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Tremella polysaccharide mitigates high-fat diet-induced anxiety-like behavior through the microbiota-gut-brain axis

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ABSTRACT

High-fat diet (HFD) consumption induces gut microbiota dysbiosis and neuropsychiatric disorders, including anxiety. Previous research found that *Tremella* polysaccharide (TP) exhibited neuroprotective effects *in vitro* and *in vivo*. This study aimed to investigate the beneficial effects of TP on HFD-induced anxiety-like behaviors and elucidate the underlying mechanisms from the point view of the microbiota-gut-brain axis. Two groups of HFD-induced obese mice were orally gavaged with low dose (TPL, 40 mg/kg) and high dose (TPH, 400 mg/kg) of TP. A 12-week administration of TPH could significantly improve anxiety-like behaviors in HFD mice. In the hippocampus, microglia activation, the expression of blood-brain barrier (BBB) markers, and the levels of two neurotransmitters (serotonin and norepinephrine) were countered by TPH in mice consuming HFD. Furthermore, TPH improved the intestinal permeability and immune response of the enterocytes in HFD-fed mice. The gut microbiota dysbiosis induced by HFD was also rebalanced by TP treatments, especially in Proteobacteria and its lower taxa. The correlational analysis also suggested that shifts of some microbial genera were closely associated with body weight and the parameters of behavioral tests. Interestingly, fecal microbiota transplantation (FMT) results indicated that fecal microbiota from TPH-treated obese mice could prevent HFD-induced anxiety-like behaviors, suppressed microglia activation and intestinal permeability. In conclusion, the present study indicated that TP intake is a promising dietary intervention strategy to prevent HFD-induced anxiety via the microbiota-gut-brain axis.

1. Introduction

High-fat diet (HFD) consumption induces many medical conditions, such as obesity, diabetes, and metabolic syndrome, which are associated with increased risk of neuropsychiatric disorders[1-3]. Converging lines of evidence have indicated that HFD exposure contributes to significantly increased anxiety-like behavior[4-5]. The microglial dysfunction is implicated in brain diseases induced by long-term consumption of HFD[6]. As mononuclear phagocytes, microglia serves crucial role to maintain the homeostasis of central nervous system and induce its pathology[7]. Microglia produce pro-inflammatory cytokines (tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-6), which subsequently induce inflammation, neuronal loss, and brain damage. Additionally, microglia secrete neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), transforming growth factor-β1, and insulin-like growth factor 1 to support neuronal health and survival[8]. Furthermore, microglia continuously extend and retract their highly ramified processes to survey the surrounding synapses, and play a

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crucial role in synaptic formation and maturation[9]. According to these functions, microglia play a critical role in anxious behaviors induced by HFD intake.

Previous studies have reported that gut microbiota dysbiosis could contribute to neuroinflammation and hippocampal neurogenesis and brain development by activating microglia[10-11]. HFD consumption could significantly alter the composition and function of gut microbiota, which serves as a critical regulator for host intestinal homeostasis and immunity. The epithelial barrier and mucus layer in the gut prevent exterior antigens from entering the host. HFD consumption could reduce the thickness of the mucus layer and attenuate the expression of epithelial tight junction proteins, thereby exacerbating both local and systemic immune response[12-13]. In addition, the changes in intestinal mucus and epithelial barrier induced by HFD lead to increased intestinal permeability, allowing translocation of bacterial endotoxin (lipopolysaccharide, LPS) into the systemic blood circulation, which is regarded as endotoxemia[14]. An increase of systemic LPS could further trigger neuroinflammatory responses[15]. Consistently, endotoxemia has been reported to activate microglia and immune cells in the central nervous system and induce inflammatory responses in the hippocampus of mice[16]. Altogether, the evidence has indicated that dysregulation of the microbiota-gut-brain axis triggered by HFD consumption results in neuroinflammation, microglial and synaptic damage, subsequently inducing brain function decline.

Given the popularity of "Western style diet" in modern life and an increasing global burden caused by emotional dysfunction, it is imperative to find effective therapeutic strategies against HFD-induced anxiety-like behavioral disorder. The Lancet Commission also reported that more than one-third of global dementia cases may be preventable through adjusting lifestyle factors, including diet[17]. Edible mushrooms have been an important part of the human diet for thousands of years, and over 100 varieties have been cultivated for their potential human health benefits. *Tremella fuciformis* is an edible medicinal mushroom well known as "Yin'er" or "Baimu'er" and has been consumed in China for thousands of years. As one of its active components, *Tremella* polysaccharide (TP) exhibits many pharmacological effects, such as hypoglycemic and hypolipidemic effects, anti-obesity effects, and, interestingly, neuroprotective effects[18-19]. For example, the purified polysaccharide of *T. fuciformis* (TL04) exhibits a neuroprotective effect on the glutamate-induced differentiated PC-12 cell damage[20]. Also, previous evidence has demonstrated that TP shows neurotrophic effects on the same neuronal cell line[21]. Apart from the *in vitro* protective effects of TP on neuronal cells, it also restores the cognitive function via regulation of the cAMP response element-binding protein signaling pathway and cholinergic system in the hippocampus of trimethyltin-induced rats[22].

Here we found that HFD-induced body weight gain linked to anxiety-like behavior. Interestingly, TP treatment in obese mice improved this neuropsychiatric deterioration. We further assessed the ability of TP to alter gut microbiota, intestinal permeability, and functions of neuronal cells in the hippocampus. This study hypothesizes that TP supplementation could prevent HFD-induced anxiety-like behavior through the microbiota-gut-brain axis.

2. Materials and methods

2.1 Animals

Male C57BL/6J mice (7 weeks old) were purchased from Weitong Lihua Co., Ltd. (Beijing, China). Five mice were housed per cage under standard conditions (12 h light-dark cycle, relative humidity at (50 ± 15)%, temperature (22 ± 2) °C). Mice were randomly divided into five groups based on the treatment they received. ND: mice fed a normal diet (ND) (10% of energy from fat, D12450J, Changzhou SYSE Bio-tec) and distilled water; ND + TPH: mice fed a ND and gavaged with 400 mg/kg of TP daily (Bo InnoHealth Biotech) in distilled water; HFD: mice fed a HFD (60% of energy from fat, PD6001, Changzhou SYSE Bio-tec) and distilled water; HFD + TPL: mice fed HFD and gavaged with 40 mg/kg of TP daily; HFD + TPH: mice fed HFD and gavaged with 400 mg/kg of TP daily. All mice underwent behavioral tests after 12 weeks of intervention. Subsequently, blood samples were drawn by cardiac puncture, and tissues were collected after euthanasia using carbon dioxide. Experimental procedures used in this study were approved by the Animal Subjects Ethics Committee of the Hong Kong Polytechnic University (ASESC No. 21-22/87-ABCT-R-OTHERS).

2.2 TP preparation

The polysaccharides were extracted and supplied by Bo InnoHealth Biotechnology Co., Ltd. (Sha Tin, Hong Kong). Briefly, 100 times pure water was added, and the fruit bodies were boiled at 100 °C for 2 h. This procedure of extraction was repeated twice. Following filtering and centrifuging, the supernatant was obtained and mixed with four times of anhydrous ethanol. The polysaccharide was precipitated using centrifuging. We resolved TP in pure water and then removed the protein using the trichloroacetic acid method. The purity of TP was 69.15% determined by NY/T 1676–2008 *Determination of crude mushroom polysaccharides*.

2.3 Behavioral measurements

The open field test (OFT), elevated plus maze (EPM), and marble burying are the approaches to evaluate anxiety- or depression-like behaviors by assessing spontaneous locomotor activity in rodents. Briefly, in OFT and EPM, the mice were carefully placed in the center area of the maze and allowed to explore for 5 min freely. The parameters, such as total distance, and the distance, time in the center or open arms were recorded with EthoVision XT video-imaging system. In marble burying test, the mice were put into a single cage with 10 glass beads on top of the bedding. The number of beads the mice buried in a 30 min session was scored. The details of behavioral tests have been reported previously[5].

2.4 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from the colon and brain were extracted using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Up to 1 μg of total RNA was reversed transcribed into cDNA using a reverse transcription kit (Takara PrimeScript RT Master Mix, Dalian,

China). Subsequently, the mRNA expression was determined by qPCR using the SYBR green PCR kit (Biotium Forget-Me-Not™ EvaGreen® qPCR Master Mix, Fremont, CA, USA) in a real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The relative mRNA level was determined by the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Table 1.

2.5 Alcian blue staining

Transverse colonic tissues were placed in methanol-Carnoy's fixative solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 3 h at room temperature, followed by dehydration and embedding in paraffin. To measure the thickness of the colonic mucus layer, the sectioned colonic samples were deparaffinized and hydrated in distilled water. They were then placed in 3% acetic acid solution for 5 min, followed by staining in alcian blue solution. The samples were rinsed in distilled water and counterstained with a filtered nuclear fast red solution.

2.6 Immunohistochemical and immunofluorescent staining

The embedded colon and brain were cut into 5 and 20 μm sections, respectively. Sodium citrate antigen retrieval solution (pH 6.0) was used to retrieve antigen, and 3% peroxide-methanol was used to quench the samples. After washing with PBS, the sections were incubated with primary antibody against ionized calcium binding adaptor molecule 1 (Iba1) (1:500, Servicebio, Wuhan, China), occludin (1:1 000, Abcam, Cambridge, UK), zona occludens 1 (ZO-1) (1:1 000, Servicebio, Wuhan, China), and mucin 2 (MUC2) (1:200, ABclonal, Wuhan, China) at 4 °C overnight. The corresponding secondary antibody was applied to incubate the samples at room temperature for 30 min. As for immunohistochemistry staining, the sections were colored with a DAB kit (Servicebio, Wuhan, China). The images were observed under a light microscope. As for immunofluorescent staining, the sections were counter-stained in PBS with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) to visualize the cell nuclei. The images were captured by a Nikon AX NIS-Elements 5.4 confocal microscope (Nikon, Tokyo, Japan).

2.7 Western blot

Hippocampal tissues were mixed with beads and homogenized in ice-cold RIPA lysis buffer containing a complete EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor. The collected supernatant was quantitated using a BCA assay. The obtained protein samples were loaded and separated in sodium dodecylsulfate-polyacrylamide gel electropheresis and transferred onto PVDF membranes, followed by blocking and incubating with primary antibody against postsynaptic density protein-95 (PSD-95) (1:1 000, Cell Signaling Technology, Boston, USA) and BDNF (1:200, Santa Cruz, Heidelberg, Germany). Subsequently, membranes were washed in TBST and incubated with HRP-linked anti-mouse IgG secondary antibody (1:2 000, Cell Signaling Technology, Boston, USA) at room temperature for 2 h. Protein bands were detected with Clarity™ ECL Western blot substrate (Bio-Rad, California, USA) and visualized using ChemiDoc Touch imaging system (Bio-Rad, California, USA).

2.8 Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-6, IL-1β, and LPS were determined using ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China). The serotonin (5-HT) and norepinephrine (NE) levels in hippocampus were measured by the ELISA kit (Xinle Biotechnology, Shanghai, China).

2.9 16S rRNA sequencing analysis

Fecal samples were collected from the rectum before euthanasia. Microbial genomic DNA was extracted using the TIANamp stool DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The extracted DNA was checked on 1% agarose gel, and DNA concentration and purity were determined with a NanoDrop UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable regions V3–V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) at Majorbio BioPharm

Table 1
The primers used in this study in detail.

