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Dietary *Lactobacillus plantarum* ST-III alleviates the toxic effects of triclosan on zebrafish (*Danio rerio*) via gut microbiota modulation

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ABSTRACT

The probiotics, *Lactobacillus plantarum* ST-III, plays an important role in modulating microbiota and alleviating intestinal metabolic disorders. Herein, we reported that *Lactobacillus* increases biodiversity of zebrafish gut flora, and attenuates toxic effects from chronic triclosan (TCS) exposure. *Lactobacillus*-feeding recovered the species and amount of microorganisms in the intestines of zebrafish, and inhibited toxin production by saprophytic bacterial growth. Abnormal physiological indexes and malonaldeyhde content resulting from TCS exposure were effectively alleviated. Additionally, lipid-metabolism disorders, such as increased triglyceride and total cholesterol levels, were attenuated by a probiotics diet. The number of $CD4^+$ T cell lymphocytes in the lamina propria of the duodenal mucosa was decreased in zebrafish receiving a *Lactobacillus* diet compared to the TCS-exposed group, showing a consistent expression trend for six immune genes (*NF-κB*, *IL-1β*, *TNF-α*, *lysozyme*, *TLR4α*, *IL-10*) in the intestinal mucosa. Histopathological observations of intestines, spleen and kidney showed that TCS exposure produced severe damage to the morphology and structure of immune and metabolism-related organs. *Lactobacillus* was capable of mitigating this damage, but bile salt hydrolase, an active extract of *Lactobacillus,* was not an effective mitigation strategy. The *Lactobacillus*-induced decrease in the number of inflammatory cells confirmed its role in preventing inflammatory injury. Three behavioral tests (T-maze, bottom dwelling and social interaction) indicated that a probiotics diet improved zebrafish movement and learning/memory capacity, effectively alleviating anxiety behavior due to TCS exposure. These findings inform development of beneficial strategies to alleviate intestinal metabolic syndromes and neurodegenerative diseases resulting from exposure to environmental contaminants through modifying gut flora with a probiotics diet.

1. Introduction

Triclosan (TCS; 5-chloro-2-[2, 4-dichlorophenoxy] phenol) is a broad-spectrum bactericide that is widely used in medical and chemical applications [1,2]. Its frequent and extensive use poses a great concern to aquatic organisms and human health. Chronic TCS exposure can lead to a variety of metabolic and endocrine disorders. For example, Hu and coworkers (2016) demonstrated that low-dosage TCS and other associated personal care products can alter gut microbiota in adolescent rats [3]. Gaulke et al. (2016) used high TCS concentrations to conduct an acute exposure experiment on adult zebrafish and found that the microbiota community was changed concomitant with an increase in TCS resistance [4]. Narrowe et al. (2015) found substantial changes in the microbiome of fathead minnows due to TCS exposure at environmentally relevant doses; however, they largely recovered after removal of TCS exposure [5]. These toxic effects of TCS and infection of pathogenic bacteria may result from disrupted mucus-bacterial interactions, which might have the potential to promote diseases associated with gut inflammation [6,7]. Past research on interactions between environmental pollutants and the microbiome has mainly focused on

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acute exposure [4,8], while there is a paucity of research examining chronic exposure of environmental contaminants on zebrafish microbiome composition and diversity.

The intestinal tract is inhabited by a diverse community of microbes collectively referred to as gut microbiota. Because gut microbiota provide important benefits to its host, especially in metabolism and immune development, disturbance of the microbiota-host balance is associated with numerous chronic inflammatory diseases, including inflammatory bowel disease and a group of obesity-associated diseases collectively referred to as metabolic syndrome and insulin resistance, such as nonalcoholic fatty liver disease (NAFLD) [9].

