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Tea polyphenols interfere with adult zebrafish lipid metabolism and gut microbiota *via* the gut-liver axis inducing hepatic toxicity

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ABSTRACT

This study investigated the hepatotoxic effects and mechanisms of high-dose tea polyphenols on zebrafish liver based on the gut-liver axis theory. An 8-week rearing experiment evaluated various aspects such as physiological and biochemical indicators, histology, gut microbiota, and liver transcriptomics of zebrafish. The results showed that feeding zebrafish a diet rich in high-dose tea polyphenols disrupted their hepatic lipid metabolism, ultimately causing liver damage. Tea polyphenols regulated signaling pathways related to "lipid metabolism and absorption" and "fatty acid degradation", promoting the uptake of fatty acids and cholesterol by liver cells, inhibiting fatty acid oxidation and excretion, and causing liver fat accumulation. Additionally, high-dose tea polyphenols altered the composition of the gut microbiota, reducing microbial diversity and decreasing the production of intestinal short-chain fatty acids (SCFAs). As a result of the gut-liver axis interaction, low levels of SCFAs and harmful bacteria from the intestine were able to enter the liver through the portal vein, activating hepatic pro-inflammatory factors and causing liver inflammation. The accumulation of liver fat further promoted the expression of pro-inflammatory factors and transported them to the intestine through the bile duct, exacerbating intestinal damage in zebrafish. Therefore, caution should be exercised when using tea polyphenols as a nutritional supplement or medication, especially at high doses and with long-term use, to be aware of their potential adverse effects.

1. Introduction

China's tea-drinking habit has a long history, extending over 4 700 years. Tea is not only a healthy beverage but also an important part of traditional Chinese culture. Through the Silk Road, tea was spread to various parts of the world and gradually gained widespread recognition and appreciation. In recent years, the scale of China's tea market has continued to expand. At the same time, the tea market has become increasingly diverse, evolving from traditional plain tea to a diversified structure that includes green tea, fermented tea, tea beverages, tea snacks, and more[1]. Additionally, the consumer base for tea is also changing, expanding from primarily older adults to increasingly include younger people and the general public[2]. The parallel development of tea culture's heritage and innovation has allowed China's tea industry to maintain its traditional charm while continuously adapting to and leading new consumption trends. This development trend not only reflects changes in market demand but also highlights the significant role and potential of China's tea industry within the global health beverage market.

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Tea drinkers generally believe that tea has various health benefits, such as refreshing effects and lipid-lowering properties[3], with tea polyphenols being identified as one of its key active components. Tea polyphenols are a class of polyphenolic compounds found in tea, mainly including (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC)[4]. Research has shown that tea polyphenols possess various biological activities such as antioxidation, anti-diabetic, anti-inflammatory, and anti-cancer[5]. For example, tea polyphenols can prevent acute kidney injury in diabetic rats after extracorporeal circulation surgery by inhibiting oxidative stress[6]; they can also protect neurons by activating phosphoenolpyruvate carboxykinase phosphorylation[7]. Additionally, green tea catechins can enhance the mineralization of mouse bone marrow mesenchymal stem cells and promote bone formation[8]. These research findings have driven the application of tea polyphenols in food and pharmaceuticals, providing the scientific basis for the long-standing Chinese tradition of tea consumption.

With the emergence of the concept of precision intake of nutrients and drugs, it has been found that in addition to the aforementioned beneficial effects, tea polyphenols may also bring about a series of toxic side effects. There are reports indicating that continuous supplementation of tea polyphenols for 6 months (720 mg/day) can induce hemolysis and acute liver failure[9]. Green tea extract enhances acetaminophen-induced liver toxicity in mice[10]. Currently, research on the relationship between tea polyphenol dosage and its effects is gradually deepening. Clinical studies have shown that oral administration of high doses of green tea extract can lead to a series of adverse reactions, such as hepatotoxicity, nausea, abdominal pain, and diarrhea[11-12]. Although multiple studies have demonstrated the toxic effects of tea polyphenols under certain conditions, as well as, the mechanism of their toxicity is still not fully understood.

The liver is the most metabolically active organ in the human body and an important detoxification organ[13-14]. The intestine is not only the main digestive organ of animals but also an important immune organ[15]. In recent years, the theory of the gut-liver axis has been widely applied in biomedical research to study the complex relationship between the occurrence of diseases and the liver and intestine. Increasing evidence suggests that dysregulation of the gut-liver axis is associated with the occurrence and development of various diseases, such as drug-induced liver injury (DILI), alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), primary sclerosing cholangitis (PSC), and primary biliary cholangitis (PBC)[16]. Currently, the gut-liver axis has gradually become an important direction in biomedical research. The gut-liver axis refers to the complex bidirectional circulation between the gastrointestinal tract and the liver[17]. Nutrients, microbial metabolites, and microbial components in the intestines can be transported to the liver through the portal vein, and these components can return to the intestines *via* the bile duct from the liver, thus influencing each other through this internal circulation[18]. In previous studies, the gut-liver axis theory has often been used to explore and explain how bioactive substances influence health and disease through the interaction between the gut and the liver, particularly in physiological processes related to nutrition, toxicology, and pharmacology. Regarding nutrition, the gut-liver axis theory reveals the importance of gut microbiota in metabolic diseases. Research has shown that sodium butyrate can alleviate fructose-induced non-alcoholic fatty liver by regulating gut microbiota[19]. In contrast, trans fatty acids can induce the release of lipopolysaccharides in the gut and the migration of pathogenic bacteria, leading to chronic liver injury[20]. These findings highlight the crucial role of dietary components in regulating gut health and demonstrate the key role of gut microbiota in this process. In toxicology, the gut-liver axis theory has been utilized to evaluate the impact of environmental toxins and chemicals on liver health through gut microbiota. For example, studies have found that chlorpyrifos has toxic effects on the liver of zebrafish[21], while exposure to triclosan (TCS) causes liver damage in mice[22]. Investigations in pharmacology that explore the gut-liver axis theory have clarified the action mechanisms of bear bile heart-saving pills in rats subjected to a high-fat diet[23]. Furthermore, by using multi-organ models to simulate the gut-liver axis, researchers can analyze the impact of the microbiome on drug metabolism[24], which is important for understanding the mechanisms of drug action in the body and the differences in individual responses.

However, regarding tea polyphenols, which are extensively used as dietary supplements, their effects on liver function, intestinal health, and overall systemic health remain unclear. Some studies have shown that tea polyphenols and EGCG can improve hyperlipidemia in rats by regulating liver metabolism and reshaping intestinal microbiota[25]. This suggests that the liver and intestines are important tissues and organs for the effects of tea polyphenols. It is plausible to apply the gut-liver axis theory to examine the mechanisms behind the toxicity of high doses of tea polyphenols on the body. According to gut-liver axis theory, there is a significant relationship between gut microbiota, their metabolites and liver injury[26]. Changes in the gut can promote or alleviate liver damage through various mechanisms, including the influence on gut microbiota[27], lipid metabolism[28], and bile acid metabolism, among others. Therefore, based on the theory of the gut-liver axis, this study explored the hepatotoxic effects and mechanisms of high-dose tea polyphenols on zebrafish. Through an 8-week breeding experiment, various aspects including physiological and biochemical indicators, histology, intestinal microbiota, and liver transcriptomics of zebrafish were evaluated, aiming to verify the hypothesis that tea polyphenols as dietary supplements may have adverse effects on the health of zebrafish and explore the mechanisms of tea polyphenols' toxicological effects on adult zebrafish. This study aims to thoroughly investigate the effects of tea polyphenols on the health of zebrafish, providing new insights into the safety and optimal intake of oral tea polyphenols. This will help formulate and optimize strategies to enhance the beneficial effects of tea polyphenols while minimizing their potential risks.

2. Materials and methods

2.1 Animal experimental design

Adult zebrafish (AB strain) were obtained from the zebrafish breeding system at Beibu Gulf University Laboratory (Qinzhou, China). During the breeding period, the light/dark cycle was set to 12 h, and the zebrafish were fed twice daily at 9:00 and 16:00 for 8 weeks. Body weight and length were measured every 2 weeks during

this period. Water temperature ((26 ± 1) °C), pH (7.4 ± 0.2), conductivity ((525 ± 25) μs), and dissolved oxygen ((7.5 ± 0.2) mg/L) were regularly monitored.

The 98% concentration of tea polyphenols was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). A total of 320 adult zebrafish were selected for the experiment and randomly allocated into 16 acrylic rearing tanks, with 20 fish per tank. The initial body weight of each zebrafish was (0.56 ± 0.02) g. The zebrafish were divided into 4 groups: control (CON) group, low-dose group, medium-dose group, and high-dose group. The CON group was fed with basic feed, while the drug groups (T100, T200, and T400) were fed with feed supplemented with 100, 200, and 400 mg/kg of tea polyphenols, respectively. Table S1 describes the formulation and basic nutritional components of the basic feed.