Gene	Forward	Reverse
Iba1	5′-TGATGAGGATCTGCCGTCCAAACT-3′	5′-TCTCCAGCATTCGCTTCAAGGACA-3′
PSD-95	5′-TCTGTGCGAGAGGTAGCAGA-3′	5′-AAGCACTCCGTGAACTCCTG-3′
BDNF	5′-CTGGATGAGGACCAGAAG-3′	5′-CCTCCAGCAGAAAGAGTAG-3′
TNF-α	5′-TAGCCAGGAGGGAGAACAGA-3′	5′-TTTTCTGGAGGGAGATGTGG-3′
IL-6	5′-CCGGAGAGGAGACTTCAC-3′	5′-TCCACGATTTCCCAGAGA-3′
IL-1β	5′-TTGAAGAAGAGCCCATCCTC -3′	5′-CAGCTCATATGGGTCCGAC -3′
MCP-1	5′-TCACTGAAGCCAGCTCTCTCT -3′	5′-GTGGGGCGTTAACTGCAT-3′
CD68	5′-AAAGGCCGTTACTCTCCTG -3′	5′-TGTGGCATGAGAAATTGTGG -3′
ZO-1	5′-ACCCGAAACTGATGCTGTGGATAG-3′	5′-AAATGGCCGGGCAGAACTTGTGTA-3′
Occludin	5′-ATGTCCGGCCGATGCTCTC-3′	5′-TTTGGCTGCTCTTGGGTCTGTAT-3′
Reg3γ	5′-TTCCTGTCCTCCATGATCAAA-3′	5′-CATCCACCTCTGTTGGGTTC-3′
CLDN7	5′-CCTGATAGCGAGCACTGCCATC-3′	5′-GTGACGCACTCCATCCAGAGC-3′
CLDN12	5′-TGCTTGGAGAAACGCTGATT-3′	5′-GTGGCTGCGTGGACATCT-3′
CLDN15	5′-ACTCCGCTGCACCAACGTGG-3′	5′-ACGGCGTACCACGAGATAGCCA-3′
CLDN2	5′-GGCTGTTAGGCACATCCAT-3′	5′-TGGCACCAACATAGGAACTC-3′
CLDN4	5′-CGCTACTCTTGCCATTACG-3′	5′-ACTCAGCACACCATGACTTG-3′
GAPDH	5′-AGAAGGTGGTGAA GCAGGCATC-3′	5′-CGAAGGTGGAAGAGTGGGAGTTG-3′

Note: *MCP-1*, monocyte chemotactic protein-1; *CD68*, cluster of differentiation 68; *Reg3γ*, regenerating family member 3γ; CLDN, claudin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Tech. Co. (Shanghai, China). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA database Silva v138 using confidence threshold of 0.7. The unifrac distance matrix performed by QIIME2 software was used for unweighted UniFrac principal coordinates analysis (PCoA). The microbial diversity in faecal samples was estimated using the α-diversity that includes the Sobs index. The 16S rRNA gene amplicon sequencing and analysis were conducted by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.10 Fecal microbiota transplantation (FMT)

For gut microbiota eradication, HFD-fed mice were treated with an antibiotic solution (ATB) containing 0.5 g/L vancomycin hydrochloride, 1 g/L neomycin sulfate, 1 g/L ampicillin sulfate, and 1 g/L metronidazole added into their sterile drinking water for 10 d. The fecal samples from donor mice in the four diet groups (ND, ND + TPH, HFD, and HFD + TPH) were collected. Subsequently, fresh fecal samples were resuspended in sterile PBS (15 mL/g of feces) and then vigorously vortexed for 5 min, followed by standing to precipitate particles for 5 min. The supernatant was used to colonize ATB-treated mice by oral gavage daily for 12 weeks.

2.11 Data analysis

Data were analyzed using the statistical package SPSS (version 20, IBM Corp., Chicago, IL, USA). All values represent the means ± standard error of mean (SEM) of six to eight independent experiments. Data were then compared using one way analysis of variance (ANOVA) with Duncan's multiple range analysis. For 16S rRNA gene sequence analysis, all reads were deposited and grouped into OTUs at a sequence identity of 97%. Statistical analyses were conducted with STAMP, and functional differences in orthologs among groups were assessed by a one-way ANOVA followed by post hoc Tukey-Kramer or Kruskal-Wallis test for multiple comparisons.

3. Results

3.1 TP improved the metabolic syndrome parameters in HFD-fed mice

Long-term HFD consumption reduces obesity and its associated metabolic syndrome. In our study, all mice were weighed just before their designated diet began and at the end of the experiment. The experimental design is shown in Fig. 1A. A 12-week supplementation of a high dose of TP (TPH) significantly decreased the body weight of HFD-fed mice, although their initial body weight was comparable among all groups (Fig. 1B). In addition, the level of fasting glucose was remarkably reduced in obese mice supplemented with TPH when compared with those of HFD-fed mice (Fig. 1C). Long-term consumption of HFD could increase the weight of several tissues, including the liver, subcutaneous and epididymal fat. However, the weight gain of those tissues in HFD-fed mice was decreased by TPH treatment (Fig. 1D). These results indicated that TP, especially the high dose, exhibited an anti-obesity effect in HFD-fed mice.

3.2 TP mitigated HFD-induced anxiety-like behavior in obese mice

Behavioral tests, including OFT, EPM, and marble burying tests, were conducted in this study before euthanasia to evaluate the anxiety-like conditions in mice. In OFT, the mice showing anxiety-like behavioral disorders spent less time in the central area than normal mice did. In the present study, HFD consumption could reduce the total distance and speed significantly in comparison with

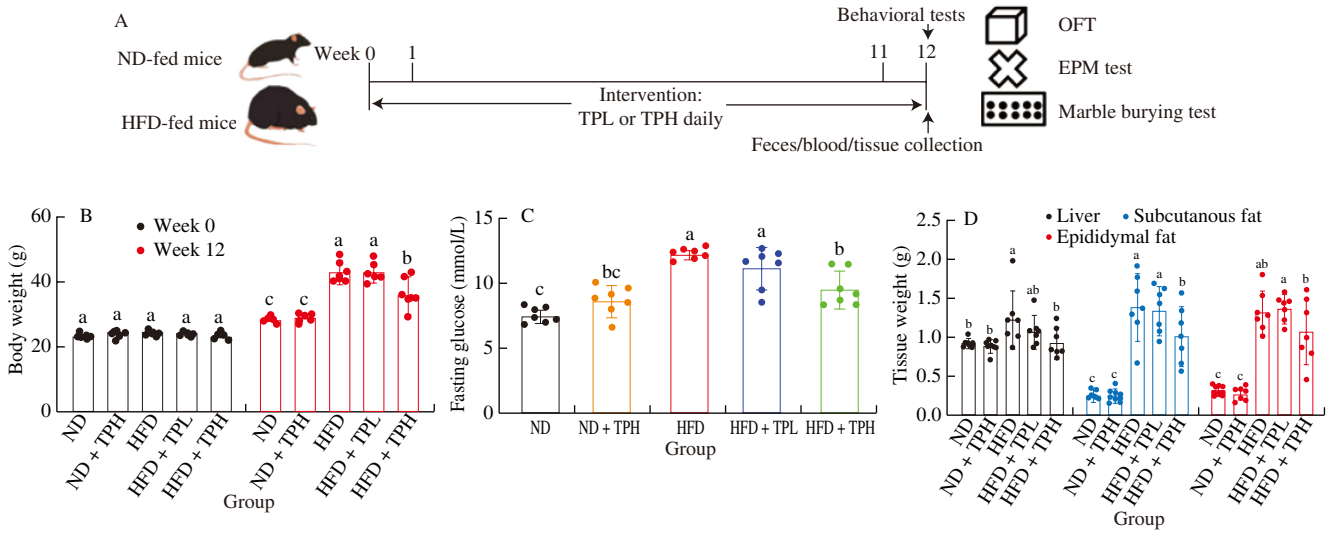


Fig. 1 TP improved the phenotype of the metabolic syndrome parameters in HFD-fed mice. (A) Experimental design; (B) Body weight (n = 6); (C) Fasting glucose (n = 7); (D) Tissue weight (n = 7). Data presented as means ± SEM. Bar values with different letters were significantly different by Tukey's multiple range test ($P < 0.05$).

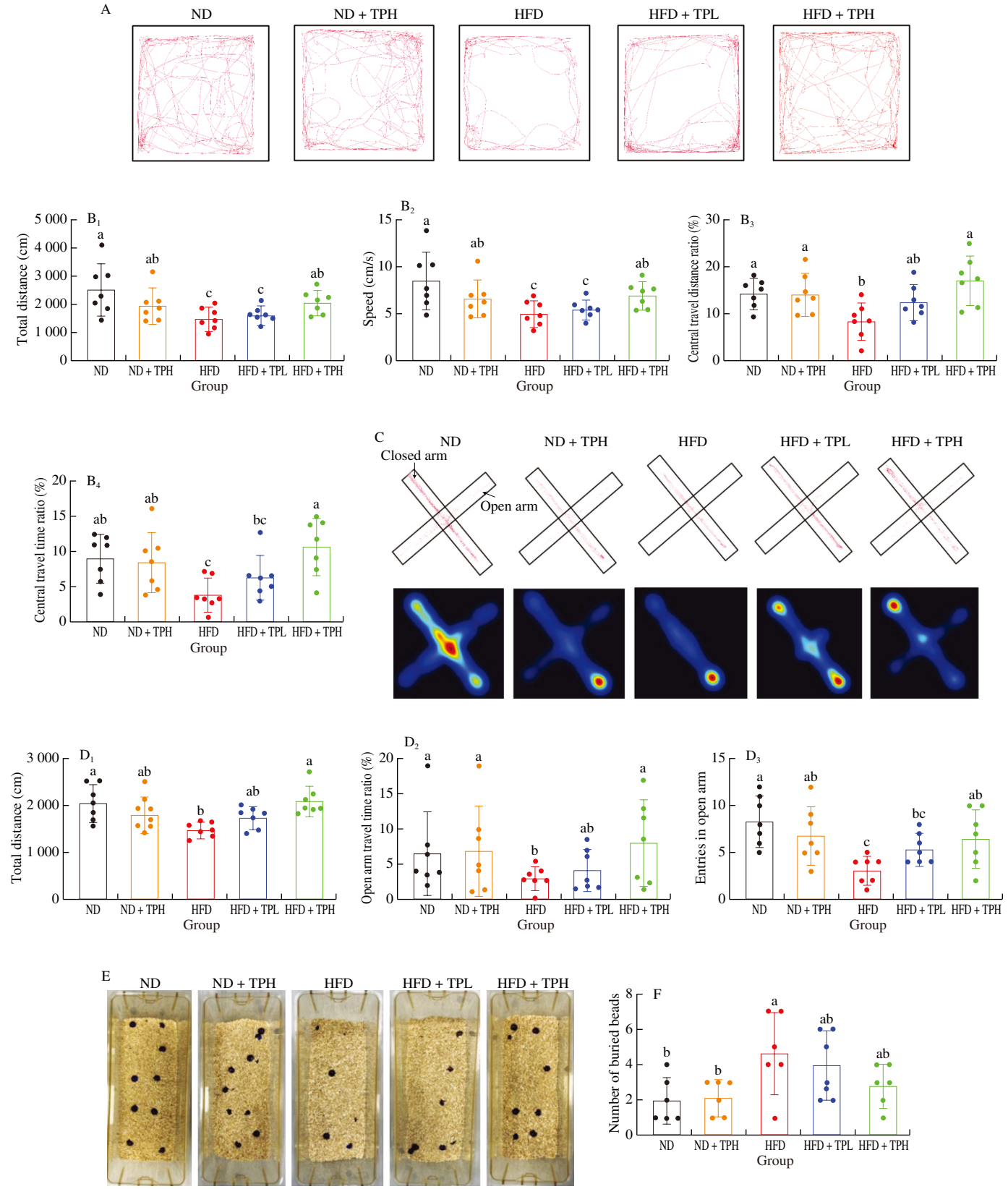


Fig. 2 TP mitigated HFD-induced anxiety-like behavior in HFD-fed mice. (A) Representative track images in OFT; (B) Quantification of total distance, speed, distance and time covered in inner zone of the arena ($n = 7$); (C) Representative track images and heatmap images in EPM test; (D) Quantification of total distance, time ratio in open arm, and entry frequency in open arm ($n = 7$); (E) Representative images in marble burying test; (F) Quantification of the number of buried beads ($n = 6$). Data presented as means ± SEM. Bar values with different letters were significantly different by Tukey's multiple range test ($P < 0.05$).