Recent research has demonstrated the role of gut microbiota in energy storage, lipid and choline metabolism, and ethanol production through different pathways [10]. Therefore, the imbalance of intestinal flora not only results in metabolic syndrome and immune damage, but also directly affects motor behavior and neural functions [11,12]. Hsiao et al. (2013) found that mice with autism spectrum disorder (ASD) receiving oral treatment of human commensal *Bacteroides fragilis* had improved gut permeability, altered microbial composition and amelioration of ASD-related defects in communicative, stereotypic, anxiety-like and sensorimotor behaviors [13]. A study on Parkinson's disease reported that gut microbes promoted α-synuclein-mediated motor deficits and brain pathology [14]. Probiotics may play an important role in modulation of the microbiota, thereby alleviating these disorders [15,16]. Probiotics are living microorganisms with immunomodulatory properties, which can prevent and treat different gastrointestinal diseases, and modify host nutrient metabolism and energy homeostasis [17,18]. The presence of bile salt hydrolase (BSH) in probiotics renders them more tolerant to bile salts, which also contributes to reduced blood cholesterol levels in the host. *Lactobacillus plantarum* ST-III was originally isolated from Chinese pickle [19] and exhibits many probiotic properties, such as cholesterol reduction due to its BSH activity [20]. Because of the susceptibility of gut microbiota to exogenous stressors, dysbiosis balance studies on intestinal microbiomes have become an emerging research area with tremendous application for improving human health and environmental safety.

Our previous studies demonstrated that chronic TCS exposure induced a series of abnormalities in lipid metabolism, immune functions and behavioral characteristics [21,22]. Probiotics can provide relief to metabolic syndrome and immune damage in rats [23], stabilize mucosal barrier function, modulate gut microflora and limit the growth of pathogenic bacteria by acidifying the gut lumen, competing for nutrients, and producing antimicrobial substances [24,25]. Additionally, probiotics may control inflammation by reducing gut pH and competing with pathogens for binding and receptor sites [26]. However, it remains uncertain whether or not *Lactobacillus plantarum* ST-III can alleviate toxic effects and dysplasia in zebrafish (*Danio rerio*) due to chronic TCS exposure, and the related mechanisms for alleviation have not been elucidated.

Based on the above considerations, we first evaluated the effects of chronic TCS exposure on lipid metabolism, intestinal mucosa immune system, behavioral indexes and gut microbiota in adult zebrafish. Using intestinal flora sequencing, the mechanisms by which *Lactobacillus plantarum* ST-III (as a model probiotic bacterium) alleviates potential dysbiosis and secondary host metabolic disruptions by regulating the gut microbiota were rigorously investigated. Results of this study assess the ecological risk of TCS and inform development of integrated strategies to modulate intestinal microbiota to alleviate metabolic syndrome and neurodegenerative diseases.

2. Materials and methods

2.1. Probiotics and diet preparation

Lactobacillus plantarum ST-III strain was isolated from fruit wine and stationarily cultivated in a MRS (Man, Rogosa and Sharpe) medium at 37°Cfor 48 h. *Lactobacilli* cells were collected into pellets by centrifugation at 2500 *g* for 10 min at 4 °C. Pellets were washed in phosphate buffer saline (pH 7.4, PBS, Solarbio, Beijing, China) three times and re-suspended at a final concentration of 1×10^8 CFU/mL. Crude BSH was extracted from *Lactobacillus* using an ultrasonic oscillator according to Grill and coworkers [27].

Dry feed supplement for adult zebrafish having a nutritional value similar to paramecium was purchased from Zeigler (Gardners, PA, USA; Larval AP100-5, 250–450 μm) [28]. The probiotics diet was prepared by supplementing the Larval AP100 food source with *Lactobacillus plantarum* ST-III at 1×10^8 CFU/g or crude BSH at a final density of 1×10^8 CFU/g of basal diet. In the control group diet, an equivalent volume of sterile PBS was added to the basal diet. Zebrafish were quantitatively fed according to their number in each treatment group. The feed was air-dried at 20°Cfor 15 h and stored at 4 °C [29]. The quality of the probiotics feed was monitored by MRS plate culture with a minimum survival rate criteria for *Lactobacilli* of 95% in the experimental diet over a two week period. Therefore, fresh feed with probiotics additives was prepared once a week to ensure high quality.

2.2. Ethics statement about animals and experimental design

All zebrafish experiments were carried out following protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University. All zebrafish surgery was performed on ice to minimize suffering.