2.2 Sample collection

After the conclusion of the 8-week rearing experiment, all zebrafish were fasted for 24 h and then euthanized using a solution of MS-222 (10 mg/L). The fish were weighed and then dissected to obtain organs such as the liver, intestines, etc., for the measurement of various parameters. The following formulas were used for calculation:

$$\text{Weight gain (g)} = \text{Final weight (g)} - \text{Initial weight (g)} \quad (1)$$

$$\text{Viscerasomatic index (VSI, \%)} = \frac{\text{Weight of heavy internal organs (g)}}{\text{Total weight (g)}} \times 100 \quad (2)$$

$$\text{Hepatosomatic index (HSI, \%)} = \frac{\text{Liver weight (g)}}{\text{Total weight (g)}} \times 100 \quad (3)$$

2.3 Analysis of liver biochemical parameters

The liver tissue of zebrafish was ground using anhydrous ethanol as the homogenization medium. Triglycerides (TG) and total cholesterol (T-CHO) were extracted from the liver tissue, and their levels were determined according to the instructions provided with the assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.4 Analysis of liver and intestinal enzyme activity

Assessment of antioxidant capacity in zebrafish was conducted by measuring total antioxidant capacity (T-AOC), catalase (CAT), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) levels in the liver. The digestive function of zebrafish intestines was evaluated by determining the concentrations of α-amylase (AMS), trypsin, and lipase (LPS). All these indicators were measured strictly following the instructions provided in the reagent kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.5 Histological observations

Based on the method reported previously[29], zebrafish liver and intestine samples were fixed in 4% paraformaldehyde, frozen in liquid nitrogen, and sectioned at a thickness of 5 μm using a cryostat (Leica, Wetzlar, Germany). Subsequently, hematoxylin and eosin (H&E) and Oil red O staining were performed separately. The stained sections were observed under an optical microscope (Nikon Eclipse TE2000-U; Nikon, Tokyo, Japan), and images were captured at 400× magnification. Fat droplets in the liver were quantified using ImageJ software. Image Pro Plus 6.0 software was used to analyze villus height and muscle thickness.

2.6 Analysis of short-chain fatty acids (SCFAs) in the intestine

Analysis of SCFAs in the intestine was performed using gas chromatography-mass spectrometry (GC-MS) with an Agilent 7890A–5975C GC-MS system (Agilent Technologies, USA). Zebrafish intestine samples were placed in 10 mL centrifuge tubes and treated with 2 mL of 2 mol/L sulfuric acid solution. After sealing, the tubes were subjected to shaking in a 60 °C water bath for 2 h. Following cooling, 3 mL of chilled ether was added, and the mixture was vortexed for 5 min. The samples were then centrifuged for 5 min, and the upper ether layer was collected. An additional 3 mL of ether was added, and the extraction was repeated. The combined ether extracts were spiked with 100 μL of 2-octanol as an internal standard, and the ether was evaporated to approximately 100 μL at room temperature before analysis. GC conditions were as follows: injector temperature, 250 °C; gas chromatography interface temperature, 250 °C; carrier gas flow rate, 1.2 mL/min; split ratio, 5:1; injection volume, 2 μL; temperature program: initial temperature, 100 °C for 5 min, then ramped at 8 °C/min to 260 °C and held for 10 min. MS conditions included: ion source temperature, 250 °C; quadrupole temperature, 150 °C; electron ionization (EI) at 70 eV; full-scan mode from m/z 29 to 450. Qualitative analysis was conducted using standard compounds, and quantification was performed using a combination of characteristic ions, internal standard calibration, and external standard curve methods.

2.7 16S rRNA gene sequencing analysis

Microbial community structure analysis was performed on zebrafish intestinal samples from the CON group and the high-dose tea polyphenols group (TP). Each group was set with 4 replicates, with 10 zebrafish intestines per replicate. Intestinal 16S rRNA sequencing was performed by Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Alpha diversity indices were evaluated using QIIME (version 1.7.0, https://qiime.org/index.html). Inter-group differences in diversity indices and principal component analysis (PCA) were analyzed using R software (version 2.15.3). Unweighted pair group method with arithmetic mean (UPGMA) clustering analysis was performed based on the weighted UniFrac distance matrix. Additionally, functional prediction analysis of bacterial communities was conducted using PICRUSt2 (https://picrust.github.com/picrust/). Specific analysis methods followed those described by Wang et al.[30].

2.8 Liver tissue transcriptome sequencing analysis

Zebrafish liver tissues from CON and TP were quickly placed into liquid nitrogen and stored at −80 °C before analysis. Three replicates were set for each group, with 6 zebrafish liver tissues collected per replicate. Zebrafish liver tissue transcriptome sequencing was conducted by BGI Genomics Co., Ltd. (Shenzhen, China). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) bioinformatics analyses were performed using the BGI Genomics DR. TOM system (including

volcano plots of differentially expressed genes (DEGs), pathway enrichment plots, cluster heatmaps, and selection of key genes).

2.9 qPCR validation of transcriptome data accuracy

To evaluate the accuracy of transcriptome analysis, 9 DEGs were selected for real-time quantitative PCR (RT-qPCR) analysis. The primer sequences used in this study (Table S2) were designed based on the mRNA sequences of zebrafish. Beta-2-microglobulin was chosen as the reference gene, and the expression levels of genes were calculated and normalized using the $2^{-\Delta\Delta Ct}$ method, with each experiment performed in triplicate. Data analysis was conducted using the least significant difference (LSD) method in the SPSS software program (version 22.0), with statistical significance set at $P < 0.05$. EasyScript® All-in-One First Strand cDNA Synthesis SuperMix was used for qPCR reverse transcription of total RNA (TransGen Biotech, China). Each reaction contained 1 μL each of forward and reverse primers, 10 μL SYBR Green Mix (TransGen, Beijing, China), and 2 μL of 1:8 diluted cDNA, with RNase-free water added to achieve a final reaction volume of 20 μL. The qPCR amplification program was conducted according to standard protocols.

2.10 Statistical analysis

A correlation analysis was carried out using the Spearman method to assess the relationship between the abundance of the top 35 species at the genus level, the SCFAs in the intestine, and the genes extracted from the liver transcriptome. Data analysis was performed utilizing GraphPad Prism (version 8.0; GraphPad, CA, USA) and SPSS software tools (version 22.0; IBM Co., NY, USA). The results were presented as the mean ± standard deviation. To compare differences among groups, one-way analysis of variance (ANOVA) was employed, followed by the LSD test for pairwise comparisons. *P*-value < 0.05 was considered statistically significant.

3. Results

3.1 Effects of different doses of tea polyphenols on the growth of zebrafish

Tea polyphenols are natural substances that exhibit a range of biological activities. Incorporating a specific dosage of tea polyphenols into the diet can accelerate the metabolism of sugars and lipids in the liver[31]. However, orally ingesting high doses of tea polyphenols can also cause toxic reactions in multiple organs[32-33]. After an 8-week experiment, it was observed that there were no significant differences in body weight between the drug group and the CON group during the first 4 weeks. However, by the 6th week, the high-dose group showed a significantly higher body weight compared to the CON group. From the 6th to the 8th week, both the medium-dose and high-dose groups experienced a decrease in body weight, with the high-dose group showing the most significant decline (Fig. 1A). The addition of tea polyphenols did not have a significant effect on the body length of zebrafish (Fig. 1B). However, both the medium and high-dose groups exhibited significantly higher HSI and VSI compared to the CON group and the low-dose group. Notably, the high-dose group had the highest

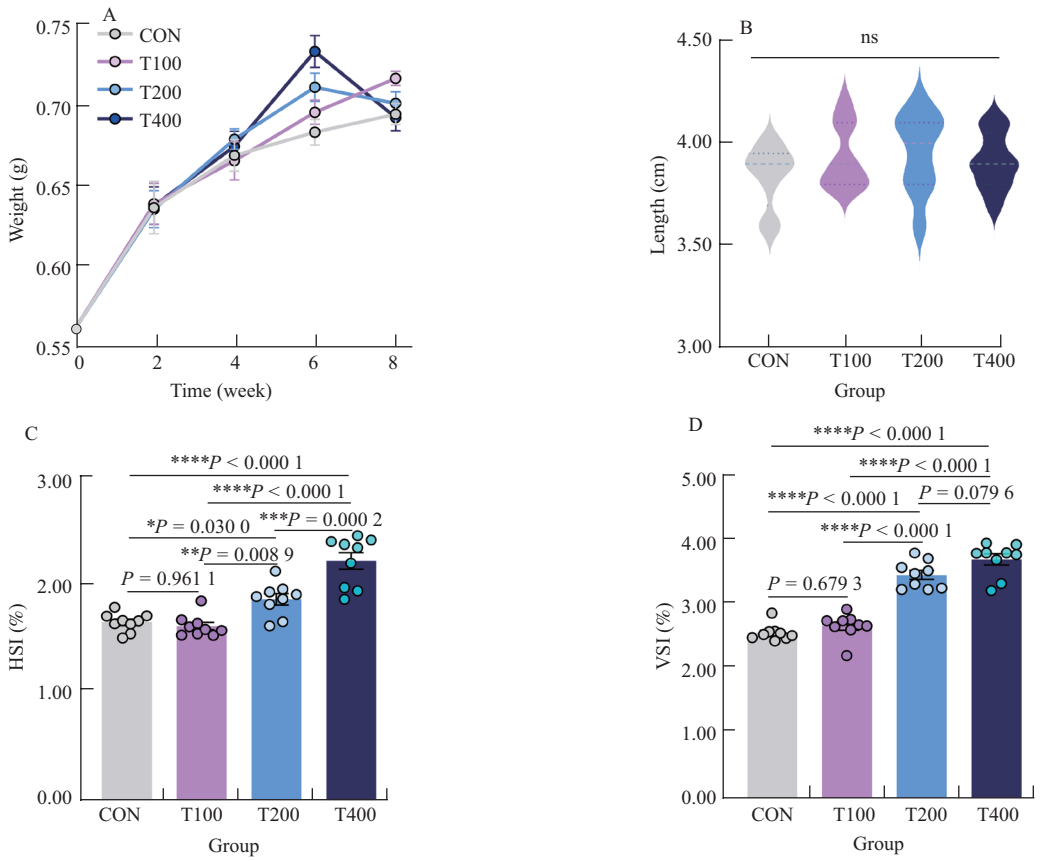

Fig. 1 Growth indicators and body composition of zebrafish. (A) Weight gain of different groups after 8 weeks. (B) Body length of different groups after 8 weeks. (C) HSI. (D) VSI. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.000\ 1$; ns, not significant ($P > 0.05$).

