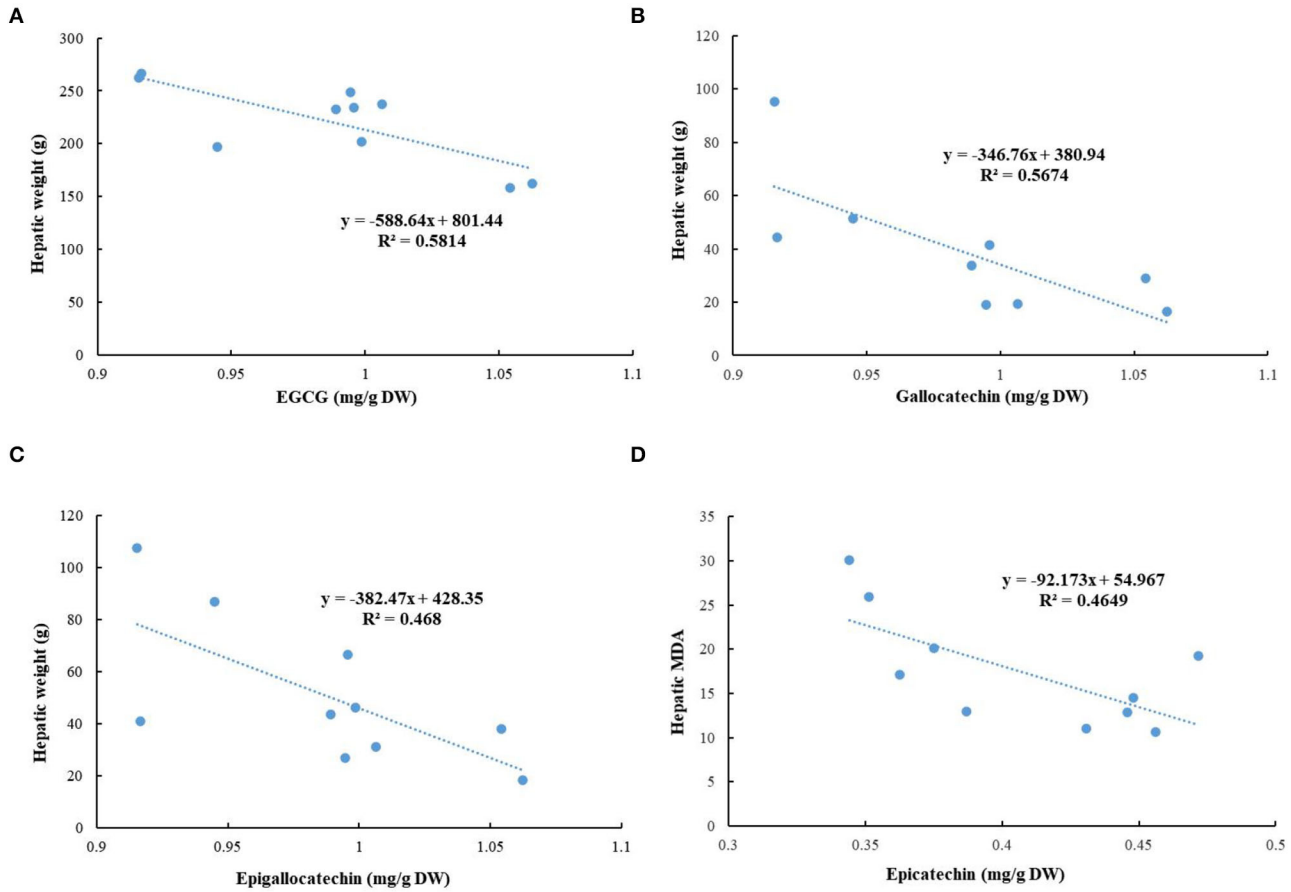


**FIGURE 10 |** Correlation analysis. **(A)** Epigallocatechin gallate (EGCG) and hepatic weight; **(B)** gallocatechin and hepatic weight; **(C)** epigallocatechin and hepatic weight; and **(D)** epicatechin and hepatic MDA.

are given in **Figure 9** and the contents of major phytochemicals in 10 green teas are shown in **Table 2**. In general, 8 types of catechins and 5 other active compounds (caffeine, gallic acid, chlorogenic acid, astragalin, and myricetin) were detected and quantified in green tea extracts (**Figure 9**). The results showed that EGCG, epigallocatechin (EGC), epicatechin, caffeine, and gallic acid could be detected in 10 green teas and most green teas contained gallocatechin gallate (GCG), epicatechin gallate (ECG), catechin, gallocatechin, catechin gallate, chlorogenic acid, and astragalin. Myricetin was only found in Dianqing Tea (GT1), Liping Xiang Tea (GT4), and Xihu Longjing Tea (GT6).

The contents of 13 phytochemicals in different green teas varied greatly (**Table 2**). EGCG was the most abundant catechin in green tea extracts (157.91 ± 5.00–266.45 ± 7.40 mg/g DW), followed by GCG (30.95 ± 1.19–103.66 ± 1.52 mg/g DW), EGC (18.40 ± 0.26–107.48 ± 3.49 mg/g DW), gallocatechin (16.44 ± 0.24–95.36 ± 1.20 mg/g DW), catechin (16.52 ± 0.30–53.15 ± 4.53 mg/g DW), ECG (9.20 ± 0.10–40.77 ± 0.43 mg/g DW), epicatechin (10.68 ± 0.65–30.07 ± 2.09 mg/g DW), and catechin gallate (2.08 ± 0.09–8.04 ± 0.27 mg/g DW). The 10 green teas had also a high content of caffeine (54.40 ± 0.28–154.22 ± 3.00 mg/g DW). However, the contents of gallic acid (1.91 ± 0.18–19.57 ± 0.25 mg/g DW), astragalin (2.05 ± 0.21–9.62 ± 0.15 mg/g DW), chlorogenic acid (1.66 ± 0.04–5.18 ± 0.06 mg/g DW), and myricetin (1.83 ± 0.06–4.17 ± 0.14 mg/g DW) were relatively low in green tea extracts.

The correlation analysis was conducted to evaluate the associations among detected phytochemicals in green tea extracts and the tested biochemical indicators in this study (**Figure 10**). The negative relationships were existed in the hepatic weight and contents of EGCG ($R^2 = 0.5814$), gallocatechin ($R^2 = 0.5674$), and epigallocatechin ($R^2 = 0.468$), hinting that these compounds might contribute to the decrease of hepatic weight. In addition, a negative association was observed between the content of epicatechin and hepatic MDA ($R^2 = 0.4649$), suggesting that epicatechin could reduce oxidative stress.