the mice fed with ND. It is of interest that those parameters in HFD-fed mice treated with TPH were notably increased. Importantly, the distance traveled and the time spent in the central zones of OFT were significantly increased in HFD mice with TPH supplementation compared to the HFD mice without TP treatment, indicating the role of TP in amelioration of HFD-induced anxiety (Figs. 2A and B). In the EPM test, mice with anxious tendencies would spend less time in open arms than normal mice. In this study, HFD-fed mice were observed to have significantly decreased total distance, and shorter time and lower entry frequency in the open arm compared with those in the ND group, while TPH greatly increased these parameters in HFD mice (Figs. 2C and D). In the marble burying test, mice with poorer social skills buried more beads than normal mice. Our results showed that mice supplemented with HFD buried more beads than ND-fed mice, while TPH treatment reduced the number of buried beads (Figs. 2E and F). In all three behavioral tests, the lower dose of TP had a limited effect on improving anxiety-like behavioral disorders, while TPH improved HFD-induced anxiety-like behavior in mice significantly.

3.3 TP suppressed the microglia activation and improved synapse in the hippocampus of HFD-fed mice

Activation of microglia is critical in the pathogenesis of brain diseases and neuroinflammation. We used double immunofluorescence staining of Iba1 and PSD-95 in all groups to determine the spatial location of microglia and synapses. The expression of Iba1 was increased while the expression of PSD-95 was decreased in the HFD group compared with those in ND and TPH groups, suggesting that TP supplementation, especially in high doses, could attenuate the activation of microglia but increase that of synapses (Figs. 3A and C_1-C_2). The immunohistochemical staining result also indicated the increased

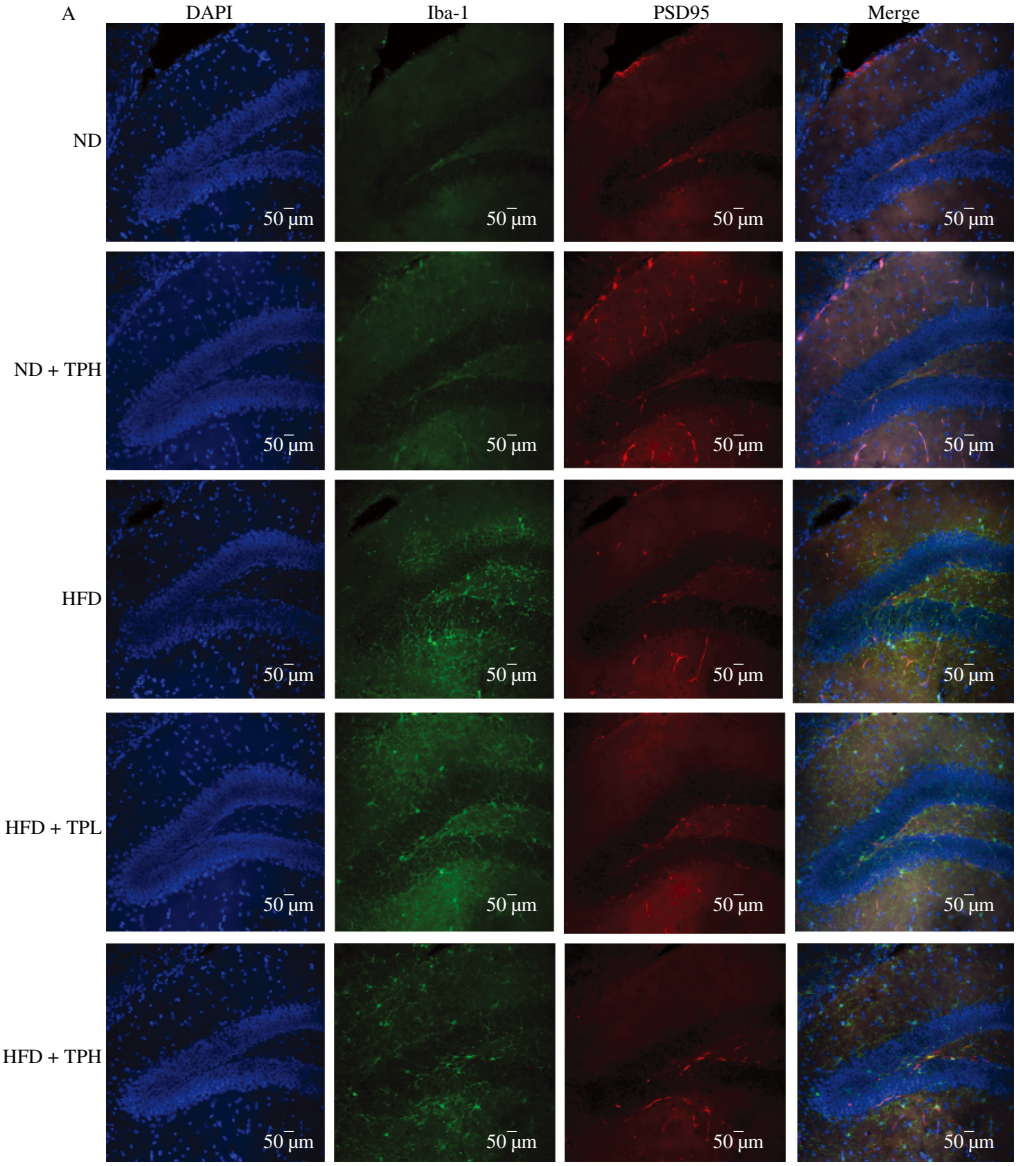


Fig. 3 Effects of TP consumption on the function of brain in HFD-fed mice. (A) The immunofluorescent staining of Iba1 and PSD-95 in hippocampus. (B) The immunohistochemical staining of Iba1 protein in hippocampus. Quantification results of (C_1 and C_2) immunofluorescent and (C_3) immunohistochemical stainings; (D) Protein levels of PSD-95 and BDNF in hippocampus; (E) Relative mRNA levels of *Iba1*, *PSD-95* and *BDNF* in hippocampus; (F) Relative mRNA levels of *Occludin*, *ZO-1*, and *Claudin 5* in hippocampus; (G) Relative mRNA levels of inflammatory parameters; (H) The levels of neurotransmitters, 5-HT and NE in hippocampus ($n = 4$). Data presented as means ± SEM. Bar values with different letters were significantly different by Tukey's multiple range test ($P < 0.05$).

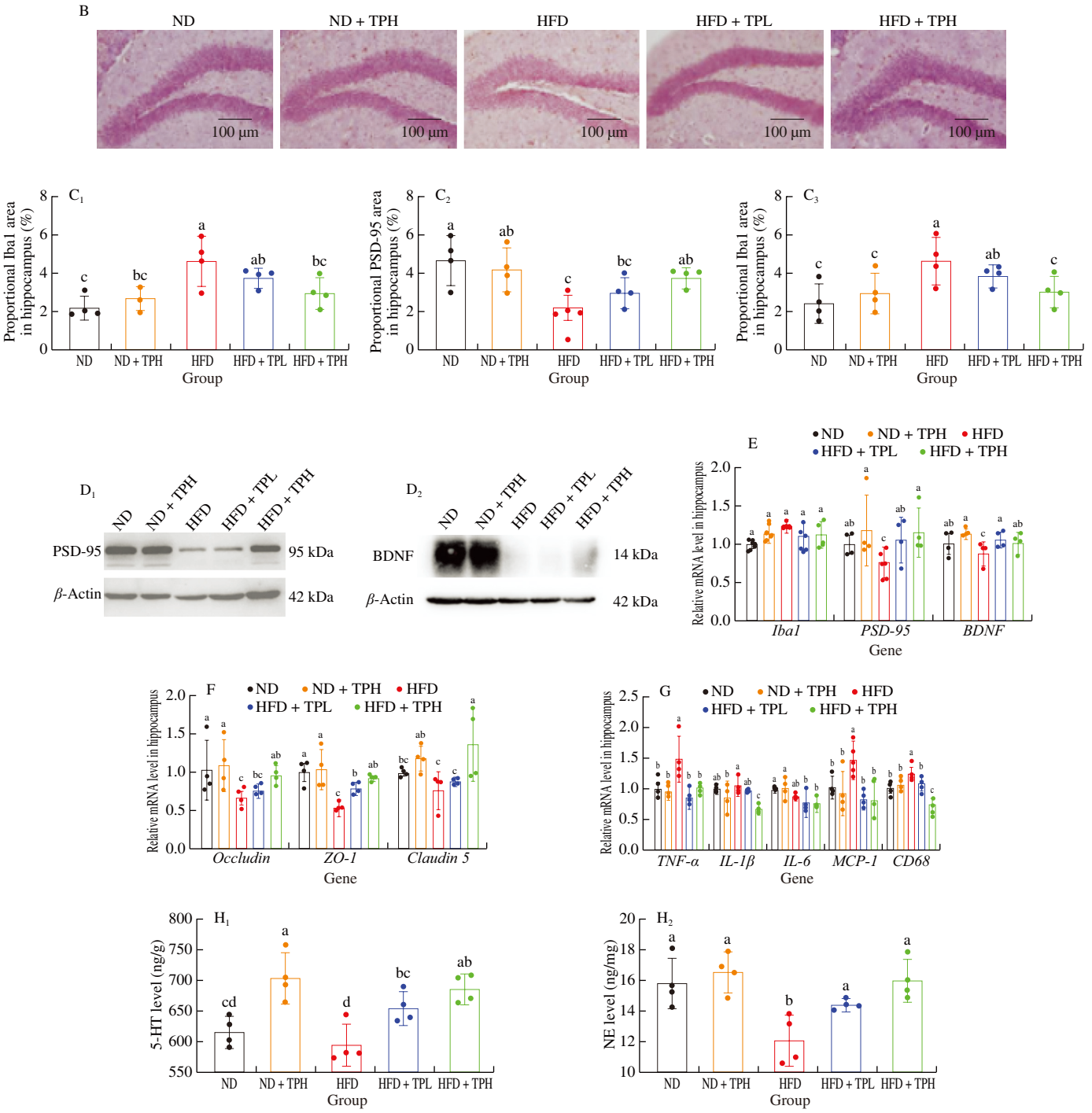


Fig. 3 (*Continued*)

microglia activation by HFD while it is decreased in obese mice treated with TPH (Figs. 3B and C_3). Western blot analysis showed decreased expression of PSD-95 and BDNF in HFD and HFD + TPL groups compared to those in ND-fed mice, whereas their expression levels were improved in obese mice with TPH supplementation (Fig. 3D). Consistent with the results in immunofluorescence, immunohistochemistry, and Western blot, the mRNA levels of *PSD-95* and *BDNF* by RT-qPCR in all five groups exhibited the same trend (Fig. 3E). Altogether, the impaired expression of microglia marker, postsynaptic dense protein, and BDNF in the hippocampus due to HFD deterioration were improved by TPH.