HSI, with a ratio of 3.15%, approximately 1.42 times higher than that of the CON group (Figs. 1C and D). This indicates that the addition of high-dose tea polyphenols caused abnormal enlargement of the zebrafish liver, suggesting protentional inflammation or metabolic abnormalities, which subsequently led to reduction in body weight.

3.2 Impact of tea polyphenols on liver lipid metabolism and antioxidant function of zebrafish

In terms of lipid metabolism, compared to the CON group and the low-dose group, the high-dose group showed significant increases in TG and T-CHO levels, with values of (2.09 ± 0.01) and (0.73 ± 0.01) mmol/g prot, respectively (Figs. 2A and B). This suggests that high-dose tea polyphenols may disrupt lipid metabolism in zebrafish liver, leading to lipid accumulation. In terms of antioxidant function, compared to the CON group, adding low-dose tea polyphenols significantly increased the T-AOC of zebrafish liver. However, in the high-dose group, T-AOC, CAT, and GSH-PX levels were significantly decreased, while MDA levels were increased (Figs. 2C−F). The outcome revealed that the antioxidant capacity of the liver in response to tea polyphenols is dose-dependent. Low concentrations may boost liver antioxidant capacity; however, as the concentration increases, higher doses of tea polyphenols may reduce liver antioxidant capacity.

3.3 High doses of tea polyphenols can lead to increased vacuolation and fat accumulation in liver cells

Liver damage and fat accumulation are usually manifested as morphological changes in tissues. To further explore the effects of high-dose tea polyphenols on liver damage, we further explored the morphology of zebrafish liver tissue using 2 methods: H&E staining and Oil red O staining. The results are shown in Figs. 2G and H. In the CON group, H&E-stained liver tissue sections showed intact cellular morphology, a higher number of cell nuclei, clear cell membrane boundaries, and abundant cytoplasm. On the other hand, treated zebrafish exhibited increased vacuoles in cells, a significantly reduced number of cell nuclei, and signs of inflammatory infiltration. The area of vacuoles was significantly larger ((80 833.73 ± 2 439.59) μm^2) than that in the CON group ((67 998.09 ± 3 764.19) μm^2) (Fig. 2I). The results of Oil red O staining of the liver (Figs. 2J and K) showed that the area of neutral fat in the liver of the treatment group was (64 242.82 ± 2 233.11) μm^2, significantly higher than that in the CON group with the value (46 194.70 ± 782.14) μm^2 (Fig. 2L). It can be concluded that feeding high-dose tea polyphenols leads to a significant accumulation of neutral lipids in zebrafish liver.

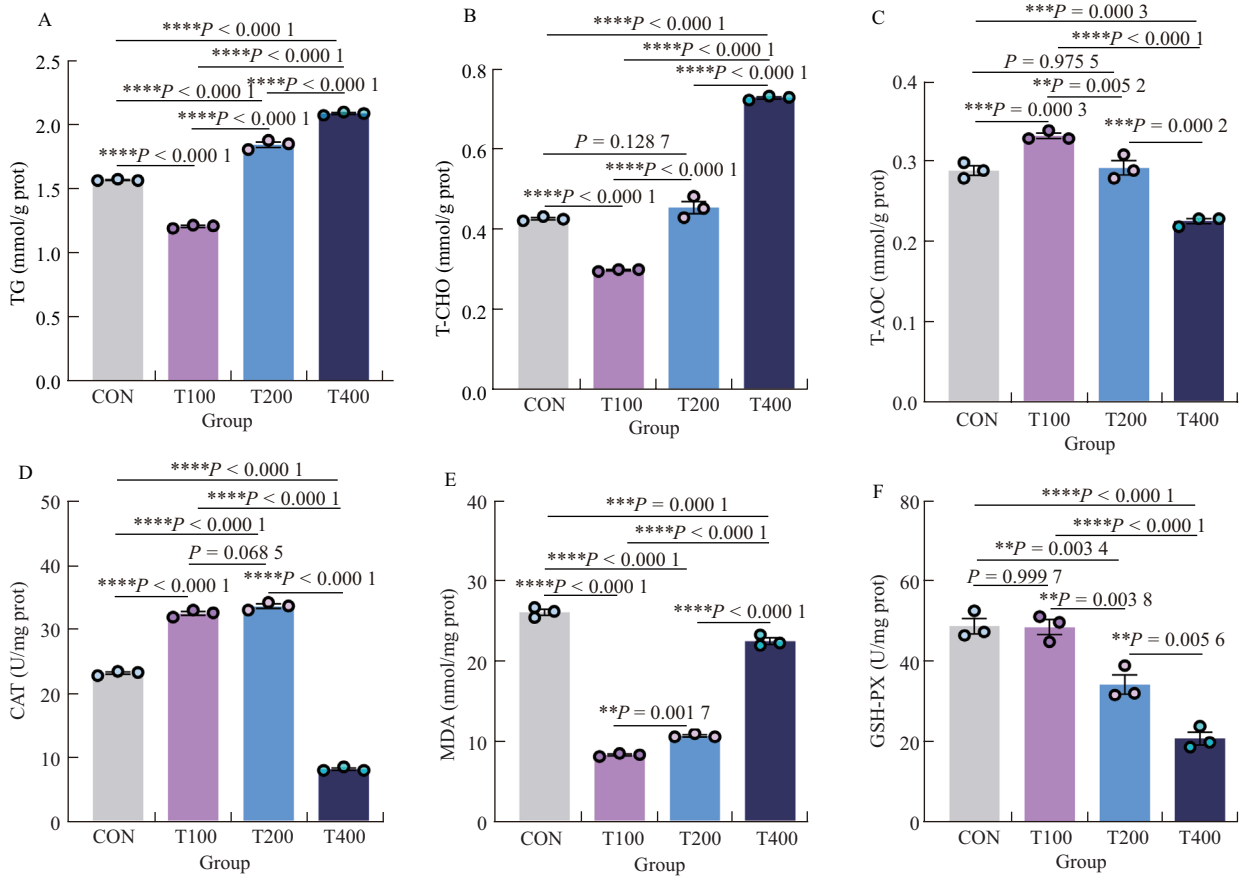

Fig. 2 Influence of 2 feed treatments on liver tissue in zebrafish. (A-F) Enzyme activity and biochemical indicators. (A) Hepatic TG. (B) T-CHO. (C) T-AOC of the liver. (D) Hepatic CAT activity. (E) Hepatic MDA. (F) Hepatic GSH-PX activity. Liver Oil red O histological sections of (G) CON and (H) TP400 group under 400× magnification (scale bars, 50 μm). (I) Number of lipid droplets in liver tissue. Liver H&E histological sections of (J) CON and (K) TP400 group under 400× magnification (scale bars, 50 μm). (L) Hepatocyte vacuolar area. Horizontal bars represented the mean ± SEM. Statistical significance was determined by using one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.000\ 1$.

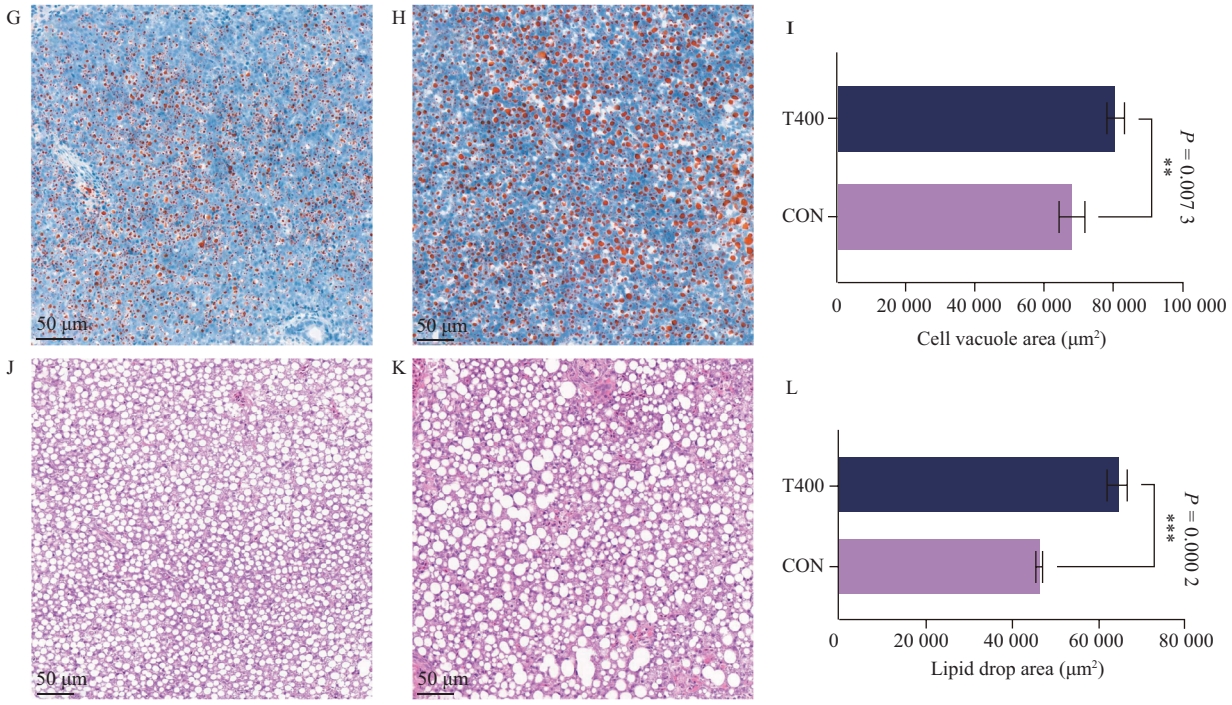

Fig. 2 (*Continued*)

3.4 High doses of tea polyphenols lead to disturbances in liver lipid metabolism and promote inflammatory responses

To investigate the mechanism of high-dose tea polyphenols toxicity on zebrafish liver, we conducted transcriptome analysis on the liver tissues of zebrafish from CON and TP. Compared to CON, TP yielded a total of 5 632 DEGs, among which 2 776 DEGs were significantly upregulated while 2 856 DEGs were markedly downregulated (Fig. 3A). KEGG pathway enrichment analysis shows that DEGs are significantly enriched in pathways such as carbon metabolism, diabetic cardiomyopathy, TCA cycle, PPAR signaling pathway, chemical carcinogenesis-reactive oxygen species (ROS), and oxidative phosphorylation (Fig. 3B, Table S3).