The rich bioactive ingredients in green tea conferred its powerful hepatoprotective and antiobesity effects, especially polyphenols. Catechins are the major phenolic compounds in green tea, such as EGCG, GCG, EGC, ECG, and catechin (39). As the most abundant catechins in green tea, EGCG was most well studied and some studies indicated the preventive properties of EGCG against obesity and NAFLD. An experimental study pointed out that the dietary supplementation with 0.4% EGCG

(w/w) for 14 weeks effectively prevented the development of NAFLD induced by a HFD in mice (21). Moreover, the coadministration of EGCG and caffeine exerted a more remarkable effect on NAFLD amelioration than their single use by suppression of body weight gain, and reduction of energy intake and white adipose tissue weight in mice (40). Therefore, green teas with high contents of EGCG and caffeine could be an excellent alternative for the prevention and management of obesity and NAFLD.

## CONCLUSION

The effects of 10 different green teas on obesity and NAFLD induced by a HFD were evaluated and compared in mice. Although all the 10 green teas showed antiobesity, their effects varied greatly. The Selenium-Enriched Chaoqing Green Tea and Jieyang Chaoqing Tea were the most effective teas in inhibiting body weight gain and the Fenggang Zinc-Selenium-Enriched Tea, Liping Xiang Tea, and Taiping Houkui Tea could markedly inhibit the increase of visceral adipose tissues. It was found that several green teas, such as Jieyang Chaoqing Tea, Taiping Houkui Tea, and Selenium-Enriched Chaoqing Green Tea, could effectively prevent the occurrence of NAFLD and underlying mechanisms were inhibiting body weight gain, reducing the accumulation of visceral fat, improving lipid profile and oxidative stress, and ameliorating hepatic steatosis. Furthermore, 13 phytochemicals in green tea extracts were separated and quantified by an HPLC method and the correlation analysis showed that EGCG, gallocatechin, and epigallocatechin could contribute to the decrease of hepatic weight and epicatechin could reduce oxidative stress. In conclusion, most of 10 green teas were first evaluated for their effects on obesity and NAFLD. Several green teas showed strong effects and could be developed into functional foods to prevent obesity and NAFLD. In addition, if the intervention dose (200 mg/kg bw) for mice in this study was transformed into that for persons, this dose could be obtained by daily drinking tea, indicating the people could prevent obesity and NAFLD by daily drinking some green teas. Therefore, the findings could also serve the public to select suitable tea for the prevention and management of obesity and NAFLD.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

All experimental procedures involving animals in this study have received the approval from the Ethics Committee in School of Public Health, Sun Yat-sen University (No. 2019–002; 28 February 2019).

## AUTHOR CONTRIBUTIONS

D-DZ, Q-QM, SL, R-YG, and H-BL: conceptualization. D-DZ, Q-QM, B-YL, ASa, S-YH, R-GX, ASh, ML, and H-YL: investigation. D-DZ: writing—original draft preparation. SL, R-YG, and H-BL: writing—reviewing and editing. H-BL: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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# Modulation effects of microorganisms on tea in fermentation

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Tea is a popular traditional drink and has been reported to exhibit various health-promoting effects because of its abundance of polyphenols. Among all the tea products, fermented tea accounts for the majority of tea consumption worldwide. Microbiota plays an important role in the fermentation of tea, which involves a series of reactions that modify the chemical constituents and thereby affect the flavor and bioactivities of tea. In the present review, the microorganisms involved in fermented tea and tea extracts in the recent studies were summarized and the modulation effects of microorganisms on tea in fermentation, including polyphenols composition and content, biological activities and sensory characteristics, were also critically reviewed. It is expected that the data summarized could provide some references for the development of microbial fermented tea drinks with specific nutrition and health benefits.

KEYWORDS

**tea beverage, microbial fermentation, tea polyphenols, biological activity, sensory characteristics**

## Introduction

Tea, one of the most popular beverages in the world, is generally made from the leaves of the *Camellia sinensis* plant through processing techniques such as curing, rolling, heaping, and drying. Tea contains various bioactive ingredients, such as polyphenols, polysaccharides, caffeine, minerals and other active substances (1). Previous studies have indicated that tea possessed antioxidant, hypoglycemic (2), antihypertensive, lipid-lowering (3), antibacterial, anti-cancer, and anti-obesity effects, which were mainly attributed to its abundant polyphenols (4).

With the rise of fermentation engineering, researchers applied microbes to tea. Unlike green tea, black tea and other teas that have not undergone microbial fermentation, microbial fermented tea has special sensory characteristics, including bright tea infusion, unique aroma, sweet, and smooth taste with low levels of bitterness

and astringency. Therefore, dark tea and kombucha, two kinds of microbial fermentation tea, are favored by consumers for their special taste and aroma (5, 6). Furthermore, tea has been fermented by microorganisms that are attracting increasing attention because of its various health benefits, including protection against hypertension and cardiovascular diseases (7, 8). At the same time, the content of its active ingredients will change (9–11). After fermentation, some molecules in tea that are not easy to be absorbed change from combined state to free state, which is conducive to the absorption of nutrients in tea and the exertion of its efficacy. Microbial fermentation is the key factor responsible for the formation of sensory attributes and the chemical components of tea.

The changes of chemical constituents and bioactivities of tea during microbial fermentation have been revealed in recent years, which were related to the bioconversion of active components by microorganisms. The purpose of this paper is to review the types of microbiota, changes of polyphenols content and composition, biological activity and sensory evaluation in tea after microbial fermentation, according to comprehensively understand the characteristics and current situation of microbial fermented tea, and look forward to its development prospects and research trends, which may provide a theoretical basis for the in-depth study of tea fermented by microorganisms.

## Typical types of microbial fermentation in tea

According to the type of microbiota, the microbial fermented tea can be divided into four types, including bacterial fermented tea, mold fermented tea, yeast fermented tea and edible and medicinal fungi fermented tea, as shown in Figure 1. After fermentation, the antibacterial activity and antioxidant activity of tea were enhanced due to the increase of phenolic substances. The reduction of caffeine after fermentation makes the tea taste better. In addition, after fermentation, the variety of aromatic substances increases, giving the tea a new aroma. As well as changes in the types and content of tea pigments, giving the tea soup has a clear color. Tea are fermented by microorganisms, a variety of active substances produced by microbial metabolism increased, thereby improving the overall quality of the tea.