To further examine the integrity of the blood-brain barrier (BBB) in the hippocampus, the mRNA levels of key tight junction proteins and inflammatory factors were measured. The transcript levels of *Occludin*, *ZO-1*, and *Claudin5* were lower in HFD-fed mice than the control ones. Nevertheless, TPH increased the levels of all three genes in obese mice, although TPL only improved the mRNA level of *ZO-1* (Fig. 3F). On the other hand, the mRNA levels of inflammatory genes, *TNF-α* and *MCP-1*, were increased in mice fed with HFD, while they were significantly decreased by TPH supplementation. However, the transcript level of other inflammatory genes, especially *IL-6*, did not show differences in those groups (Fig. 3G). Furthermore, the levels

of two neurotransmitters derived from gut microbiota, namely 5-HT and NE, were measured in this study. Though the levels of 5-HT were similar in ND and HFD mice, TP supplementation increased the level of 5-HT at both dosages in mice fed with either ND or HFD. The level of NE was decreased in HFD-induced obese mice but improved significantly by TPH and TPL in HFD-fed mice (Fig. 3H). Overall, these results indicated that TP improved the parameters of BBB at mRNA level, attenuating inflammatory responses, and modulating the pathways of neurotransmitters.

3.4 TP improved intestinal permeability and immune response of the enterocytes in HFD-fed mice

In this study, HFD resulted in a shorter colon length in the mice than that in the ND-fed mice, whereas TPL and TPH could increase the colon length by 9.64% and 20.25% with significant differences (Figs. 4A and B). Moreover, HFD decreased the thickness of colonic mucus compared with those of ND-fed mice, as evidenced by mucus-staining and MUC2 immunofluorescence staining of the enterocytes. TPL and TPH treatments increased the thickness of the mucus layer in HFD-induced mice (Figs. 4C and D). Additionally, both doses of TP increased the expressions of occludin and ZO-1 at both protein and transcript levels in the colon in comparison with those of HFD-fed mice (Figs. 4E-H). Apart from *Occludin* and *ZO-1*, the mRNA levels of other colonic-specific tight junction genes, including *CLDN2*, *CLDN4*, *CLDN7*, *CLDN12*, *CLDN15*, *ICAM-1*, and *VCAM-1*, were also determined. The transcript levels of *CLDN12*, *CLDN15*, and *VCAM-1* were significantly decreased by HFD supplementation when compared with those in ND-fed mice, whereas they were partially elevated after TPH supplementation (Fig. 4I). The deterioration of colonic mucus and tight junction proteins might affect the expression of antimicrobial peptide Reg3γ and inflammatory parameters. We observed that TPH increased the mRNA expression of *Reg3γ* relatively but not significantly while it significantly decreased the endotoxemia (LPS) in plasma in comparison with the mice in the HFD group (Figs. 4J-K). Furthermore, the mRNA expressions of inflammatory parameters, *TNF-α*, *IL-1β*, *IL-6*, *MCP-1*, and *CD68*, were attenuated by both TPL and TPH in obese mice (Fig. 4L). Consistently, plasma inflammatory factors were also lowered by TPL and TPH in HFD-induced mice (Fig. 4M). Therefore, we concluded that TP could protect the colon from HFD-induced deterioration.

3.5 TP rebalanced HFD-induced gut microbiota dysbiosis

A growing body of evidence suggests that the community of microorganisms throughout the gastrointestinal tract is associated with anxiety disorders. 16S rRNA gene sequencing was conducted to evaluate the effect of TP on the gut microbiota in HFD-induced anxious mice. The composition of microbiota in all groups was similar at OTU level before treatments at week 0, while distinct separation of OTU clusters among the five groups at the last week was observed (Fig. 5A). Similarly, the α-diversity based on Shannon index did not show much difference in mice at week 0, whereas HFD-fed mice with or without TP treatments exhibited less diversity than ND-fed mice at week 12 (Fig. 5B). At the phylum level, all the HFD-fed mice, with or without TP treatments, showed an

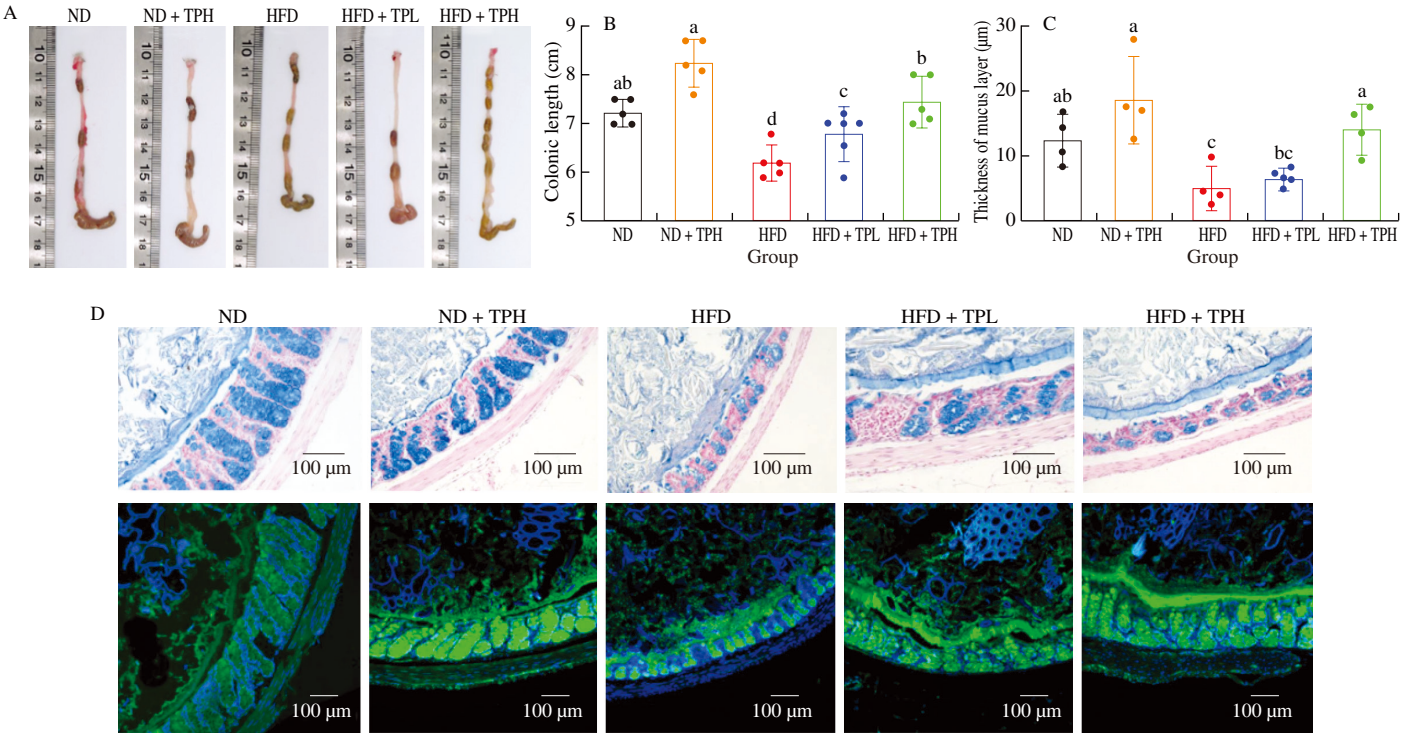
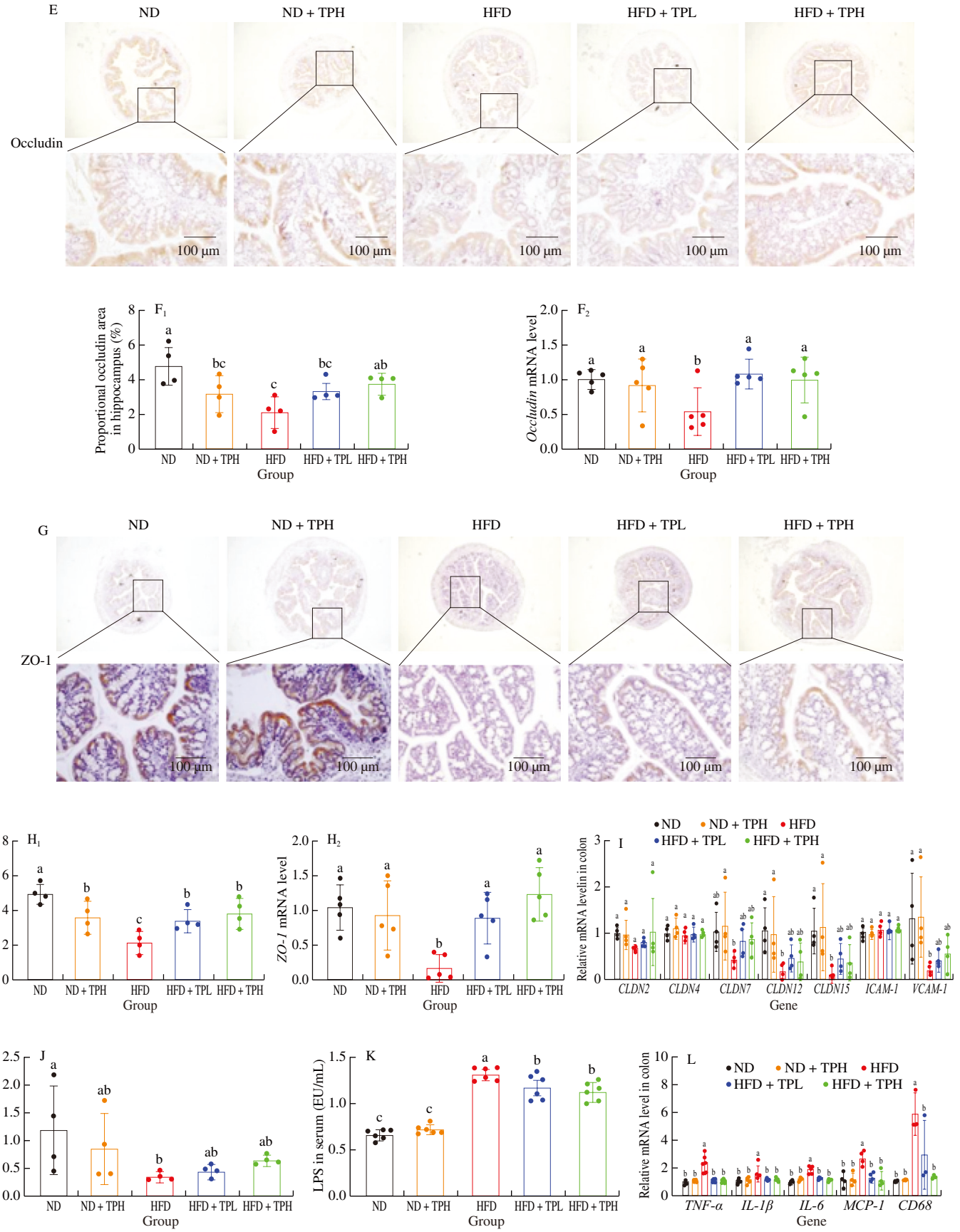


Fig. 4 Effects of TP consumption on the structural and functional changes of colon in HFD-fed mice. (A) Representative colon image for each group; (B) Quantification of colon length ($n = 5$); (C) Quantification of mucus thickness ($n = 5$); (D) The alcian blue staining and immunofluorescent staining of mucus layer; (E) The immunohistochemical staining of the protein occludin; (F) Relative mRNA level of *Occludin* ($n = 5$); (G) The immunohistochemical staining of the protein ZO-1; (H) Relative mRNA level of *ZO-1* ($n = 5$); (I) Relative mRNA levels of tight junction proteins in colon ($n = 4$); (J) Relative mRNA level of *Reg3γ* ($n = 4$); (K) Levels of serum LPS ($n = 6$); (L) Relative mRNA levels of inflammatory parameters in colon ($n = 5$); (M) Levels of inflammatory parameters in serum ($n = 5$). Data presented as means ± SEM. Bar values with different letters were significantly different by Tukey's multiple range test ($P < 0.05$).