To explore the causes of lipid accumulation in the zebrafish liver, we further filtered the enriched pathways related to glycolipid metabolism from the enrichment results. We plotted a heatmap of DEGs in each pathway (Table S4), and combined with the observed phenomenon of lipid accumulation, selected the top 10 genes with the highest fold change in each pathway to plot the signaling pathway diagrams (Figs. 3C-E). The results show that in the fatty acid metabolism pathway, genes involved in fatty acid synthesis, such as *acsbg1*, *acsl4b*, and *acsl1b*, were activated, while the rate-limiting enzymes involved in fatty acid *β*-oxidation, CPT1, and ACAA2, were inhibited. In the fat digestion and absorption pathway, fatty acid-binding protein FABP, fatty acid transport proteins CD36 and the scavenger receptor, class B type 1 (SR-B1) were activated. Additionally, the gene *ABCA1* involved in fatty acid efflux was inhibited. Regarding carbon metabolism pathway, the key rate-limiting enzymes glucokinase (GCK) and hexokinase (HK) were both activated. Furthermore, the tumor necrosis factor alpha (TNF-α) and the pro-inflammatory cytokine interleukin 1 beta (IL-1β), which act in multiple pathways, were also activated. Moreover, genes from the B-cell lymphoma-2 (*bcl2*) family involved in promoting cell apoptosis, such as *badb* and *PMAIP1*, were activated. To verify the accuracy of the transcriptome data, qPCR was performed for a total of 9 target genes including *Scarb1*, *IL-1β*, *Apoea*, *Apoeb*, *Fabp1b1*, *Badb*, *HK2*, *GCK*, and *CD36*. The outcome disclosed that the qPCR results were consistent with the liver transcriptome results (Fig. 3F), indicating the reliability of the transcriptome data.

3.5 Effects of different doses of tea polyphenols on digestive enzyme activity in zebrafish intestine

Animal growth rate and intestinal digestive enzyme activity are highly correlated. LPS, AMS, and trypsin are the main enzymes in the human body responsible for the digestion and absorption of fats, carbohydrates, and proteins[34-36]. To investigate the effects of different doses of tea polyphenols on the digestive capacity of zebrafish, we measured the activity of 3 intestinal digestive enzymes in zebrafish. The results showed that the addition of tea polyphenols significantly reduced the activities of LPS and AMS in the zebrafish intestine, with the highest dose group showing the lowest enzyme activity (Figs. 4A and B). Furthermore, the activity of trypsin was significantly increased in the medium dose group and decreased in the high dose group (Fig. 4C). This indicates that the inhibitory effect of tea polyphenols on digestive enzymes increases with the supplementation dose.

3.6 High doses of tea polyphenols led to intestinal tissue damage in zebrafish

Intestinal enzyme inactivation is a key factor in increasing intestinal susceptibility[37]. Therefore, we analyzed the zebrafish intestine to further investigate whether the addition of high-dose tea polyphenols affects intestinal tissue morphology. The results, as

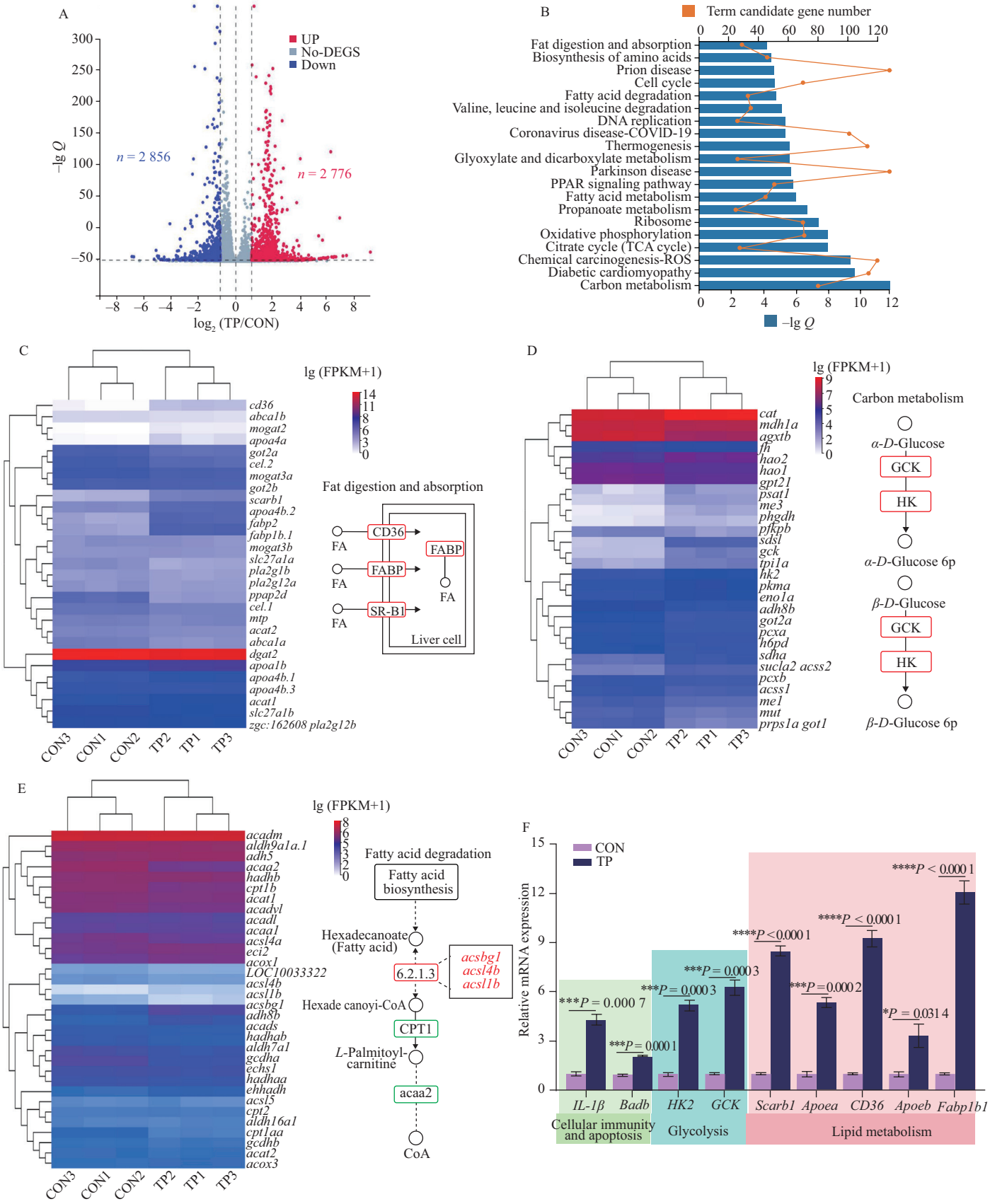

Fig. 3 Transcriptome analysis of hepatic metabolic disruption induced by high-dose tea polyphenols. (A) Volcano map of differential genes. (B) Differential gene enrichment KEGG pathway analysis. (C) DEGs heat map of fat digestion and absorption signaling pathway. (D) DEGs heatmap of the carbon metabolism signaling pathway. (E) DEGs heatmap of the fatty acid degradation signaling pathway. (F) Real-time fluorescence quantification validation analysis. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.000\ 1$.