### Bacteria fermentation in tea

In recent years, the research on using bacteria to ferment tea is mainly focused on kombucha. During the fermentation process of kombucha, changes in sugar and acid content give the tea a new taste, the production of aromatic substances increases the aroma of the tea, and changes in phenolic substances increase the antioxidant capacity of the tea.

Zhao et al. reported that *lactic acid bacteria* fermentation may be an effective method to improve the bioavailability of phenols and protect cells from oxidative stress (12). Furthermore, kombucha is fermented by *acetic acid bacteria* and yeast, during which the content of beneficial ingredients such as vitamin C and glucuronic acid increases (13). Simultaneously kombucha has significant changes in antioxidant potential, pH, acetic acid, alcohol and sugar contents, and beneficial ingredients such as organic acids, minerals and vitamins, amino acids and polyphenols can be produced in the fermentation process (14).

### Mold fermentation in tea

On account of tea polyphenols in tea have no inhibitory effect on mold, and some metabolites produced by mold will produce a series of reactions in tea, including degradation, oxidation, methylation, etc., which can improve the quality of tea. Therefore, when tea is fermented with mold, some researchers selected *Streptomyces*, *Aspergillus niger,* and *Mucor* to conduct fermentation studies (15).

The results showed that compared with fresh tea, the polyphenol content of tea fermented by *Streptomyces bacillaris* strain R9 and *Streptomyces cinereus* strain Y11 strains was higher, and the total phenol content was 32.9 ± 0.1 mg/mL and 31.9 ± 0.1 mg/mL, respectively, after 42 days of fermentation (16). In another set of experiments, the contents of polyphenols and purine alkaloids in solid state fermentation system of *Aspergillus niger* and *Aspergillus fumigatu* were experimentally studied, which provided a reliable basis for dynamic data description and metabolic pathway of tea polyphenols in fermented pu-erh tea (17). Similarly, when molds and yeasts were used in the fermentation of black and green teas, molds fermentation increased caffeine content, which are related to the methylation process and the increase of the premise substance of caffeine, while yeasts fermentation decreased caffeine content. It was found that *Aspergillus niger* had the best fermentation performance of the three molds in this study (18).

In addition, *Eurotium cristatum* was also used for tea fermentation in recent years. The bacteria secrete amylase and oxidase, which can catalyze the transformation of protein and starch in tea to monosaccharides, catalyze the oxidation of polyphenol compounds, and transform them into substances beneficial to human body, so as to improve and optimize tea taste and other characteristics. When the extract of raw dark tea was fermented by using *Eurotium cristatum*, the dry weight of mycelia increased by about 10 times after the fermentation, the mass concentrations of tea polyphenols, total protein and water extract were decreased, the concentrations of total flavonoids,

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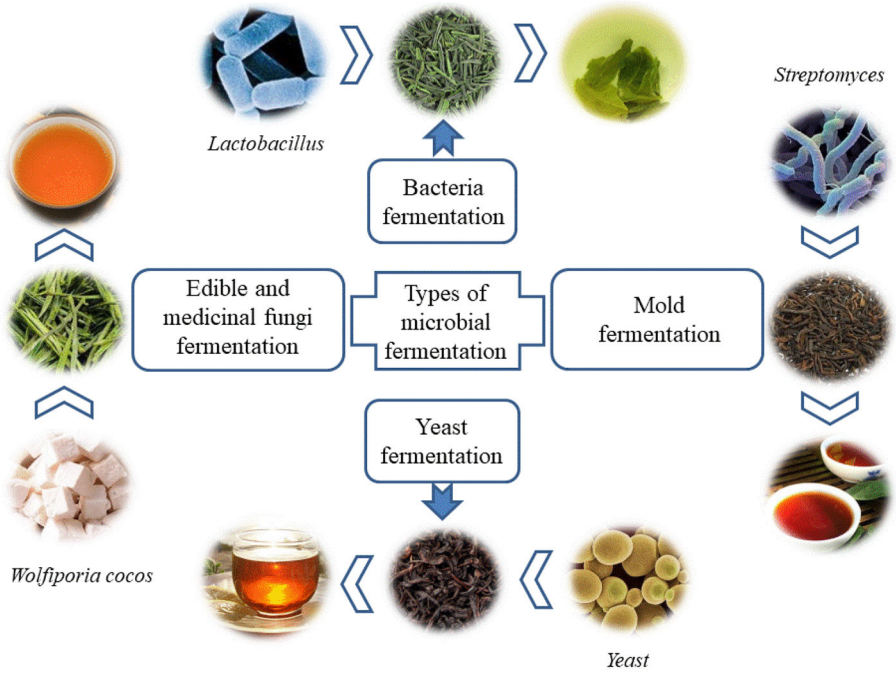


FIGURE 1
Typical types of microbial fermentation in tea.

free amino acids and theabrownin were increased, and 12 kinds of aromatic components were increased, most of which were esters and alcohols (19).

## Yeast fermentation in tea

Using yeast to ferment tea can not only improve the activity of tea fermentation, but also the complex biochemical reactions in the fermentation process will produce ethanol, acids and esters and other flavor substances to improve the sensory quality of the microbial fermentation of tea.

Studies have shown that the fermentation of black tea by *Dabaryomyces hansenii* results in the reduction of caffeine and a large amount of tannins, and improves its nutritional and medicinal value, so the ingestion of fermented tea is more advantageous than black tea (20). Additionally, when yeast strains were isolated from pu-erh tea and fermented raw tea samples of pu-erh tea, the study found that after yeast fermentation, the contents of tea polyphenols, theaflavins and catechins in tea were increased, while the contents of amino acids, caffeine, flavonoids, thearubigins and theabrownin were decreased. The contents of amino acids, catechins and caffeine affect the taste of tea, and the content of tea pigment determines the color of tea soup. The decrease of theanine content improved the bitter taste of tea, while the increase of theaflavins improved the color, aroma and taste of tea. Therefore, yeast has a great influence on the quality formation of pu-erh tea (21).

## Edible and medicinal fungi fermentation in tea

With the in-depth study of tea and modulation effects of microorganisms on tea beverage in fermentation process, many researchers also introduced edible and medicinal fungi for tea fermentation. With the fermentation of edible and medicinal fungi, the tea fermented by microorganisms goes through biochemical reaction to obtain aroma substances such as esters and alcohols. There were also changes in substances such as polyphenols and proteins. Improved the stale taste, sour taste and astringency of tea, and gave tea a new aroma and taste.