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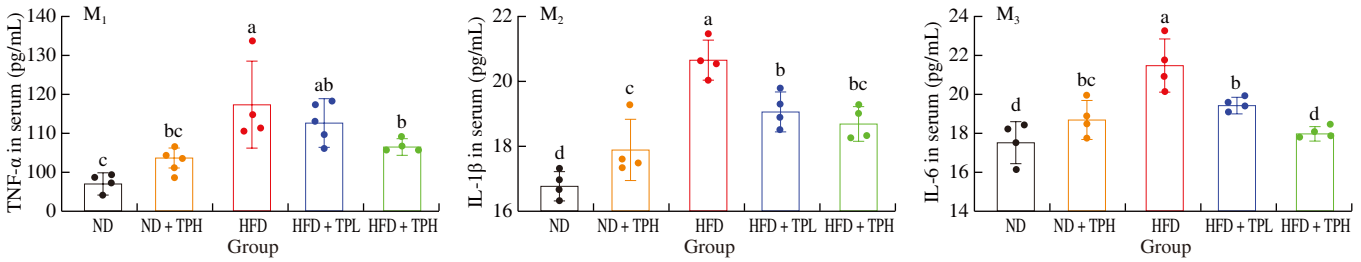

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increased abundance of Firmicutes and a decreased abundance of Bacteroidetes. The abundance of Actinobacteria in the HFD group was decreased, while TPH treatment increased their abundance of HFD-fed mice instead. Proteobacteria containing various pathogenic bacteria were enriched in HFD-fed mice without TP treatments. However, TPH could rebalance their abundance in HFD-induced mice (Figs. 5C and D). Many species in Proteobacteria, such as Enterobacteriaceae and *Desulfovibrio*, have been characterized as proinflammatory species and may be related to anxiety-like behavioral disorders. We also observed that many communities at the genus level changed significantly between ND- and HFD-fed mice (Fig. 5E). The abundance of *Lactobacillus* was significantly decreased in the HFD group compared to that in the ND group, although TPL and TPH treatments could not rebalance their abundance in obese mice. The abundance of *Bifidobacterium*, a probiotic genus, was much lower in the HFD group than that in ND-fed mice, implying the HFD did not favor the growth of *Bifidobacterium*. Interestingly, TPH induced a relative increase in their abundance, albeit not reaching statistical significance. We also found that TPH could rebalance the abundance of *Allobaculum*, an observation that was not widely

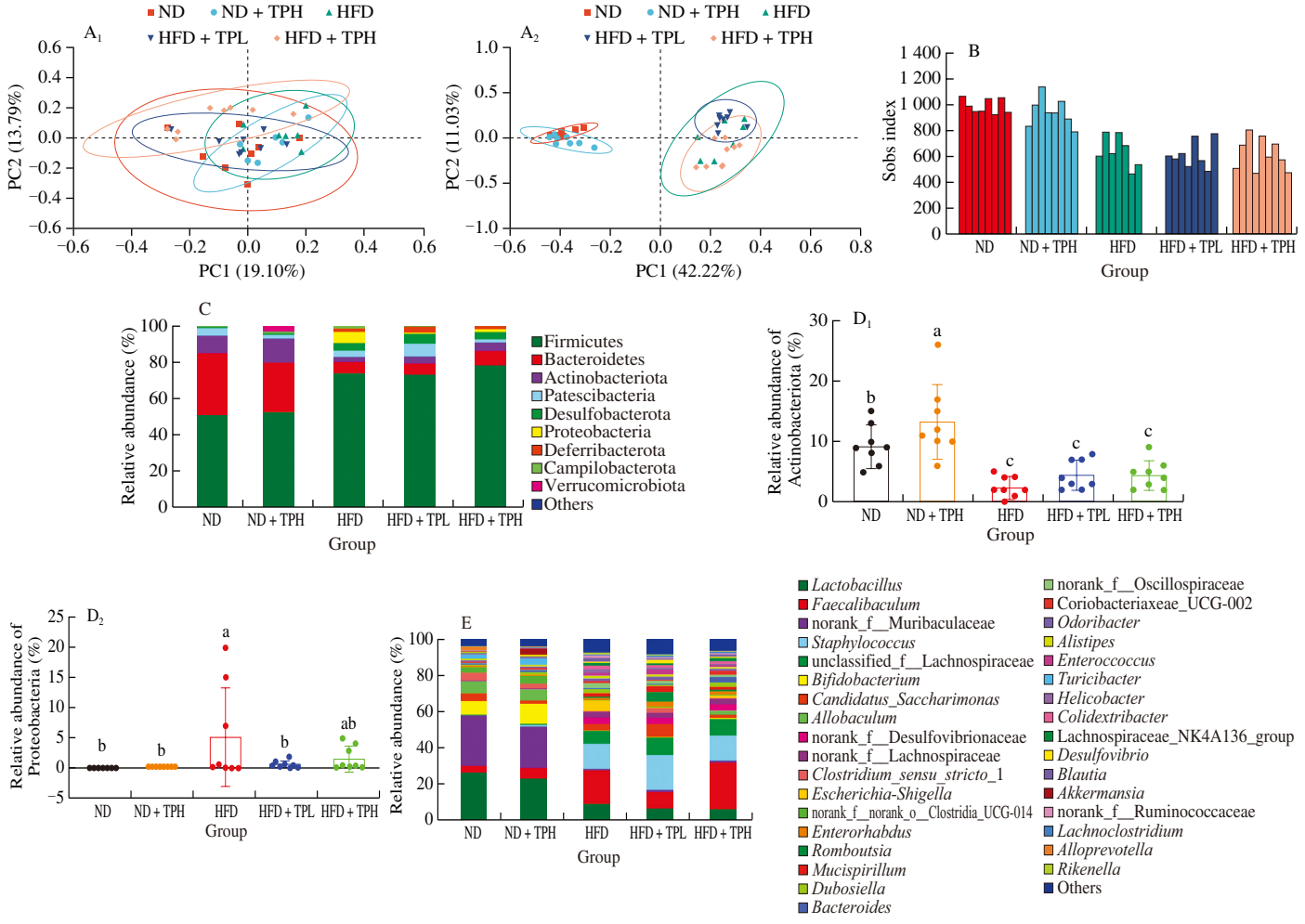
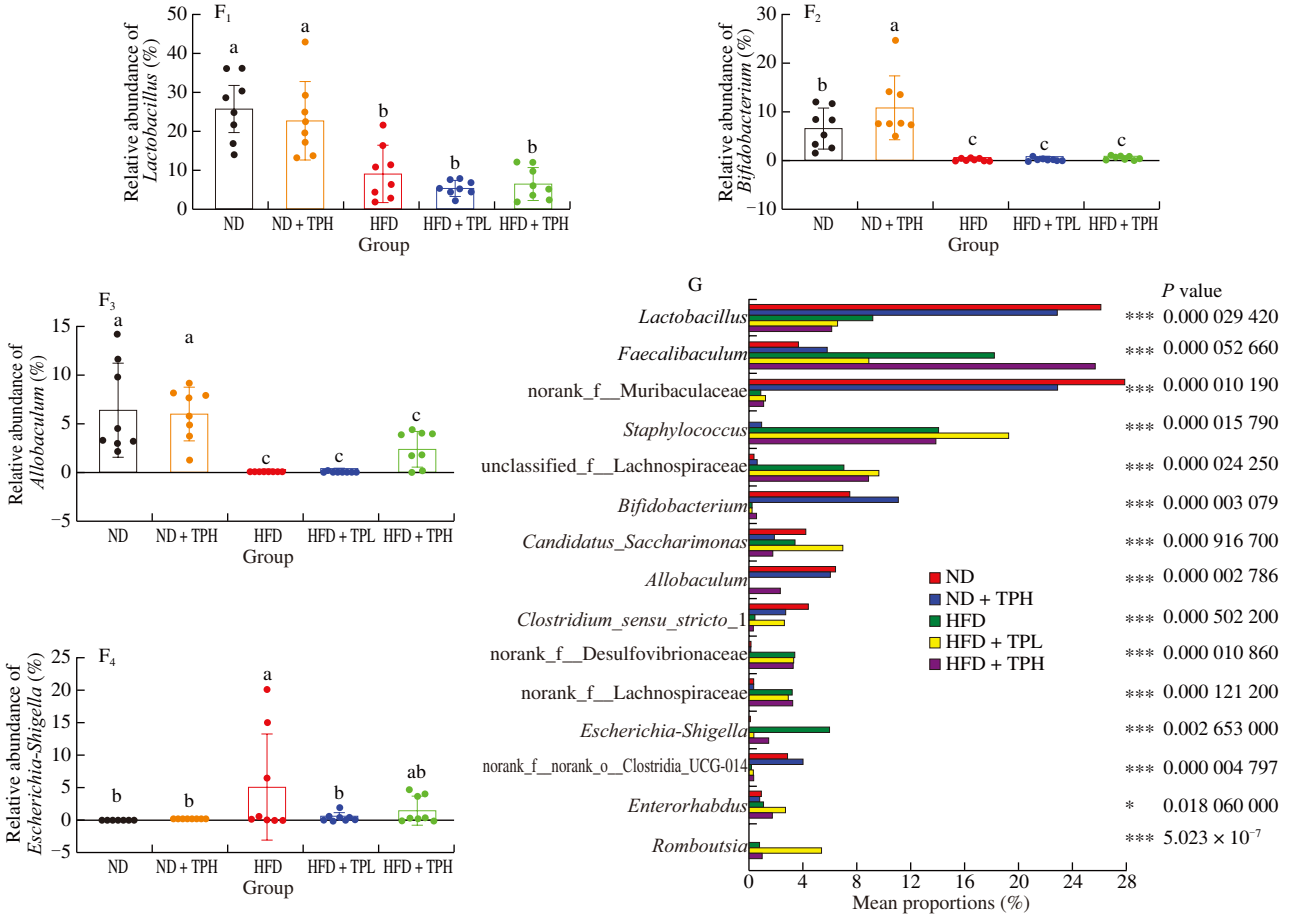


Fig. 5 Effects of TP consumption on the structural alterations of gut microbiota in HFD-fed mice. (A) Principal coordinates analysis plot of unweighted UniFrac distances (PCoA) at (A_1) week 0 and (A_2) week 12; (B) α-Diversity measured by Sobs index at week 12; (C) Community alterations at phylum level; (D) Relative abundance of phyla, Actinobacteria and Proteobacteria; (E) Community alterations at genus level; (F) Relative abundance of genera, *Lactobacillus*, *Bifidobacterium*, *Allobaculum*, and *Escherichia-Shigella*; (G) Differential abundance analysis at genus level. One-way ANOVA with a post hoc Kruskal-Wallis *H* test. * $P < 0.05$; *** $P < 0.001$ ($n = 8$). Data presented as means ± SEM. Bar values with different letters were significantly different by Tukey's multiple range test ($P < 0.05$).