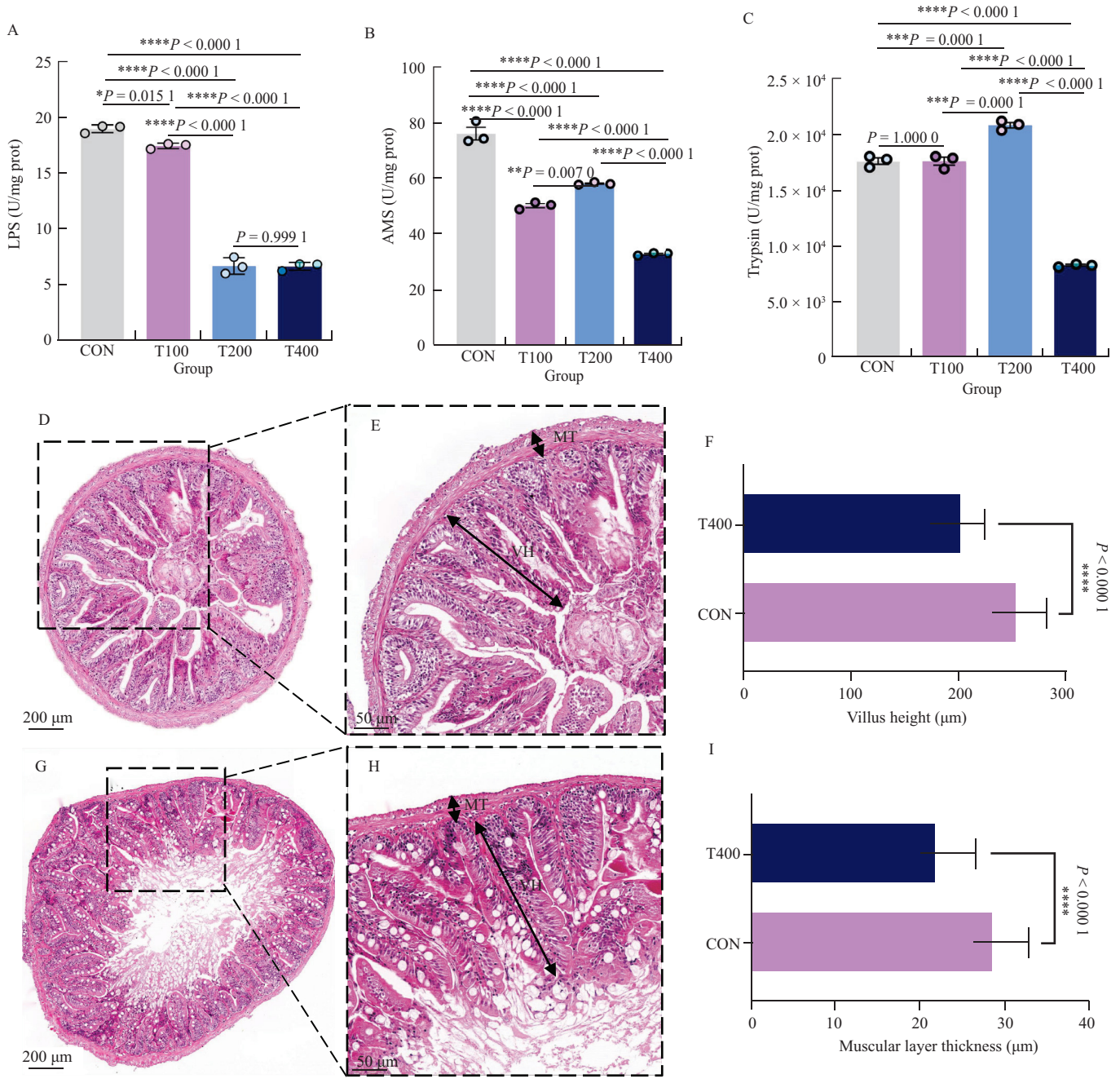

Fig. 4 The influence of 2 feed treatments on intestinal tissue in zebrafish. (A-C) Enzyme activity and biochemical indicators: (A) Intestinal LPS. (B) Intestinal AMS. (C) Intestinal trypsin. (D-E) Intestinal histological sections of CON group under 100× and 400× magnification (scale bars: 200 and 50 μm). (F) Average height of villus. (G-H) Intestinal histological sections of TP group under 100× and 400× magnification (scale bars: 200 and 50 μm). (I) Muscular layer thickness. Statistical significance was determined by using one-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.000\ 1$.

shown in Figs. 4D-I, indicate that the intestinal villus height in the high-dose group of zebrafish was (202.11 ± 20.04) μm^2, significantly lower than that in the CON group ((253.31 ± 10.95) μm^2) (Fig. 4F). Regarding the thickness, the intestinal muscle layer was significantly reduced (Fig. 4I). Furthermore, it was observed that there was more mucus in the intestines of the high-dose group of zebrafish. This suggests that high-dose tea polyphenols inhibit zebrafish intestinal enzyme activity, leading to changes and damage in intestinal tissue morphology.

3.7 High doses of tea polyphenols lead to intestinal microbiota dysbiosis and decreased levels of SCFAs

To analyze the relationship between high-dose tea polyphenols and intestinal damage in zebrafish, we selected the top 10 species at the phylum and genus levels in terms of abundance for both TP and CON. We generated bar charts of species relative abundance to visually observe the species with higher relative abundances at different taxonomic levels in each sample. The most dominant species in both groups were Proteobacteria, with a proportion of

55.11% in CON and 93.34% in TP. Additionally, the abundance of Firmicutes in TP was only 1.75%, significantly lower than the Firmicutes abundance in CON (12.99%) (Fig. 5A). At the genus level, the highest abundance in CON was *Stenotrophomonas*, accounting for 15.20% of the total species, while in TP, the highest abundance was *Plesiomonas*, accounting for 27.82% (Fig. 5B). The Venn diagram showed the overlap of genus-level species among samples (Fig. 5C), where 801 species were unique to CON, 174 species were unique to TP, and 408 species were shared between the 2 groups. The PCA plot displayed the distribution between the 2 groups, showing significant inter-group differences (Fig. 5D). Statistical analysis of the α-diversity indices for the 2 groups of samples revealed that in the CON, Shannon, Simpson, and Inverse Simpson indices were all significantly higher than those in the TP group, while there were no significant differences in Chao1 and PD indices (Fig. 5E). Species difference analysis indicated a significant increase in the abundance of *Plesiomonas* at the genus level in TP (Fig. 5F).

PICRUSt2 was used to predict functions based on the KEGG database, and a clustered heatmap of the top 30 functions at the KEGG-LEVEL 2 was generated (Fig. 5G). Since each function at the LEVEL 2 belongs to various functions at the LEVEL 1, we summarized and classified them to obtain the relative abundances of the 2 sample groups at the KEGG-LEVEL 1. These included 6 functional gene pathways: metabolism (36.67%), human diseases (16.67%), cellular processes (13.30%), organismal systems (13.30%), genetic information processing (13.30%), and environmental information processing (6.67%) (Table 1). KEGG functional prediction revealed differences in microbial communities between the 2 groups in metabolic pathways.

SCFAs are bacterial metabolic products derived from the fermentation of dietary oligosaccharides and play a crucial role in maintaining gut health[38]. To investigate the mechanism by which high doses of tea polyphenols cause intestinal damage in zebrafish, we measured the levels of 13 SCFAs and the intestinal microbiota 16S rRNA in zebrafish intestines. The results showed that all 13 SCFAs were detected in the intestines of both groups of zebrafish. Among them, isobutyric acid, isovaleric acid, 4-methylvaleric acid, and tetradecanoic acid in the TP group did not significantly differ from those in the CON. However, the levels of the remaining 9 SCFAs were found significantly lower in the TP group compared to the CON. Among these, the most pronounced change was observed in acetic acid, with a concentration of (9.88 ± 0.60) mg/kg, representing only 69.23% of the concentration in the CON (Table 2). This indicates that the addition of high doses of tea polyphenols reduces the levels of SCFAs in the intestines.

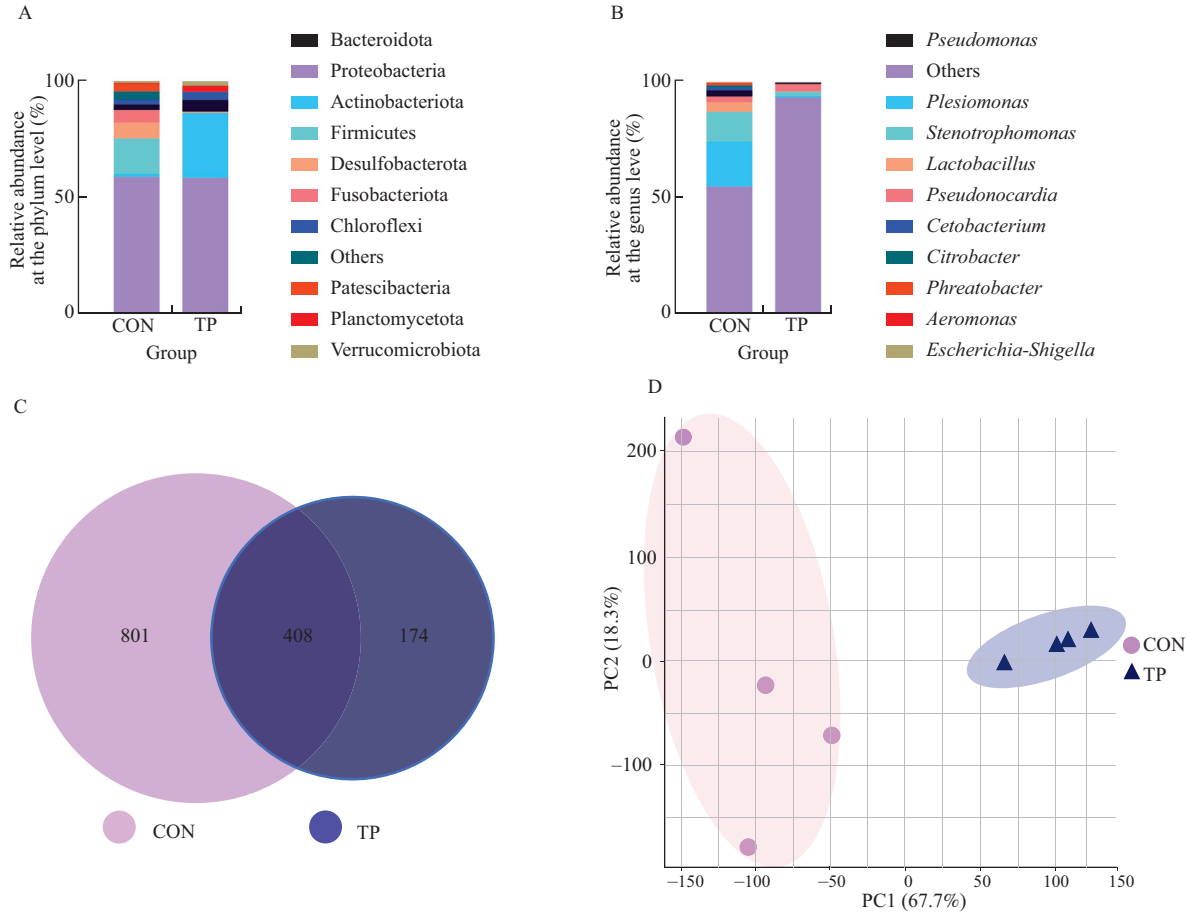

Fig. 5 Comparison of abundance and diversity of gut microbiota between 2 groups. (A and B) Relative abundance of gut microbiota at the phylum and genus level in TP and CON of zebrafish. (C) Venn graph. (D) Principal coordinate analysis of intestinal microbiota at the genus level. (E) α-diversity analysis of intestinal microbiota. (F) Comparison of CON and TP group microbial communities. (G) Relative abundance of functional genes predicted by KEGG analysis in zebrafish microbial communities.