Rigling et al. used *Poria cocos* to adjust the smell of green tea. The study found that after immersion and fermentation for 17 h, due to the formation of methyl anthranilate, linalool, 2-phenylethanol and geraniol, *Poria cocos* changed the unique smell of green tea into jasmine flower and slightly citrus flavor. Meanwhile, the antioxidant activity of green tea is retained (22).

In addition, after the fermentation of Jinxuan oolong tea with medicinal mushrooms *Grifola frondosa* and Tianzhi (new variants of *Ganoderma lucidum*), the contents of polysaccharide, free amino acid and protein of the fermented tea were significantly increased, and the taste of the fermented tea was fresher and mellow. The contents of tea polyphenols, caffeine and water extract in the fermented products were significantly reduced, which reduced the turbidity of tea juice, reduced the bitterness, and gave it sweet taste and aroma (23).

## Changes of polyphenols content in tea after microbial fermentation

Tea polyphenols are the main components that determine the color, aroma, taste and efficacy of tea. They are classified as flavonoid (flavonols, flavanols, flavones, flavanones, isoflavones, and anthocyanins) and non-flavonoid molecules (phenolic acids, hydroxycinnamic acids, lignans, stilbenes, and tannins) (24). Tea polyphenols have anti-inflammatory, antiviral, antibacterial, hypolipidemic, hypoglycemic, weight loss and other effects. After microbial fermentation, the content of polyphenols changes in the tea (25–28).

### Changes of flavonoid content in tea

In recent years, researches on flavonoids polyphenols in tea mainly focus on flavanols, flavonols, and flavones. This section will review the changes of flavonoid content of tea before and after microbial fermentation.

Catechins are synthesized from sugars through shikimic acid pathway through the action of a series of enzymes to form benzene ring compounds. Catechins are typical flavanols, accounting for about 18 to 36% of the dry weight of tea (29). As shown in Figure 2, there are four important structures of catechins, namely (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (-)-Epicallocatechin (EGC) and (-)-Epicatechin (EC). In the process of tea fermentation, the content of catechin varies with the degree of tea fermentation, fermentation time, fermentation temperature and other factors (30). In another study, Qin et al. using the method of quantitative analysis and high performance liquid chromatography to study the change of tea polyphenols content of pu-erh tea in solid-state fermentation system, it was found that the content of ester-catechins increased slightly at the initial stage of fermentation, and then the ester-catechins gradually degraded to produce catechin and gallic acid. In the initial stage (0 to 8 h), the content of EGCG increased slightly, from 23.392 and 23.431 mg/g to 24.983 and 24.897 mg/g (17). According to another set of study, used the dominant strain of qingzhuan brick tea to conduct solid fermentation of qingzhuan brick tea, and after 6 days of fermentation, the catechin content decreased by 27.6% under the action of *Aspergillus fumigatus* M1 (25). The content of catechins in most teas decreases during fermentation, possibly due to the breakdown of catechins into substances such as theaflavins during fermentation (31).

The main flavonols and flavones in tea include kaempferol, quercetin, myricetin, and apigenin. Wang et al. identified flavonoid glycoside (quercetin-3,4'-O-di-β-glucoside, quercetin 3-O-galactosyl rutin, myricetin 3-galactoside, luteolin 6-C-glucoside, vitexin (apigenin-8-C-glucoside), kathinol 7-O-glucoside) in green tea extracts fermented by *Lactiplantibacillus plantarum* 299 V significantly were decreased, which may be related to the absorption and utilization of flavonoids by *lactic acid bacteria* cell wall (32). Seven kinds of tea fungis were used to ferment the sun-dried green tea, which promoted to the accumulation of kahenol and myricetin. It was found that the antioxidant activity of tea increased after fermentation, and the positive correction of gallic acid and kamanol to the antioxidant activity of fermented tea was observed (33). In addition, Ma et al. found that *Aspergillus palladium* PT-3 and *Aspergillus sesamae* PT-4, two tea fungis, could promote the biosynthesis of various flavonoids such as neferol, quercetin, and myricetin in the metabolic process of phenolic compounds, thus increasing the content of flavonoids in tea during fermentation (34). Furthermore, other researchers have used bacterial strains to ferment black tea to make drinks. For example, when *Starmerella davenportii* strain Do18 was used to fermented black tea extract, the research results showed that the flavonoid content of fermented tea drinks was higher, and the total flavonoid content of fermented drinks was significantly higher than that of unfermented samples, and reached the highest level after 36 h of fermentation (35).

Moreover, changes in acids in the fermentation environment also lead to the release of bound flavonoids (36–38). However, in the study of inoculated fermented tea, the flavonoid content of fermented tea was higher than that of unfermented tea.

### Changes of non-flavonoid molecules content in tea

Phenolic acids in tea are secondary metabolites of aromatic substances and non-flavonoids, which have many biological characteristics. The phenolic acids in tea mainly include gallic acid, chlorogenic acid, salicylic acid, vanillic acid and so on. During the fermentation of tea, the content of phenolic acid will vary with the degree of fermentation (39, 40).

Gallic acid is one of the important active ingredients in tea. Fermentation of tea will affect the content of gallic acid in tea. When studying the fermentation of loose tea by *Eurotium cristatum* at different temperatures, it was found