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reported. Moreover, both TPL and TPH attenuated the abundance of *Escherichia-Shigella*, which was enriched in HFD group and reported to be associated with many diseases, suggesting their beneficial effect on maintaining a healthy microbial gut flora (Figs. 5F and G).

The community heatmap analysis further showed that the microbiota in the control mice of ND and ND + TP groups were closely linked to each other, while the relationship of gut microbiota among HFD-fed mice, especially the two groups treated with TP, was close (Fig. 6A). KEGG functional orthologs predicted by PICRUSt identified potential functions at level 3 (Fig. 6B). HFD-fed mice, with or without TP treatments, were associated with biosynthesis of secondary metabolites, microbial metabolism in diverse environments, biosynthesis of amino acids, carbon metabolism, and ATP-binding cassette (ABC) transporters (Fig. 6B). Several amino acid metabolites, such as tryptophan and glutamate, were considered as precursors of neurotransmitters participating in the gut-brain axis. We further investigated the correlations between gut microbiota and other parameters, including body weight and parameters in behavioral tests. Unclassified_k_norank_d_Bacteria, *Turicibacter*, norank_f_norank_o_*Clostridia*_UCG-014, *Allobaculum*, and *Bifidobacterium* were negatively correlated with body weight and number of buried beads in marble burying test. However, norank_f_Peptococcaceae, unclassified_f_Ruminococcaceae, norank_f_Ruminococcaceae, *Colidextribacter*, *Enterococcus*, norank_f_Oscillospiraceae, norank_f_Desulfovibrionaceae, and *Staphylococcus* were positively correlated with these two parameters. We also observed that there was a negative correlation between Erysipelatoclostridium and OFT, while *Allobaculum* was positively correlated to the parameter of OFT. Only *Desulfovibrio* was found to negatively correlate with EPM test (Fig. 6C).

3.6 *Effect of FMT on HFD-induced mice*

To further evaluate the causative effect of microbiota on brain function in HFD-induced mice, we transplanted fecal microbiota of donors from ND-fed mice (dND), ND-fed mice with TPH treatment (dND + TPH), HFD-fed mice (dHFD), and HFD-fed mice treated with TPL (dHFD + TPL) and TPH (dHFD + TPH) into HFD-fed recipients (rHFD). The timeline and experimental design are shown in Fig. 7A. The results in behavioral tests indicated that the mice in dHFD → rHFD and dHFD + TPL → rHFD spent more time in the peripheral zones or in the closed arms in OFT and EPM test than the recipients transplanted with microbiota from dND, dND + TPH and dHFD + TPH (Figs. 7B-E). In the marble burying test, the microbiota from dHFD + TPH also reduced the number of buried beads in rHFD when compared with that in dHFD → rHFD and dHFD + TPL → rHFD (Figs. 7F-G). Thus, the microbiota from obese mice treated with TPH could attenuate the anxiety-like behavior in HFD-induced mice.

In addition, microbiota from dHFD + TPL and dHFD + TPH attenuated the expression of Iba1 and increased the expression of PSD-95 at both protein and mRNA levels in the hippocampus of HFD-induced recipients using double immunofluorescent staining and RT-qPCR (Figs. 8A-C). The mRNA level of *BDNF* was also improved in mice of dHFD + TPL → rHFD and dHFD + TPH → rHFD groups (Fig. 8C). The mice receiving microbiota from dHFD + TPL and dHFD + TPH exhibited significantly decreased mRNA level of *TNF-α* in the hippocampus in comparison with that in dHFD → rHFD mice, while only the microbiota from dHFD + TPH mice significantly attenuated the transcript level of *IL-1β*. On the other hand, microbiota from dHFD + TPL and dHFD + TPH exhibited a limited effect on reducing the *IL-6* mRNA levels in rHFD mice with no significant difference (Fig. 8D). In the colon, FMT from dHFD + TPH significantly increased the thickness of the mucus layer, the mRNA expression of tight junction proteins and anti-microbial peptide *Reg3γ* when compared with the mice in dHFD → rHFD (Figs. 8E-I). In short, these results indicated that FMT from mice treated with TP could improve anxiety-like behavioral disorders via attenuating neuroinflammation and protecting colonic integrity.

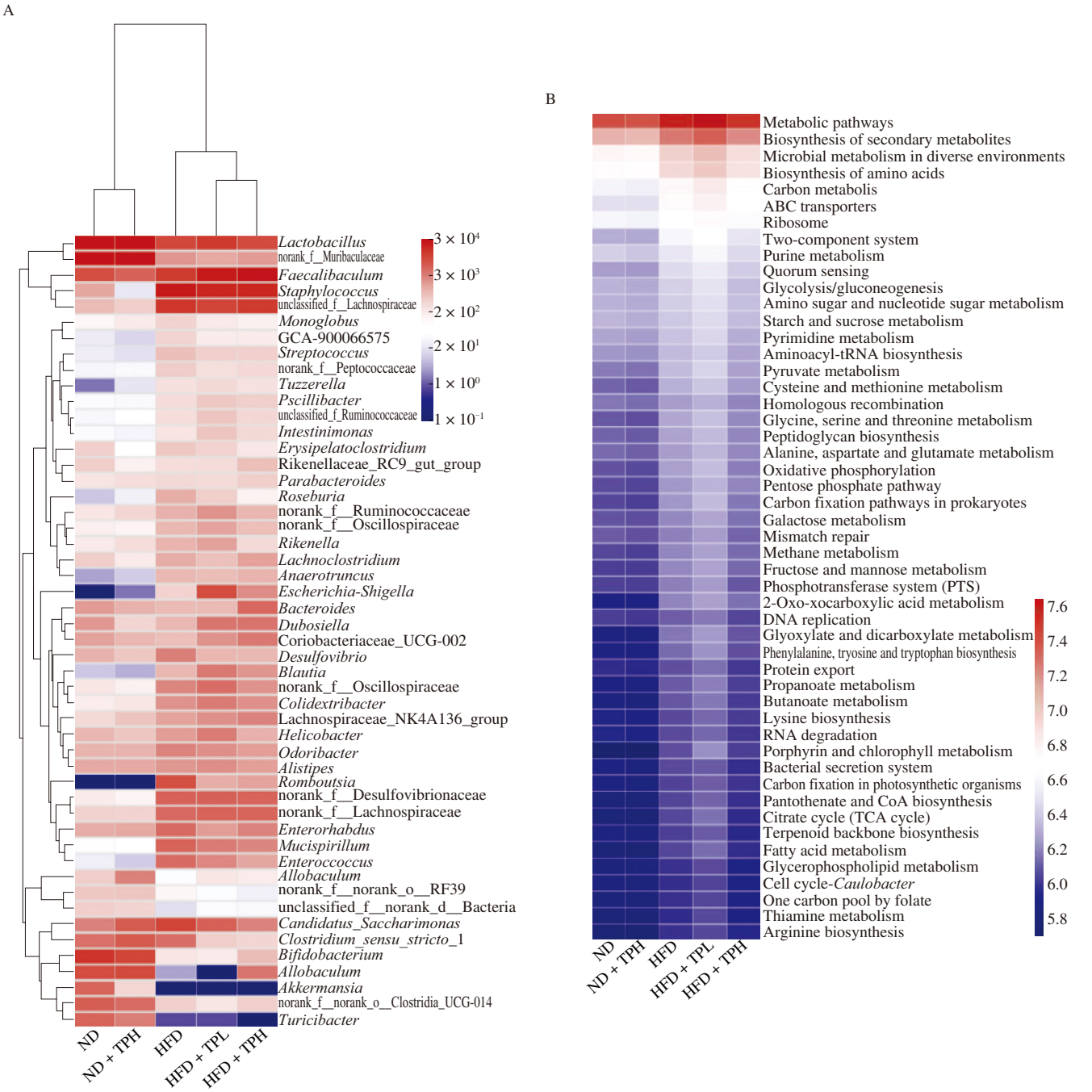
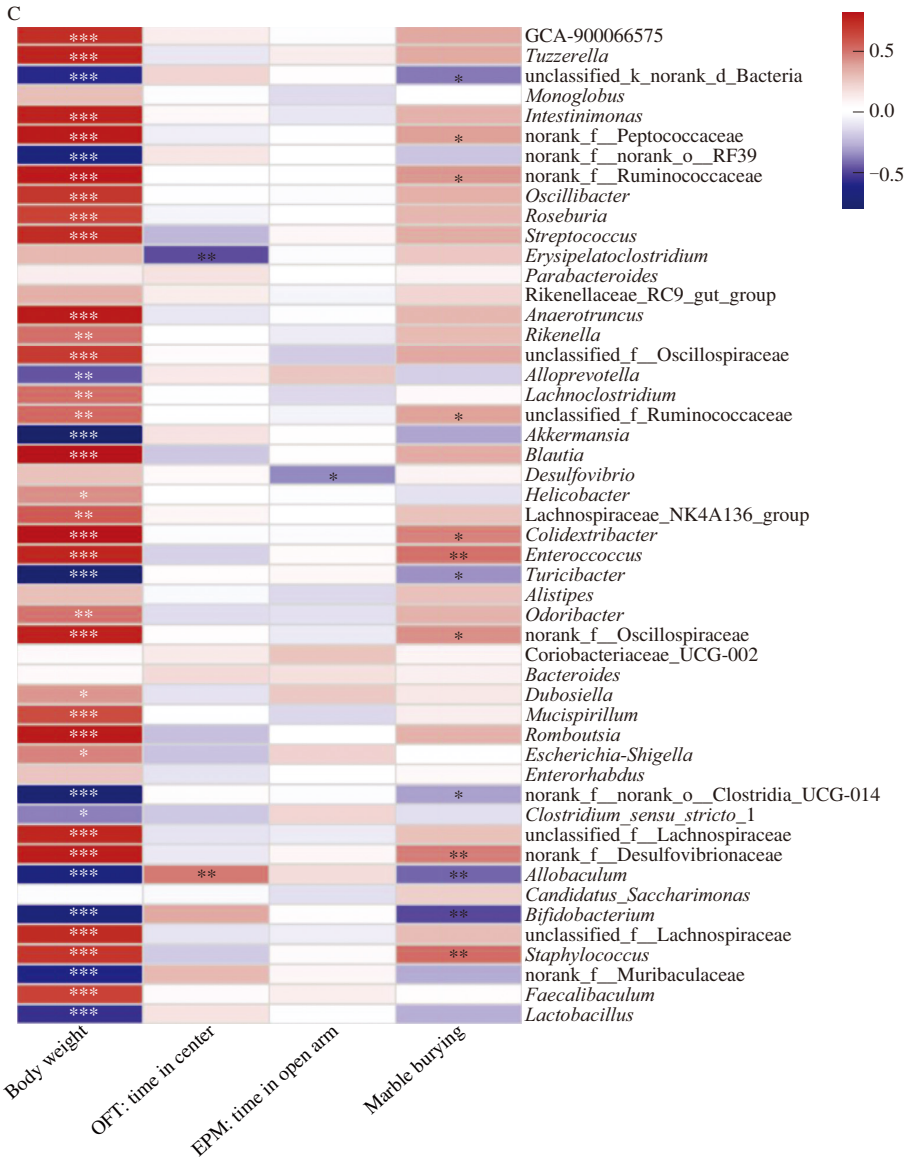


Fig. 6 Effects of TP consumption on the functional alterations of gut microbiota in HFD-fed mice. (A) Community heatmap analysis at genus level in mice; (B) Predicted KEGG functional pathway differences using PICRUSt2; (C) Spearman's rank correlation heatmap showing the correlations between neurobehavioral alterations and specific microorganism.