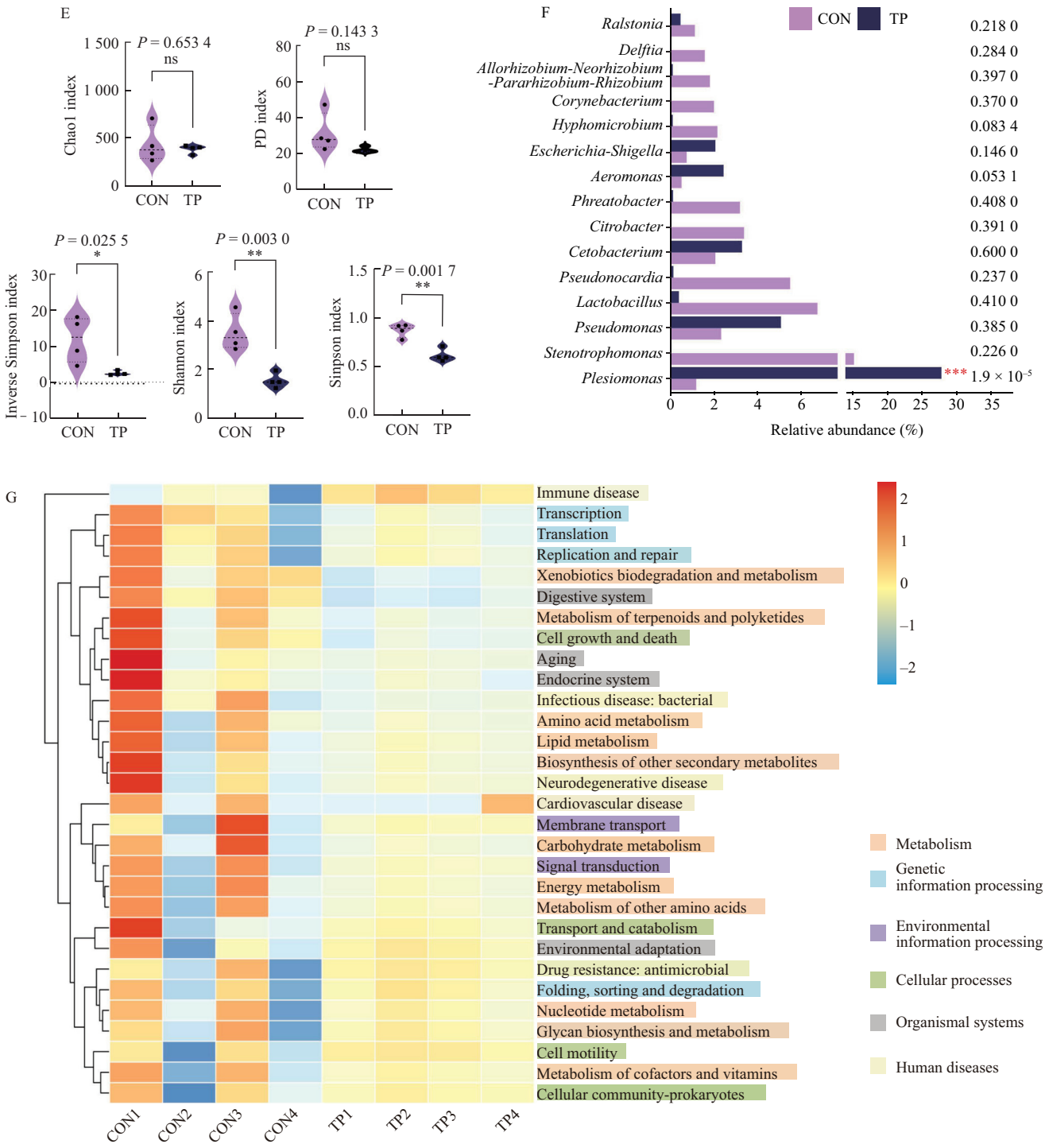

Fig. 5 *(Continued)*

3.8 Analysis of the correlation between gut microbiota, SCFAs, and hepatic target genes

The gut microbiota not only produces metabolites such as SCFAs to promote host health but also changes in the gut microbiota are closely related to liver health[39-40]. Therefore, we utilized Spearman's algorithm to investigate the mutual relationship between the top 10 species at the genus level in the gut microbiota of 2 zebrafish groups and 13 SCFAs as well as the target genes selected from the transcriptome and plotted heatmaps accordingly. As shown in Fig. 6, *Pseudonocardia* and *Allorhizobium* were significantly positively correlated with most SCFAs ($P < 0.05$). *Chryseobacterium* was significantly positively correlated with 4-ethyloctanoic acid ($P < 0.05$).

Table 1
Function of intestinal microbiota predicts the proportion of KEGG pathway.

KEGG-LEVEL 1	Percentage (%)	KEGG-LEVEL 2
Metabolism	36.67	Xenobiotics biodegradation and metabolism
		Metabolism of terpenoids and polyketides
		Amino acid metabolism
		Lipid metabolism
		Biosynthesis of other secondary metabolites
		Carbohydrate metabolism
		Energy metabolism
		Metabolism of other amino acids
		Nucleotide metabolism
		Glycan biosynthesis and metabolism
		Metabolism of cofactors and vitamins
Human diseases	16.67	Immune disease
		Infectious disease: bacterial
		Neurodegenerative disease
		Cardiovascular disease
		Drug resistance: antimicrobial
Cellular processes	13.3	Cell growth and death
		Transport and catabolism
		Cell motility
		Cellular community-prokaryotes
Organismal systems	13.3	Digestive system
		Aging
		Endocrine system
		Environmental adaptation
Genetic information processing	13.3	Transcription
		Translation
		Replication and repair
		Folding, sorting and degradation
Environmental information processing	6.67	Membrane transport
		Signal transduction

Table 2
Intestinal short-chain fatty acid content.

Determine the concentration (mg/kg)	Groups		*P* value
	CON	TP	
Acetic acid	14.27 ± 0.68	9.88 ± 0.60**	< 0.01
Propanoic acid	2.50 ± 0.09	1.71 ± 0.06***	< 0.01
Propanoic acid, 2-methyl-	0.19 ± 0.01	0.20 ± 0.05	0.89
Butanoic acid	0.71 ± 0.03	0.54 ± 0.02***	< 0.01
Butanoic acid, 3-methyl-	1.42 ± 0.01	1.68 ± 0.46	0.38
Pentanoic acid	0.24 ± 0.02	0.20 ± 0.01*	0.02
Hexanoic acid	1.41 ± 0.06	1.00 ± 0.09**	< 0.01
Heptanoic acid	1.85 ± 0.09	1.25 ± 0.05***	< 0.01
Octanoic acid	4.51 ± 0.15	3.64 ± 0.09***	< 0.01
4-Ethyloctanoic acid	0.96 ± 0.04	0.58 ± 0.04***	< 0.01
4-Methylnonanoic acid	2.51 ± 0.53	0.77 ± 0.97	0.06
n-Decanoic acid	1.92 ± 0.13	1.46 ± 1.44*	0.01
Tetradecanoic acid	108.42 ± 6.83	108.59 ± 20.38	0.99

Note: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* CON group.

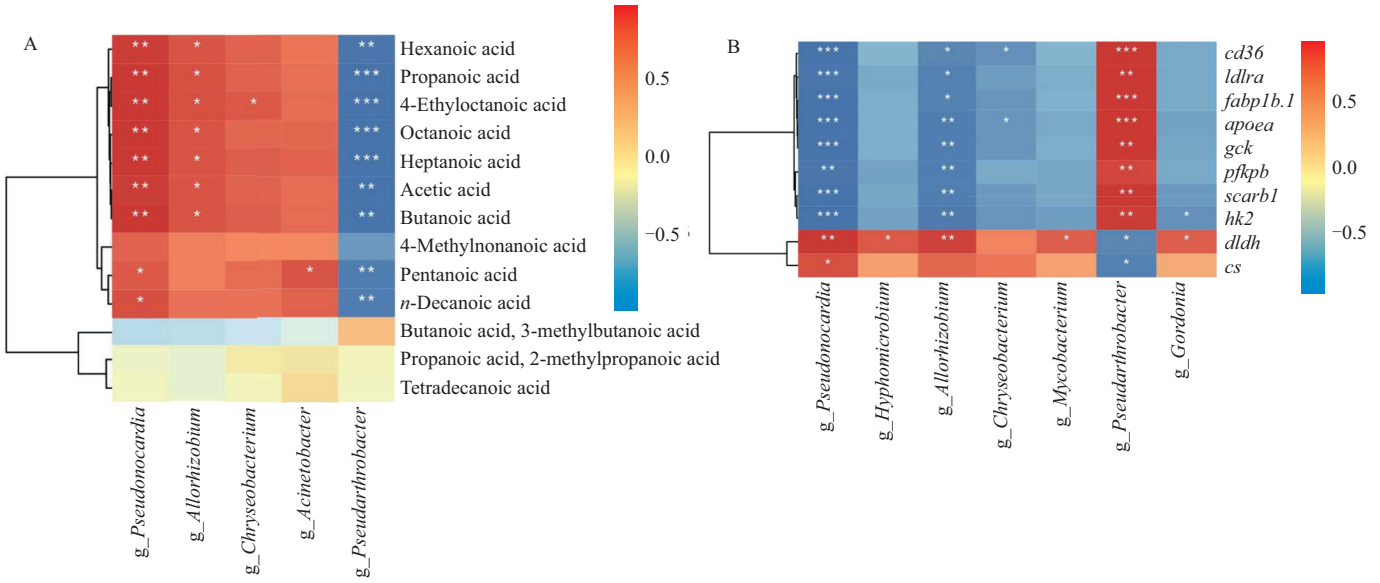

Fig. 6 Correlation analysis based on Spearman's method. (A) Correlation analysis of intestinal SCFAs and intestinal microbiota. (B) Correlation analysis of key genes and intestinal microbiota in the liver.