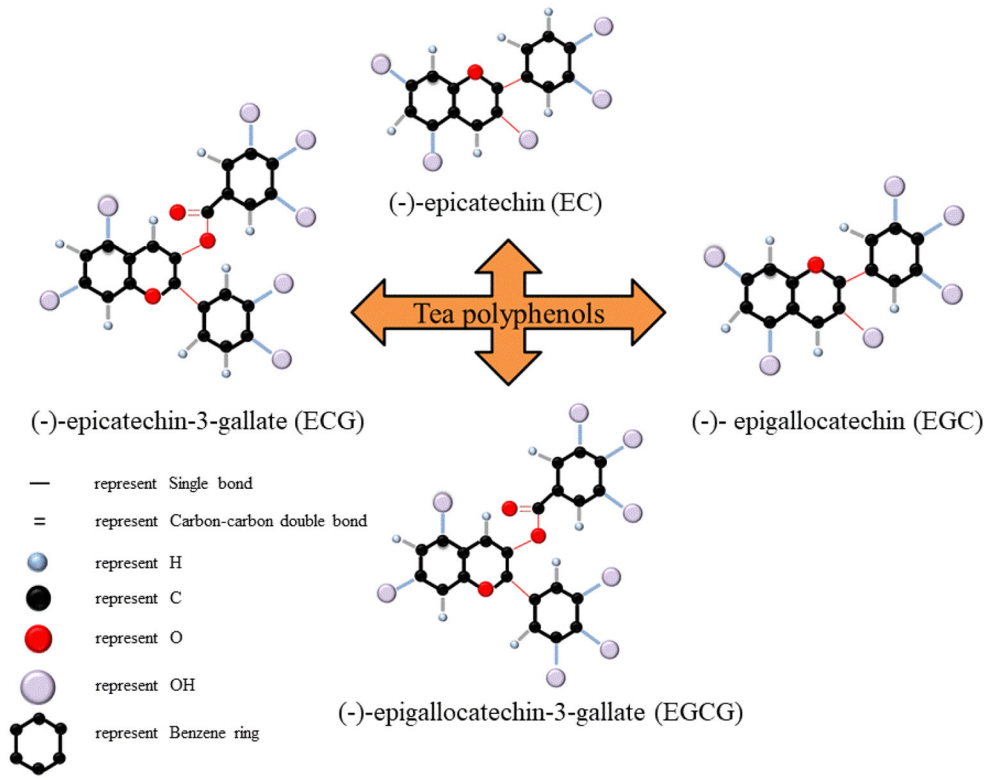


FIGURE 2
The main structure of tea polyphenols.

that appropriately increasing the fermentation temperature was beneficial to increase the content of gallic acid in fu brick tea (41).

Liu et al. used *Aspergillus Niger* to ferment tea. It was found that tannase was involved in the metabolism of gallic acid during the fermentation of pu-erh tea, which increased the content of gallic acid during the fermentation (42). However, Gallic acid showed a decreasing trend in the early fermentation stage of dark tea fermented by *M. coronoid* (43). In addition, in the fermentation process of black tea extract, with the increase of fermentation time, it is not conducive to the accumulation of phenolic acids such as gallic acid and cinnamic acid (44). In the fermentation process of tea, the change of phenolic acid content should be analyzed in combination with the specific situation (45).

## Changes of biological activity in tea after microbial fermentation

Tea has biological activities such as antibacterial, antioxidant, hypoglycemic, weight loss, anticancer and so on (46–48). With the fermentation of tea, its active function will change. The antibacterial, anti-oxidant, hypoglycemic, anti-lipid, anti-inflammatory, anti-toxin and anti-cancer activities of microbial fermented tea can be applied to the food, medicine and cosmetics industry and have a good development prospect (49–52). In this section, the antibacterial, antioxidant, hypoglycemic, lipid-lowering and other activities of tea fermented by microorganism would be elaborated.

### Changes in antibacterial activity

With the deepening of fermentation, the metabolites of some fungi in tea have the function of inhibiting intestinal microorganisms (53, 54). Comparing the inhibitory effects of black tea extracts before fermentation and black tea extracts after fermentation on *Escherichia coli*, the research results showed that among the three different concentrations of non-fermented black tea extracts, only the tea extract at a concentration of 25 mg/mL can inhibit *Escherichia coli*. The fermented black tea extracts at concentrations of 5, 10, and 25 mg/mL can significantly inhibit the growth of *Escherichia coli*. Figure 3 shows the damage of 25 mg/ml fermented black tea extract to *Escherichia coli* (55). When studying unfermented black tea

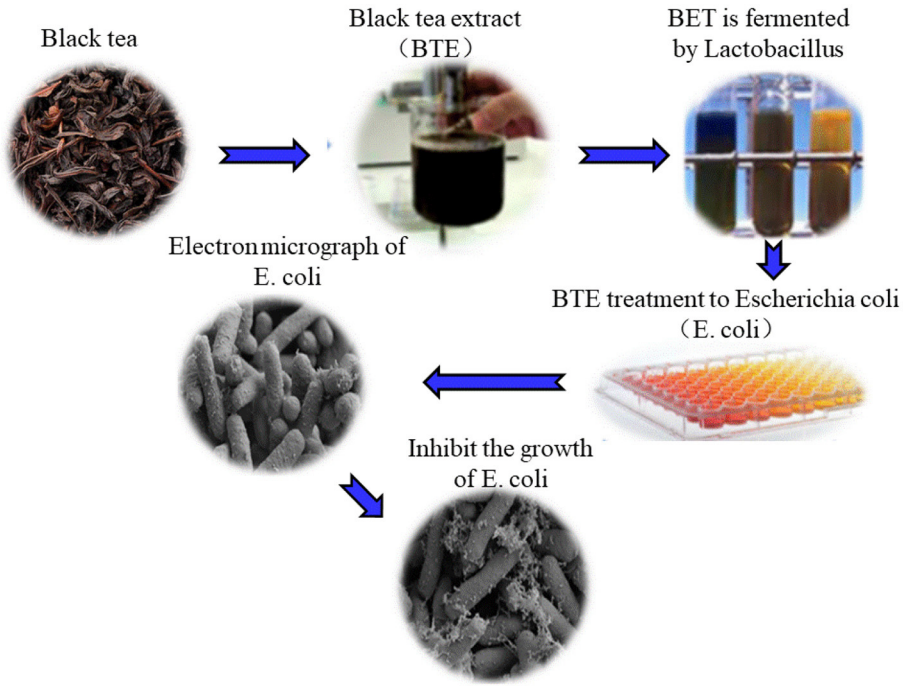


FIGURE 3
The inhibitory effect of fermented black tea extract on *Escherichia coli*. The cell membrane and cell division of *Escherichia coli* were damaged by 25 mg/ml fermented tea extract.

extracts and fermented black tea extracts, it was found that as the fermentation time increased, the antibacterial effect of black tea extracts increased. The results showed that under the condition of PH 7.0, black tea extracts had no inhibitory effect on *S. typhimurium* when it was not fermented; when fermented for 14 days, the inhibition of *S. typhimurium* by fermented tea could reach 30–35 mm in diameter; Unfermented black tea extracts has no inhibitory effect on *Escherichia coli*, and after 14 days of fermentation, the diameter of the inhibitory zone of fermented tea on *Escherichia coli* can reach 30–35 mm; when fermented for 0–4 days, fermented tea has no inhibitory effect on *Candida albicans*. When fermented for 4–14 days, the inhibition halo diameter of fermented tea on *Candida albicans* reaches 10–15 mm (56). Studies have found that the fermented fu brick tea contains a class of triterpenoids with 6-hydroxy-7-one function. The antimicrobial activity of compound enteric pathogenic *Escherichia coli*, *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae*, and *Salmonella typhi* was evaluated by plate diffusion. The test results show that the compound has weak antibacterial activity against enteropathogenic *Escherichia coli* (EPEC) and *Salmonella typhi*. The microbial fermented tea has a certain inhibitory effect on harmful bacteria, and the inhibitory ability may be related to the fermentation time, pH and other factors of the microorganisms (57).