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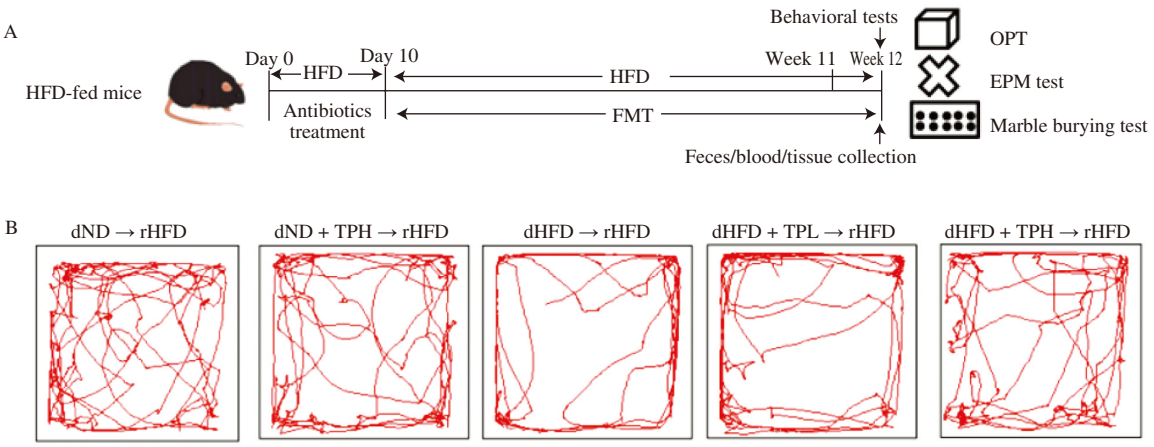
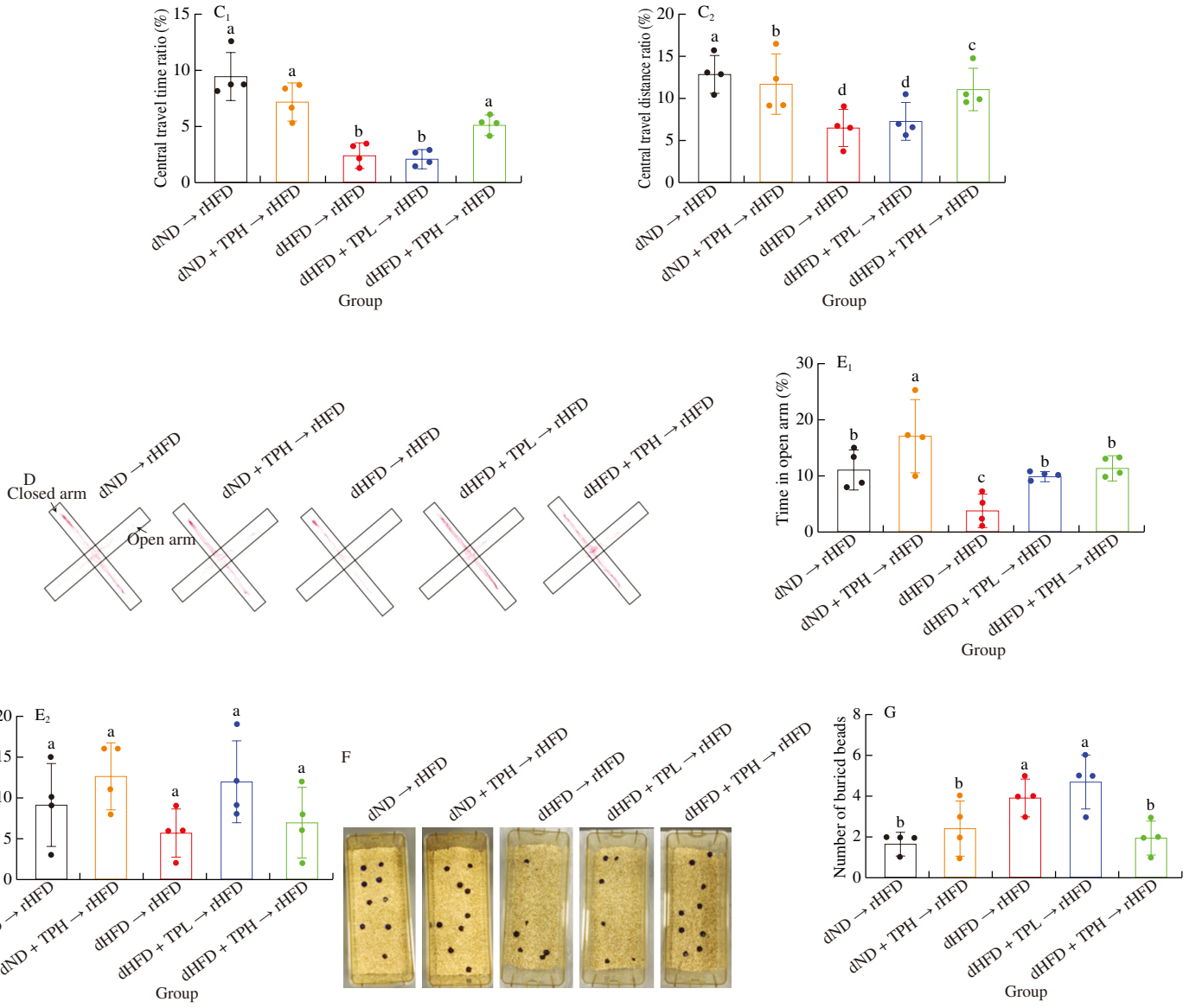


Fig. 7 Effect of FMT on HFD-induced anxiety-like behavior. (A) Experimental design; (B) Representative track images in OFT; (C) Quantification of distance and time covered in inner zone of the arena; (D) Representative track images in EPM; (E) Quantification of time ratio in open arm and entry frequency in open arm; (F) Representative images in marble burying test; (G) Quantification of the number of buried beads ($n = 4$). Data presented as means ± SEM. Bar values with different letters were significantly different by Tukey's multiple range test ($P < 0.05$).



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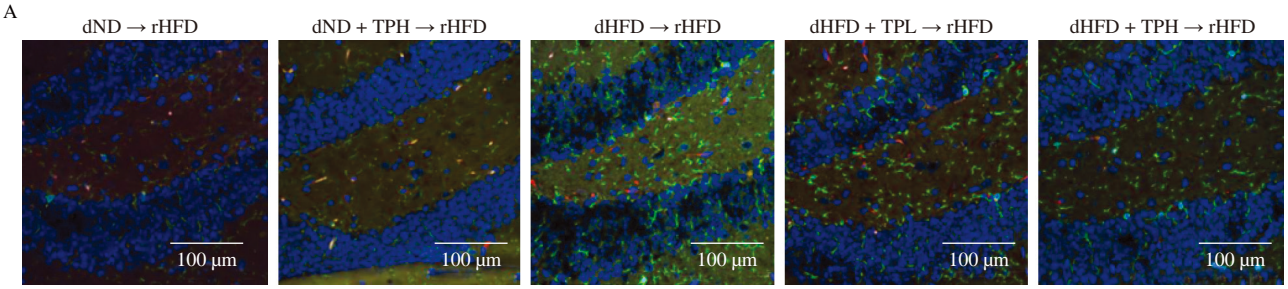
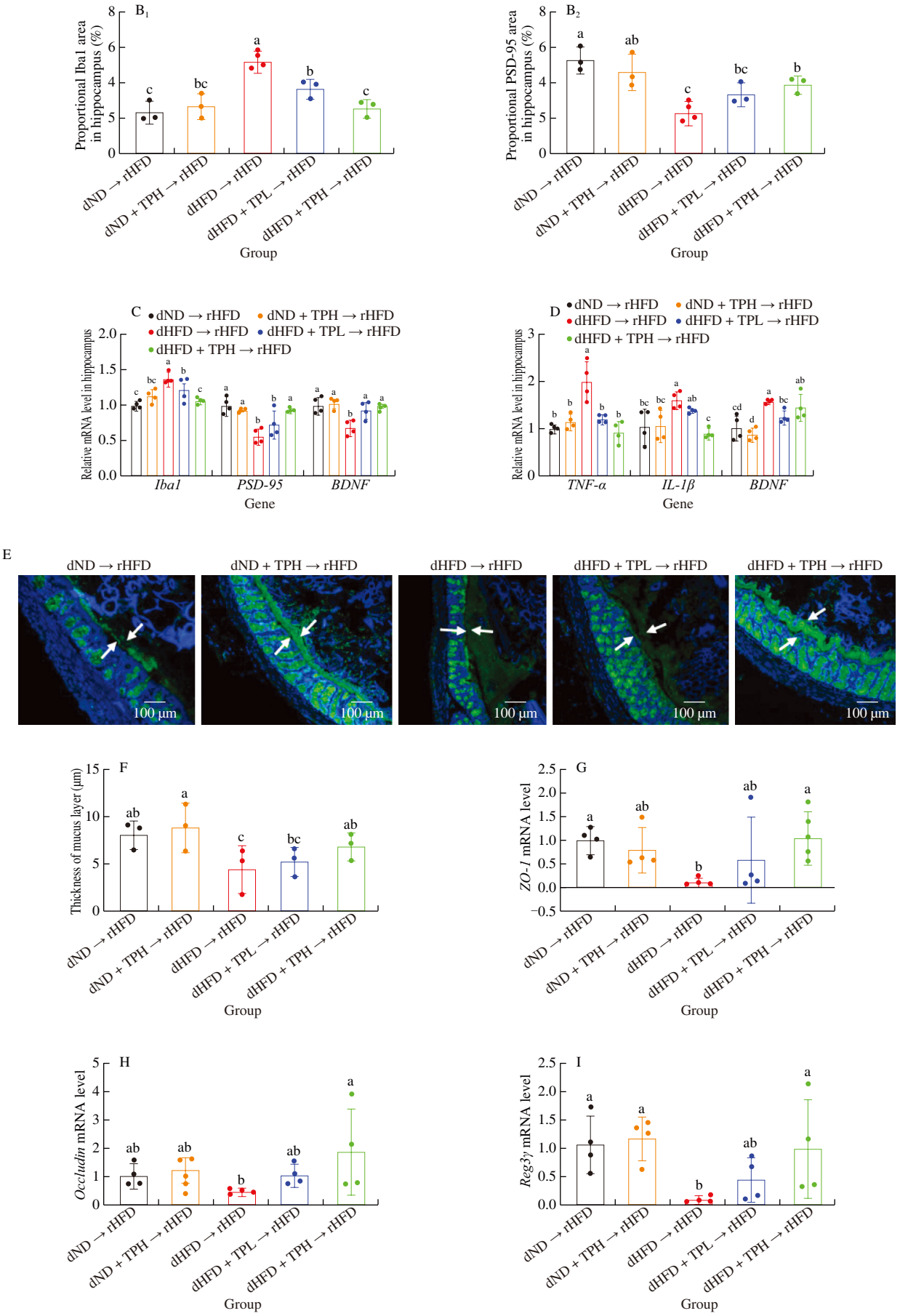


Fig. 8 Effect of FMT on brain and colon in HFD-fed mice. (A) The immunofluorescent staining of Iba1 and PSD-95 in hippocampus; (B) Quantification for the expression of Iba1 and PSD-95 in hippocampus; (C) Relative mRNA levels of *Iba1*, *PSD-95* and *BDNF* in hippocampus; (D) Relative mRNA levels of inflammatory parameters in hippocampus; (E) The immunofluorescent staining of mucus layer; (F) Quantification of mucus thickness; (G-I) Relative mRNA level of *ZO-1*, *Occludin*, and *Reg3γ* in colon ($n = 4$). Data presented as means ± SEM. Bar values with different letters were significantly different by Tukey's multiple range test ($P < 0.05$).