4. Discussion

Tea polyphenols, as a widely studied natural substance, possess various biological activities and have a positive impact on human health, showing promising potential in disease prevention and wound healing. Studies have demonstrated that tea polyphenols can regulate cellular signaling pathways to prevent and inhibit cancer cell growth[41], promote wound healing[42], and inhibit lung injury[43]. Additionally, tea polyphenols play an important role in weight management, alleviating metabolic syndrome, and preventing diabetes and cardiovascular diseases[44]. In the context of weight control, tea polyphenols suppress the function of various digestive enzymes, resulting in reduced absorption of fats and sugars, thereby decreasing calorie intake and fat storage in the body[45]. For example, white tea polyphenols can lower blood sugar and lipid levels by reducing glucose and cholesterol intake[46]; green tea polyphenols reduce the weight of female rats by regulating obesity-related genes, anti-inflammatory activity, antioxidant stress capacity, and estrogen-related effects[47]. Continuous supplementation of 0.25% caffeine-free red tea polyphenols and green tea polyphenols in the diet for 4 weeks can alleviate mouse obesity by regulating the AMPK pathway[48]. In addition, studies have indicated that tea polyphenols may have certain effects on the liver such as to inhibit hepatic fat synthesis[49], alleviate hepatic steatosis[50], and mitigate liver fibrosis[51]. The feeding of 1% green tea polyphenols in the diet can exacerbate cholesterol-induced hepatic steatosis in mice[52]. As a model organism, zebrafish have digestive systems and biochemical pathways that are fundamentally similar to those of humans[53]. In this study, the effects of tea polyphenols on the digestive enzymes in the intestines of zebrafish are largely consistent with previous research, showing that the enzyme inhibitory effect gradually increases with higher concentrations of tea polyphenols. Since changes in body weight are related not only to intestinal absorption but also to hepatic glucose and lipid metabolism, tea polyphenols play a significant role in both body weight regulation and liver metabolism. Our research has also found that low-dose tea polyphenols have a promoting effect on zebrafish body weight, hepatic enzyme activity, and lipid metabolism. Contrarily, high doses of tea polyphenols (400 mg/kg) resulted in a significant reduction in zebrafish body weight and liver abnormalities, including increased liver coefficient and noteworthy changes in liver tissue morphology, such as markedly increased cell vacuole area and lipid droplet area. This finding aligns with earlier studies that indicated tea polyphenols worsen hepatic steatosis in mice. These studies suggest that although tea polyphenols may be beneficial for fat metabolism within a certain dosage range, high doses or long-term use may lead to liver fat accumulation and abnormalities. Consequently, it is important to exercise caution when using tea polyphenols as a nutritional supplement or medication, particularly at high doses and with prolonged use, in order to be aware of their potential adverse effects. Tea polyphenols play a significant role as natural antioxidants in maintaining health and preventing diseases. Structures like the dihydroxy and trihydroxy groups in tea polyphenols can capture and neutralize free radicals, protecting cells from oxidative stress damage[54]. Green tea polyphenols have been proven to alleviate glutamate excitotoxicity through antioxidative and anti-apoptotic pathways, exhibiting a protective effect on primary cultured cortical neurons[55]. Furthermore, studies have found that daily oral gavage administration of 400 mg/kg of tea polyphenols can reduce infection-induced ileitis inflammation and oxidative stress, enhance the expression of tight junction proteins in the ileal mucosal barrier, and ameliorate ileal damage in mice infected with *Salmonella typhimurium*[56]. Therefore, the role of dietary tea polyphenols is closely associated with alleviating oxidative stress in organisms[57]. Under certain pathological conditions, oxidative stress occurs when cellular antioxidant defenses are insufficient to neutralize the surge of ROS. CAT is a typical antioxidant enzyme that plays a crucial role in protecting cells from the harmful effects of oxidative stress and inflammation[58]. MDA in the liver is a product of liver stress and lipid oxidation, characterized by pro-inflammatory and pro-fibrotic properties[59]. Glutathione (GSH) is a hydrophilic

intracellular antioxidant that protects the body from oxidative damage caused by free radicals and ROS[60], and it has various metabolic and physiological functions. In this study, low doses of tea polyphenols enhanced the T-AOC of zebrafish and reduced indicators such as MDA enzyme activity, consistent with previous research results. However, high doses of tea polyphenols may inhibit the activity of antioxidant enzymes such as CAT and GSH-PX, while increasing MDA enzyme activity. This suggests that high doses of tea polyphenols may impair the antioxidant defense mechanism of zebrafish liver, leading to oxidative stress. The results of H&E staining and Oil red O staining also showed evident liver damage in the high-dose group of zebrafish, including significant fat accumulation, cell vacuolation, and inflammatory infiltration. This suggests that high doses of tea polyphenols may have a pronounced toxic effect on the zebrafish liver. This phenomenon is not unique; studies have found that high concentrations of EGCG have pro-oxidative capabilities, leading to liver damage and GSH depletion[61-62]. In addition, EGCG exhibits pro-oxidative effects[63-64] and inhibits cellular CAT activity by increasing ROS levels[65]. These results indicate that the action of tea polyphenols is bidirectional; their antioxidant function under certain conditions (such as high concentrations) may lead to pro-oxidative effects, exerting negative effects on biological health.

Further analysis of the toxic effects of high-dose tea polyphenols on abnormal lipid accumulation in zebrafish liver revealed that tea polyphenols may affect lipid metabolism through multiple pathways, leading to abnormal neutral lipid accumulation in zebrafish liver. Firstly, tea polyphenols may promote lipid synthesis and absorption by activating genes involved in fatty acid synthesis (such as *acsbg1*, *acsl4b*, and *acsl1b*) and fatty acid-binding proteins (such as FABP). At the same time, tea polyphenols may inhibit rate-limiting enzymes involved in fatty acid β-oxidation (such as CPT1 and ACAA2), leading to inefficient metabolism and utilization of fatty acids. Secondly, tea polyphenols may also activate lipid transport proteins (such as CD36 and SR-B1) and inhibit proteins involved in fatty acid efflux (such as ABCA1), leading to increased lipid absorption and decreased efflux, further exacerbating the abnormal accumulation of neutral lipids in the liver. Additionally, the accumulation of cholesterol may be attributed to the oxidative reaction of EGCG found in tea polyphenols, resulting in the production of toxic phenoxyl radicals subsequently damage mitochondria[52,66]. This damage may lead to the inhibition of the rate-limiting enzyme CPT1 in the fatty acid degradation pathway, thereby affecting fatty acid β-oxidation and exacerbating liver fat accumulation. Research suggests that the activation of the glycolytic pathway may be associated with the self-repair process following cell damage[67-68]. In this study, 2 key enzymes (GCK and HK2) associated with the glycolytic pathway were activated, which may represent a stress response of injured cells to the harm inflicted by tea polyphenols. These changes may lead to abnormalities in energy metabolism, thereby affecting lipid metabolism and accumulation.

The gut-liver axis theory suggests that the gut microbiota plays a regulatory role in liver damage. The stability of the gut microbiota is crucial for the smooth operation of the immune system, as it can affect the host's immune status by producing metabolites such as SCFAs[69]. When the gut microbiota is disturbed, this dysbiosis can result in the development of various diseases by affecting adjacent or distant organs, including the liver[16]. Previous studies have indicated that tea polyphenols may exert toxicity on the intestines and kidneys of experimental animals at higher concentrations[70]. Our outcomes found that the high-dose tea polyphenol group increased the abundance of Proteobacteria while decreased the abundance of Firmicutes. In fact, an excess of Proteobacteria may be strongly linked with the activation of inflammatory responses[71], and the abnormally increased abundance of Proteobacteria could serve as a the potential diagnostic markers for dysbiosis and disease risk[72]. These fluctuations in the microbial community may trigger the activation of inflammatory responses. Therefore, at the phylum level, dysbiosis of the gut microbiota may be an important factor leading to intestinal inflammatory responses. Changes in the composition of the gut microbiota were also observed at the genus level in the high-dose tea polyphenol group, with an increase in the abundance of *Aeromonas* and *Pseudomonas*, while the abundance of *Lactobacillus* decreased. *Aeromonas* is a bacterium found in the environment that has the ability to infect both animals and humans[73]. *Pseudomonas* is a common pathogen of aquatic animals, responsible for causing various diseases and affecting the growth and health of fish[74]. In contrast, *Lactobacillus* is a probiotic with well-documented anti-inflammatory properties[75]. These changes further support the idea that elevated levels of tea polyphenols can cause dysbiosis of the gut microbiota in zebrafish. The increase in *Aeromonas* and *Pseudomonas* may increase the intestinal burden against pathogenic microorganisms, negatively impacting zebrafish health. Meanwhile, the decrease in *Lactobacillus* may reduce the intestinal anti-inflammatory capacity, further exacerbating the unfavorable state of the intestines. These results suggest that tea polyphenols may influence the homeostasis of the gut-liver axis by affecting the composition of the gut microbiota, thereby impacting liver health.