In addition, black tea extracts fermented by *acetic acid bacteria* and yeast showed obvious inhibitory effects on *Staphylococcus aureus* ATCC6538 (*S. aureus*) and *E. coli* ATCC11229 (*E. coli*) (58), which suggest that fermented tea might be a potential source of preservatives. The antimicrobial activity of fu-brick tea after fermentation by *bursa corundum* is obviously improved compared with that without fermentation. When the concentration of fermented tea extract was 5 mg/mL or less, the growth of intestinal pathogenic *bacteria Shigella* and *Staphylococcus aureus* could be reduced by 50%, and the minimum inhibitory concentration of fermented tea extract against *Staphylococcus aureus* was 0.625 mg/mL (59). After microbial fermentation, the antibacterial activity of tea was improved, and its antibacterial effect on *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* and other pathogenic bacteria had improved significantly.

## Changes in antioxidant activity

During the fermentation of tea, the antioxidant capacity of tea will also change with the changes in content of secondary metabolites (12, 60, 61). A single strain isolated from pu-erh tea was used to inoculate fresh tea to study its fermentation effect. The results of the study showed that fresh tea was inoculated

with these strains, and the antioxidant capacity was significantly enhanced after 42 days of fermentation. Among them, the polyphenol content of tea inoculated with *Streptomyces bacillus* strain R9 was 3.3 mg/100 g, and the scavenging ability of DPPH free radicals reached 92%, the total polyphenol content was the highest, and the antioxidant capacity was the strongest (11). Fermentation of fu brick tea using *Eurotium cristatum* has found that fermented tea at 28 and 37°C has strong antioxidant capacity (26). Compared with unfermented tea, fermented tea has better antioxidant properties, and fermentation temperature has a significant impact on the antioxidant properties of fermented tea. Furthermore, using kombucha to ferment black tea, and study the changes in its antioxidant activity with fermentation time, the results of the study found that as the fermentation time increases, the antioxidant activity of fermented tea increases. After 8 days of fermentation, the antioxidant activity of fermented tea can reach up to 89.69% (62). And the DPPH scavenging ability of green tea reached 94.38% after fermentation of GABA-producing *lactic acid bacteria* for 5 days (63).

Combined with the above research, when studying the changes of antioxidant activity of microbial fermented tea, we can improve the antioxidant activity of tea through microbial fermentation, so as to provide a theoretical basis for the research and development of antioxidant functional food and the food industry.

## Changes in hypoglycemic activity

In recent years, many researchers have used animal experiments to study the biological activity of tea and found that after microbial fermentation, it is demonstrated that the activity of tea to reduce blood glucose has been improved (64–66).

The regulation effect of fermented tea on glucose metabolism is mainly related to the metabolites of fermented tea. For example, the relative contents of 10 kinds of polyphenol metabolites (4 kinds of fatty acids, 1 kind of artemisylline derivative, 3 kinds of lysophosphatidylcholine and 2 kinds of triterpenoids) increased while the relative contents of the other 5 kinds of polyphenol metabolites decreased after the fermentation of dark tea by *Cystis canopetiformis*. These metabolites are related to the hypoglycemic activity of fermented tea (67). It was found that the kombucha has obvious therapeutic effect to diabetic rats after fermentation. The findings from the histopathological analyses revealed that those of the alloxan-induced diabetic rats showed clear atrophy of β-Cells. The pancreas of the diabetic rats that were treated with fermented black tea, on the other hand, noted to undergo a marked amelioration. Mainly because after fermentation of tea extract on plasma and alpha amylase and lipase activity in pancreas have better inhibitory effect, at the same time improve the pancreas of diabetic mice structure, have better inhibitory effect on blood sugar levels rise, as shown in Figure 4 (68). The green tea extracts fermented by *acetic acid bacteria*, *yeast* and *lactic acid bacteria* can improve the intestinal flora of mice. The improvement of intestinal flora reduces the damage of intestinal barrier, thus reducing lipopolysaccharide replacement and inhibiting the occurrence of insulin resistance *in vivo*. In addition, increasing the number of SCFAs-producing bacteria can increase the number of SCFAs, improve the function of islet β cells, and reduce blood glucose by promoting the secretion of gastrointestinal hormones (69).

## Changes in lipid-lowering activity

At present, research on the lipid-lowering activity of microbial fermented tea is mainly focused on lowering blood lipids and weight loss (70, 71). For example, the fu brick tea was fermented by *Eurotium cristatum*, and the water extract of the fermented fu brick tea was fed to high-fat zebrafish. The results showed that when the concentration was 500 μg/mL, the lipid level of the zebrafish was compared with the control group, it decreased by 51.49%. The water extract of fermented *Eurotium cristatum* showed effective lipid-lowering activity on high-fat zebrafish (72). In addition, *Lactobacillus paracasei subsp* was used to fermentation *Houttuynia cordata* leaf tea and green tea, and the anti-obesity activity of fermented tea was studied through in *vivo* and in *vitro* experiments. Wang et al. demonstrated that the fermented tea contained epigallocatechin gallate, epigallocatechin, and chlorogenic acid, which inhibit lipogenesis in mature 3T3-L1 adipocytes by stimulating adipose decompose (73). Qin et al. fermented Anhua black tea with *Monascus* and *Cystaphylococcus coronatum*, resulting in many active substances in the fermented products, such as lovastatin, which had certain lipids lowering effects (74). Therefore, long-term drinking of microbial fermentation tea has a good role in medical care.