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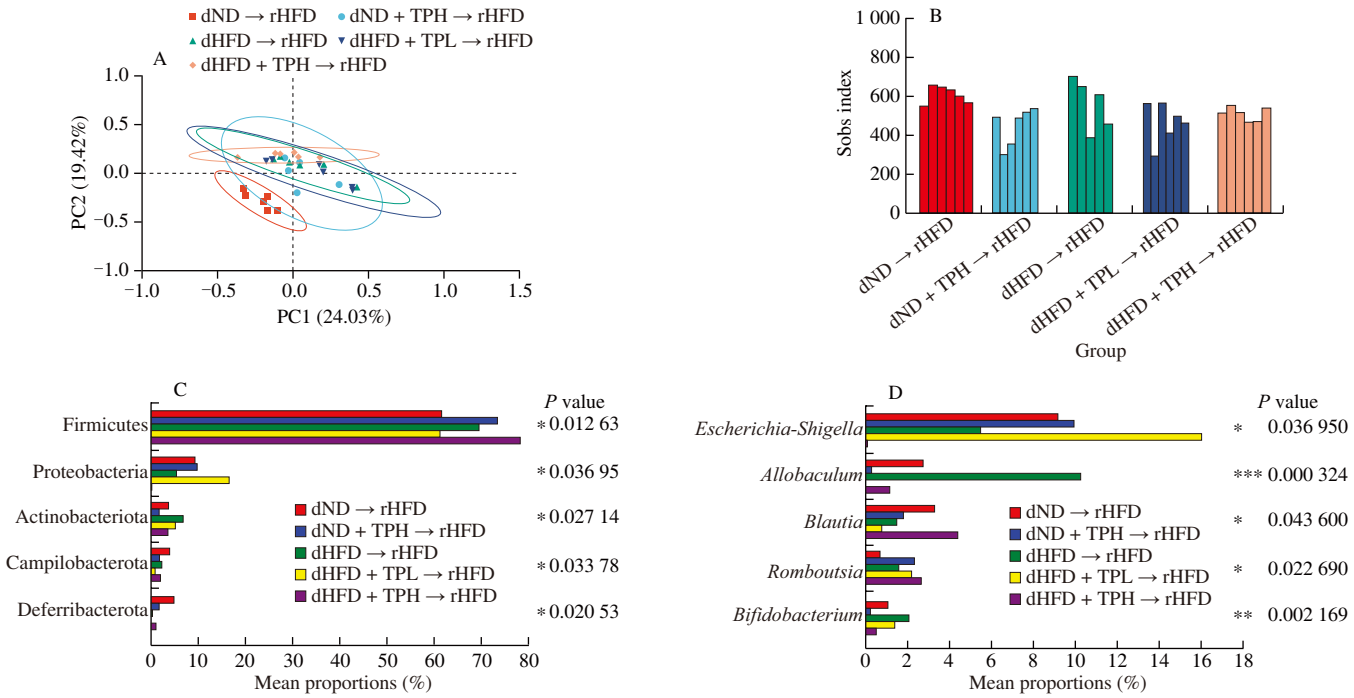

Fig. 9 Effect of FMT on gut microbiota in HFD-fed mice. (A) Principal coordinates analysis plot of unweighted UniFrac distances (PCoA); (B) α-Diversity measured by Sobs index; (C) Comparison of dominant phyla; (D) Comparison of dominant genera. One-way ANOVA with a post hoc Kruskal-Wallis H test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 5$).

The changes in the gut microbiota of mice receiving fecal microbiota from different donor groups were also analyzed. The microbiota in dND → rHFD and dHFD + TPH → rHFD groups were separated from that in other groups (Fig. 9A). However, the α-diversity of microbiota in all the groups did not show much difference (Fig. 9B). The structural analysis of communities in gut microbiota at the phylum and genus levels indicated that mice in dHFD + TPH → rHFD showed the lowest abundance of Proteobacteria and its genus *Escherichia-Shigella*. Nevertheless, the abundance of phylum Firmicutes, and genus *Blautia* and *Romboutsia* were the highest in mice from the dHFD + TPH → rHFD group. Interestingly, the abundance of the probiotic genus *Bifidobacterium* was the lowest in dHFD → rHFD, while the level was rebalanced by FMT from other groups (Figs. 9C and D). Thus, our results suggested that FMT from mice treated with TP rebalanced the abundance of pathogenic Proteobacteria while increasing the abundance of probiotic *Bifidobacterium* in HFD-fed mice.

4. Discussion

In the present study, TP administration decreased the body weight gain and reduced liver and fat weight in HFD-fed obese mice. The behavioral tests revealed that anxiety-like behavioral disorders due to HFD consumption were prevented by TP intake. For the first time, we presented evidence that TP treatment suppressed neuroinflammation, microglia activation, and synaptic impairment, whereas the mRNA levels of tight junction proteins that were involved in the BBB and levels of neurotransmitters were increased by TP intake in HFD-fed mice. Moreover, leaky gut and gut microbiota dysbiosis caused by HFD were improved by TP. FMT results also indicated that the rebalanced gut microbiota by TP could directly ameliorate anxiety-like behaviors.

TP shows multiple physiological and healthy-promoting effects including hypoglycemic and hypolipidemic effects, and reducing body weight[19,23]. Similarly, the body weight gain, weight of fat and liver, and fasting blood glucose level were reduced by TP intake in HFD-fed obese mice. In addition, the anti-obesity effects of TP suggest a possible use of TP for treating obesity-associated complications. Long-term consumption of HFD is a risk factor of neuropsychiatric disorders, including anxiety[24]. In the present study, HFD-induced anxiety was evaluated in mice using OFT and EMP tests. For the first time, TP was found to alleviate anxiety behavioral disorders in this HFD-fed obese model.

The microbiota-gut-brain axis, the bidirectional communication between gut bacteria and the brain, is essential for maintaining homeostasis of the gastrointestinal, central nervous, and microbial systems in animals and humans[25]. There is growing evidence that gut microbiota is crucial for the fundamental structure and function of the brain. For example, germ-free mice have been reported to show myelination alterations, whereas this phenotype is reversed by transferring it to a conventional microbiota[26]. In several neuropsychiatric disorders, including Alzheimer's disease, anxiety, and autism, the gut microbiota diversity is reduced when compared with healthy controls[27]. Here, HFD-fed mice with anxiety-like behavioral disorders exhibited lower diversity of gut microbiota (Sobs index) and structural dysbiosis, while the dysbiosis of gut microbiota was prevented by TP intake. Interestingly, transferring the microbiota from donors of TP-treated obese mice to HFD-fed recipients could alleviate the behavioral disorders. Furthermore, the results in our current study showed that the transcriptional level of tight junction proteins in the colon of HFD-induced mice was significantly decreased, and the LPS concentration in serum was increased in obese mice, indicating impaired intestinal barrier function. These observations are consistent with previous findings

that HFD supplementation could disrupt intestinal barrier function through dysregulation of gut microbiota in mice[27]. Collectively, the evidence in the present study suggested that TP could ameliorate these parameters through rebalancing gut microbiota dysbiosis.

The communication pathways in the microbiota-gut-brain axis include direct signaling via chemical transmitters. The 5-HT, which is mainly produced by gut bacteria, is crucial in regulating brain function[28]. Previous studies have reported that the biosynthesis of 5-HT is decreased in germ-free mice and antibiotic-treated mice, while this phenotype is rescued by transplanting spore-forming bacteria that increase tryptophan metabolism by enterochromaffin cells[29]. NE is regarded as one neurotransmitter that induce a wide range of responses in bacteria. Host production of NE contributes to the induction of bacterial virulence genes, subsequently leading to infection and mortality[30]. In addition, NE has been reported to show a chemotactic effect, thereby increasing bacterial migration to the intestinal mucosa in the host[31]. NE concentration in blood is higher in germ-free mice than in conventional rodents[32]. Though the level of 5-HT did not change significantly between ND-fed and HFD-fed mice, the level of 5-HT in the hippocampus of HFD-induced mice was increased by TP treatment with a significant difference. The concentration of NE was reduced by HFD consumption, while TP intake significantly improved the NE level in the hippocampus of HFD-fed mice.

The immune system is regarded as one of the communication pathways in the microbiota-gut-brain axis. The gut microbiota, various immune cells, and neural nerves are in close proximity of intestinal lining, forming entangling relationships and affecting immune response via trilateral interactions. The gut microbiota is vital in regulating the development and function of the innate immune cells in the brain[33-34]. Moreover, it is necessary to mature and activate microglia in the brain. The number of immature microglia in specific brain regions is higher in gnotobiotic and antibiotic-treated mice than that in conventional mice[10]. Increasing evidence revealed that microglia play an important role in cognitive dysfunction through synaptic over-pruning in Alzheimer's disease[35]. A high-fat and fiber-deficient diet could induce microglia activation in the hippocampus[36]. In our study, the expression of microglia marker Iba1 was significantly increased in HFD-fed mice when compared with that in ND mice, while TP notably improved its expression in obese mice.

The gut microbiota also interacts with the brain via the systemic immune system. For example, the changes in circulating cytokines within systemic immunity lead to altered inflammation in the brain. Many studies have reported that neuropsychiatric diseases, such as depression, anxiety, and autism, are associated with peripheral inflammation[37]. The circulating cytokines and chemokines can be directly transported through the BBB and access the central nervous system. Gut microbiota can affect the permeability of the BBB due to reduced expression of tight-junction proteins[38]. Moreover, previous studies also suggested that the permeability of the BBB is increased in the obese mouse model[39]. Freeman et al.[40] demonstrated that the excessive exposure of the brain to inflammatory cytokines, such as LPS, IL-1β, IL-6, and TNF-α, could aggravate neuropsychiatric disorders in HFD-induced obese mice. Our finding further demonstrated that HFD induced early signs of neuropsychiatric disorders in the hippocampus, including reduced tight junction transcript levels, and systemic and local inflammation. Interestingly, the treatment with TP could alleviate these undesirable effects, exert an anti-inflammation effect, and restore anxiety-like behavioral disorders induced by HFD.

Institutional review board statement

All the experimental protocols were in accordance with the Chinese Council on Animal Care Guidelines and approved by the Animal Subjects Ethics Committee of The Hong Kong Polytechnic University (ASESC No. 21-22/74-ABCT-R-OTHERS).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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