SCFAs are considered to have the potential to improve chronic inflammatory conditions through mechanisms that involve the inhibition of pro-inflammatory factors, including the production of TNF-α and IL-6. Specifically, propionic acid can reduce hepatic cholesterol synthesis, improve lipid metabolism, and possess anti-inflammatory and antimicrobial properties[76]. Butyric acid can also enhance the production of IL-10 in T cells of patients with inflammatory bowel disease (IBD)[77]. Therefore, SCFAs can prevent inflammation and protect intestinal health. In this study, a decrease in SCFAs such as propionic acid and butyric acid was observed in the intestines of zebrafish in the high-dose tea polyphenol group. This could be a reason for the decreased intestinal anti-inflammatory capacity and the imbalance in intestinal homeostasis. This modification was also reflected in intestinal tissue damage. Hence, high concentrations of tea polyphenols may inhibit the production of SCFAs in the intestines of zebrafish, thereby promoting the occurrence of intestinal inflammation. The correlation analysis results indicated that *Allorhizobium*, a type of *Rhizobium*[78], is positively associated with most SCFAs. Previous studies have also indicated that rhizobia can promote the production of SCFAs in the intestines of zebrafish[30]. Thus, the reduction in rhizobia abundance observed in the TP group in this study may be linked to the decline in SCFAs levels. The dysbiosis of the intestinal microbiota in the TP group resulted in an overall decrease in SCFAs levels and triggered intestinal damage. Therefore, we believe that high concentrations of tea polyphenols may cause intestinal damage and inflammatory responses by altering the composition of the intestinal microbiota and inhibiting the production of intestinal SCFAs.

Chronic inflammation resulting from various gastrointestinal diseases and oxidative stress can accumulate and lead to endotoxin production, thereby affecting liver function[79]. Dysbiosis of the gut microbiota can lead to the accumulation of products in the portal circulation, resulting in increased TNF expression in the liver and promoting liver inflammation[80]. Increased levels of cytokines such as TNF-α, IL-1β, and IL-6 in the liver may further increase intestinal permeability and exacerbate intestinal damage[81]. The liver transcriptome of zebrafish showed activation of *TNF-α*, *IL-1β*, and pro-apoptotic genes from the *bcl2* family, including *badb* and *PMAIP1*. The activation of TNF-α may limit the fat storage capacity of adipose tissue, resulting in fat accumulation in non-adipose tissues like the liver. Therefore, we speculate that the activation of pro-inflammatory genes in the high-dose tea polyphenol group may be due to the interaction of the gut-liver axis caused by intestinal damage. The activation of pro-inflammatory factors intensifies liver damage and disrupts lipid metabolism. Additionally, the accumulation of neutral fat in the liver promotes the expression of these pro-inflammatory factors through oxidized tea polyphenols, further worsening liver damage. These findings deepen our understanding of the toxicological mechanisms of tea polyphenols and also suggest that the impact of tea polyphenols on organismal health is a complex process involving the interaction of multiple metabolic pathways and cellular signaling pathways.

5. Conclusion

This study, for the first time, explored the hepatotoxic effects and mechanisms of high-dose tea polyphenols on zebrafish liver based on the gut-liver axis theory (Fig. 7). The results indicate that the addition of high-dose tea polyphenols to the zebrafish diet leads to lipid metabolism disorders in the liver, reduced antioxidant capacity, activation of pro-inflammatory and apoptotic factors, resulting in liver damage. Meanwhile, tea polyphenols regulate signaling pathways related to lipid metabolism and absorption and fatty acid degradation, promoting the uptake of fatty acids and cholesterol by liver cells, inhibiting fatty acid oxidation and excretion, causing liver fat accumulation, promoting liver inflammation, and leading to liver damage. On the other hand, high-dose tea polyphenols can alter the composition of the gut microbiota, reduce intestinal species diversity, increase the abundance of harmful

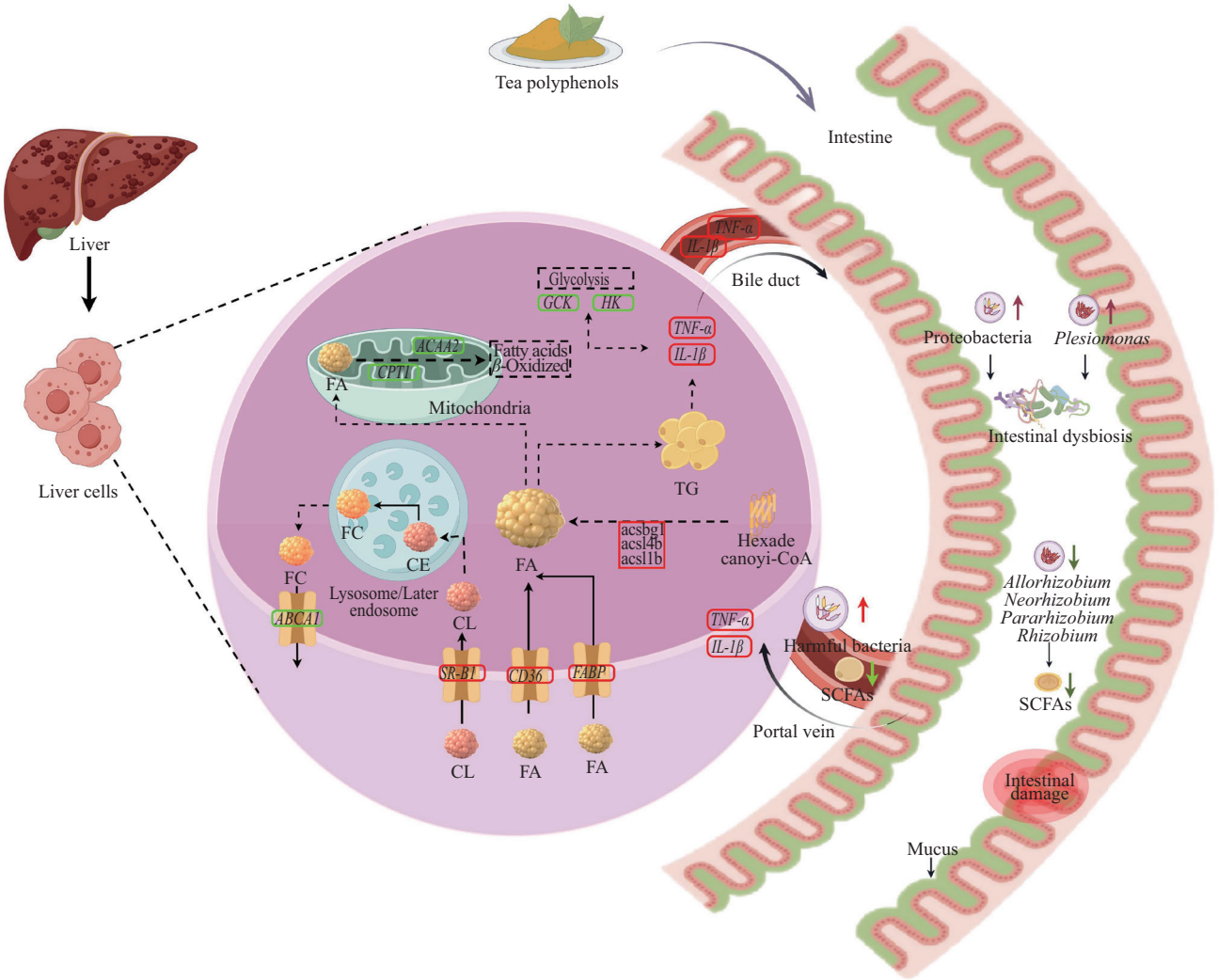

Fig. 7 Analysis of the hepatotoxic effects and mechanisms of high-dose tea polyphenols on zebrafish liver based on the gut-liver axis theory.

bacteria, and decrease the production of intestinal SCFAs. Due to the interaction of the gut-liver axis, the low levels of SCFAs and harmful bacteria in the intestines will enter the liver through the portal vein, thereby activating hepatic pro-inflammatory factors and causing liver inflammation. The accumulation of fat in the liver further promotes the expression of pro-inflammatory factors and transports them to the intestine through the bile duct, further exacerbating intestinal damage in zebrafish. In conclusion, our study suggests that long-term consumption of tea polyphenols can have negative effects on the intestines and liver, resulting in damage to both organs and accumulation of fat in the liver. However, since model organisms of different species have varying tolerance levels to tea polyphenols, further exploration is needed to determine the safe concentration of orally administered tea polyphenols in clinical settings.

Data availability

The sequence data supporting the result of this article in the NCBI (National Center for Biotechnology Information) under accession number PRJNA1107713 and PRJNA1107785.

Declaration of competing interests

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

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Compliance with ethical standards

This study adhered to the guidelines set forth by the Ministry of Science and Technology of the People's Republic of China in their "Guidelines for the Care and Use of Experimental Animals" (approval number: 2006-398) and received approval from the Animal Research and Ethics Committee of Beibu gulf University for all experimental animal manipulations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250398.

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