## Changes in other biological activities

The tea was fermented by microorganisms has antibacterial, antioxidant, hypoglycemic, lipid-lowering, anti-inflammatory and anti-cancer activities (75, 76). Zhang et al. found that lovastatin produced by monascus fermentation of pu-erh tea can induce neutrophil apoptosis by phosphorylation of ERK/AKT and reduce neutrophil recruitment to inflammatory sites, thereby reducing inflammation in zebrafish (77). In addition, Assam tea after fungal fermentation, compared with unfermented tea, microbial biological conversion of phenol makes total polyphenol, total tannin content in the fermented tea enhancement and enrichment of condensed tannins, thus as a target, the function of the bioactive ingredients in anti-inflammation mediated diseases such as cancer and

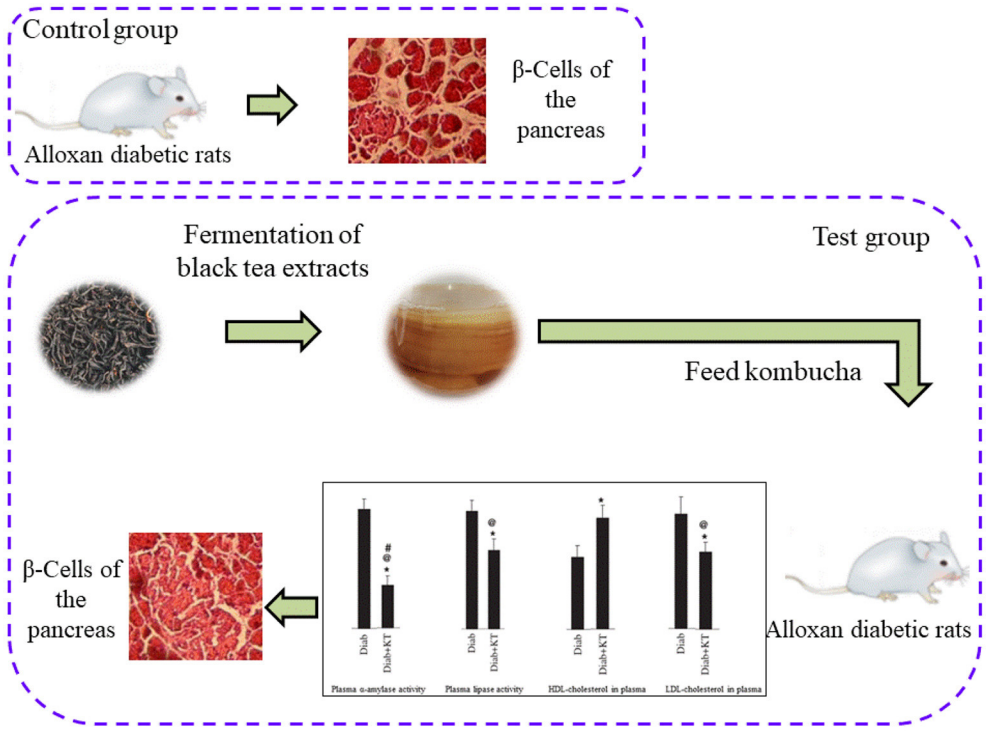


FIGURE 4
Effect of fermented black tea extract on β-cells of the Pancreas in Alloxan diabetic rats.

cardiovascular disease found in the corresponding application (78). Moreover, Villarreal-soto et al. studied the bioactivity of extracts from black tea fermented by kombucha bacteria. After 21 days of fermentation, the anti-inflammatory bioactivity was enhanced, with $IC_{50}$ values up to 9.0 μg/mL (79). Li et al. used *Nigrospora sphaerica* HCH285 to ferment the sun-dried green tea, so as to develop a kind of melanospora fermented tea. With the increase of fermentation time, after 45 days of fermentation, the content of bostrycin in the fermented tea reached 3.18 g/kg, and the content of bostrycin was the highest in the whole fermentation process. Bostrycin has good anticancer activity, so the anticancer activity of green tea is enhanced in the fermentation process (80). Tea are fermented by microorganisms, most of the biological activity of tea will be enhanced.

## Changes of sensory evaluation in tea after microbial fermentation

Not only there is a change in the content of tea polyphenols and bioactive functional, the sensory properties of tea changed after fermentation (81, 82). For example, the change of tea taste and the color and brightness of tea juice are mainly caused by the change of amino acid content in the fermented tea and the formation of theaflavins and thearubigen (83, 84).

## Change in taste

Compared with unfermented tea, the taste of tea is enhanced by microbial fermentation. This is because, after tea fermentation, the protein in tea is decomposed into free amino acids, increasing the flavor of tea. At the same time, catechins were degraded and the bitter taste of tea beverage was reduced. Besides, sugar content increased and pH value decreased, making fermented tea beverage taste sour and sweet.

*Laphet*, a fermented tea from Myanmar, has a bitter taste in the raw tea without fermentation, but the bitter taste of the tea is reduced after fermentation (85). Nishioka et al. used *Lactobacillus* to ferment Awaban tea and found that the free amino acids of fermented tea contained large amounts of theine and glutamate, which enhanced the umami taste of tea (86). At the same time, the quality of Awaban tea is improved by some other ingredients produced by lactic acid bacteria. In addition, Zhao et al. used tea bacteria to discover the extract of pu-erh tea and studied the flavor of tea in the fermentation process. It was found that the total amount of free amino acids increased slightly with the increase of fermentation time, and these free amino

acids, as the most important aromatic precursors of fermented tea, could significantly improve the flavor quality of fermented tea. At the same time, due to the presence of *acetobacter*, the PH of fermentation liquid is reduced, tea polyphenols are oxidized, catechins are degraded, and bitterness is reduced, and fermented tea is endowed with sweet and sour taste (87). Moreover, it was found that the sensory properties of black brick tea were changed during fermentation by liquid chromatography-mass spectrometry. Based on liquid chromatography-mass spectrometry of metabonomics analysis revealed that microbial fermentation of the tea samples and microbial fermentation before samples have significant differences, a total of 102 compounds were identified as the key to lead to metabolic changes and green tea processing metabolites, catechins content decreased significantly, and form new phenolic acids and catechins derivatives. The sensory quality of the green brick tea is mainly formed in the process of microbial fermentation, which greatly reduced the astringency and bitterness of raw tea and produced its characteristic woody and stale aroma as well as mellow taste (88). When the dark tea was fermented by *Aspergillus Niger,* the bitterness and astringency of tea were reduced significantly due to the catechin content was decreased in the fermentation process. In addition, bitterness and umami taste were also changed due to amino acids were changed in tea (89). After the fermentation of bacteria, the pH of black tea decreases at the initial stage of fermentation, during which sucrose is hydrolyzed and lactic acid content increases, and tea is endowed with a sour and sweet taste (5).

## Change in aroma

Tea has been fermented by microorganisms. Aroma substances in tea are produced, including alcohols and aldehydes. Besides enzymatic and non-enzymatic reactions occur in tea, and the hydrolysis of some substances, such as glycoside hydrolysis, has a great influence on the formation of tea aroma (90–92).

For example, in the post-fermentation process of dark tea, ketones are formed. These ketones are very important flavor compounds with special floral and woody odors. Therefore, the fermented dark tea has a special fragrance (93). In the fermentation process of pu-erh tea extract, the aroma of tea becomes weaker under the action of microorganisms, while the compositions of fermented tea such as camalool, phenylacetaldehyde, heptanaldehyde and 2, 4-dimethylbenzaldehyde are produced, which makes the tea fermentation liquid have the fruit flavor (60). In addition, the study used four non-Saccharomyces cerevisiae strains to ferment green tea slurry, and the proper fermentation of these four strains changed the characteristics of aroma compounds, so that the aroma of fermented green tea changed. In the microbial fermentation process of tea, the aroma composition

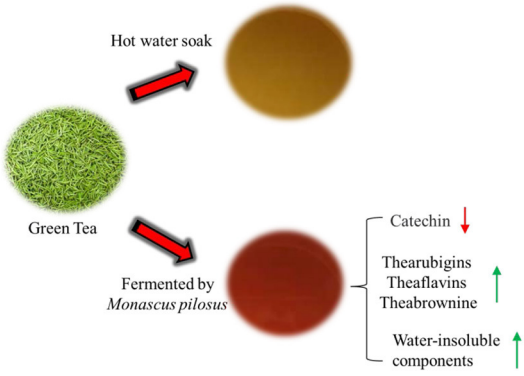


FIGURE 5
The color change of tea soup before and after fermentation of Green Tea.

and aroma will change benignly, which makes the sensory sense of tea more abundant (94). After the fermentation of green tea with four kinds of mixed bacteria, a fruity ethyl ester was produced, which increases the content of aroma compounds such as methyl salicylate, geraniol and 2-phenylethanol, giving tea a special aroma (32). Furthermore, the presence of D-limonene (27.71%) and β-cinnamene (11.55%) in fu brick tea was fermented by *Eurotium cristatum* gives the tea a fruity and special aroma compared with the unfermented green tea (72). Besides, *Cyberlindnera aturnus var. mrakii* NCYC 2251 was used to ferment green tea pulp. With the extension of fermentation time, glycosylated aroma substances such as methyl salicylate, benzyl alcohol and 2-phenylethanol increased (95).

## Change in color

After the tea was fermented, the taste and aroma would be changed. What's more, the color of tea will also be changed. Under the action of microbial fermentation, the content and type of tea pigment in tea change, and then change the color of tea soup. Therefore, the color of tea beverage will become bright, and the tea soup will be improved after microbial fermentation.

For example, the brown pigment content affects the color of black tea. After fermentation with *Aspergillus fumigata M1*, the brown pigment content of pu-erh tea extracts increases by 110.6%, which improves the color of pu-erh tea (25). Kim et al. conducted sensory evaluation on *Monascus Pilosus* fermented green tea and found that the brightness evaluation of fermented tea soup was as high as 4.26, significantly higher than that of unfermented tea. Therefore, microbial fermentation of tea can improve the color of tea soup and the overall quality of tea (96), as shown in Figure 5. After fermentation, the color of the tea soup mostly become translucent and the overall quality will be improved.

## Concluding remarks

In the process of microbial fermentation of tea, the content of catechins and flavonoids in tea mostly showed a decreasing trend, mainly due to the oxidation or degradation of catechins and flavonoids into gallic acid during the fermentation process. The biological activity of unfermented tea and microbial fermented tea is also different. After the tea is fermented, its antibacterial and antioxidant capacity would be enhanced. In addition, microbial fermented tea also has anti-inflammatory and weight loss effects. Long-term drinking of microbial fermented tea has good medical and health care effects. Compared with unfermented tea, microbial fermented tea has improved sensory performance, reduced bitterness and astringency, stronger aroma, and brighter tea juice. At present, researchers have not done much research on the fermentation of tea with inoculated microorganisms. In the future, the fermentation of tea with inoculated microorganisms may have a good development prospect. At present, there are many researches on fermented tea in the field of food, such as kombucha, which uses mixed strains to ferment different kinds of tea. Tea fermented by microorganisms has a good prospect in functional food research and development because of its antioxidant, hypoglycemic and lipid lowering properties. The antibacterial properties of fermented tea have a good application prospect in food preservation. In recent years, with the development of fermentation engineering, bostrycin and lovastatin obtained by microbial fermentation of tea have anti-cancer and anti-inflammatory effects, and are expected to be applied in medical clinical research. This article will provide a theoretical basis for future researchers to explore more the functional activities of microbial fermented tea, and provide a certain scientific basis for the research and development of tea in the field of functional health products, food and medicine.

## Author contributions

TH: conceptualization, project administration, writing—original draft, and writing—review and editing. SS: investigation and writing—original draft. QM: conceptualization and writing—review and editing. All authors contributed to the article and approved the submitted version.

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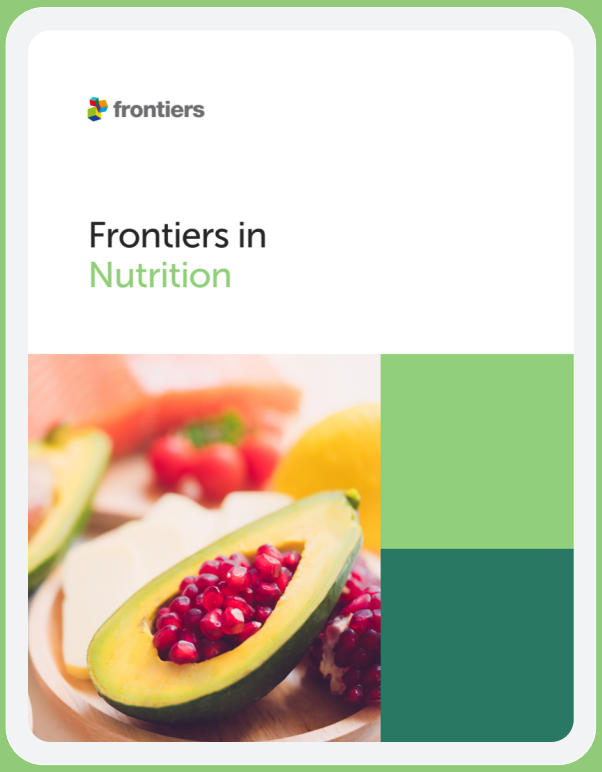


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