Adult wild-type (AB strain) zebrafish (*Danio rerio*) were cultured according to our previous studies: aerated tanks at 28 ± 2 °C, a 12 h light/dark cycle and fed twice daily [30]. All of the experiments were divided into six treatment groups (Fig. S1): control group (control), received unsupplemented feed; triclosan-exposure group (TCS; Sigma-Aldrich, St. Louis, USA), received unsupplemented feed and triclosan exposure; *Laetobacillus plantarum* ST-III (LAB) group, received LAB-supplemented feed; BSH group, received BSH-supplemented feed; triclosan and probiotics combined treatment (TL) group, received LAB-supplemented feed and triclosan-exposure; and triclosan and BSH combined treatment (TB), received BSH-supplemented feed and triclosan-exposure. The above 6 groups were abbreviated as control, TCS, LAB, BSH, TL and TB, respectively. A 0.002% (v/v) TCS solution was added to the water of the TCS, TL and TB groups (Fig. S1). Water in TCS, TL and TB group tanks was replaced every 2 days to ensure stable triclosan concentration and the water in the control, LAB and BSH groups was similarly replaced every 2 days. TCS-exposure concentrations were chosen on the basis of our previous study [21]. Embryos in control groups were treated with 0.002% acetone (the highest acetone content found in the 200 μg/L TCS-exposure treatment) [31].

2.3. Intestinal flora sequencing

2.3.1. Sample collection

After 90-day TCS exposure, intestinal contents were collected 24 h after the last feeding. Twelve zebrafish were randomly selected from each treatment, and all experiments were performed under aseptic conditions. According to a previous report [32], DNA was extracted from 72 adult zebrafish (12 for each group) intestines using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA concentration and purity were determined by running the extracts on 1.2% agarose gels (Figs. S2A–B).

2.3.2. Illumina library preparation and 16S rRNA gene sequence analysis

Sequencing of the V4-V5 region of the 16S rRNA gene was performed on the Illumina MiSeq platform. The V4-V5 region of bacterial 16S-rRNA genes was amplified using universal primers 515F (GTGCC AGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTGAGTTT). Primers were selected for their high coverage of almost all phyla in conventional and metagenomic studies [33]. The primers also

contained the Illumina 5′ overhang adapter sequences for two-step amplicon library building, following manufacturer's instructions for overhang sequences. Prior to library pooling, the barcoded PCR products were purified using a DNA gel extraction kit (Axygen-Corning, Shanghai, China) and quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). Libraries were sequenced by 2300 bp paired-end sequencing on the MiSeq platform using the MiSeq v3 Reagent Kit (Illumina) from Tiny Gene Bio-Tech Co. (Shanghai, China).

2.3.3. Bioinformatics workflow

Raw FASTQ files were demultiplexed according to the barcodes. Paired-end reads for all samples were run through Trimmomatic (Ver. 0.35) [34] to remove any low quality base pairs using the following parameters: SLIDINGWINDOW: 50:20 MINLEN: 50. Trimmed reads were merged using the FLASH program (Ver. 1.2.11) with default parameters. Low-quality contigs were removed via the screen.seqs command using the following filtering parameters: maxambig = 0, minlength = 200, maxlength = 580, and maxhomop = 8. 16S rDNA was analyzed using mothur (Ver. 1.33.3) (http://www.mothur.org), UPARSE (USEARCH Ver. 8.1.1756, http://drive5.com/uparse/), and R (Ver. 3.2.3 https://www.r-project.org/) software in combination. The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) using the UPARSE pipeline. OTU representative sequences were assigned for taxonomy against the Silva 119 database with a confidence score ≥0.8 by the classify.seqs command in mothur. OTU taxonomies (from phylum to species) were determined based on National Center for Biotechnology Information data (https://www.ncbi.nlm.nih.gov/).

For alpha diversity analysis, Shannon, Simpson, Chao1, ACE index, and rarefaction curves were calculated using mothur and then plotted with R statistical software [35]. For beta diversity metrics, the weighted and unweighted UniFrac distance matrices were calculated using mothur and visualized using heatmap in R statistical software.

2.4. qRT-PCR analysis of immune-related genes

To determine differentially expressed genes of zebrafish at the transcriptional level *in vivo*, *q*RT-PCR was performed for control, TCS, BSH, LAB, TL and TB groups. Total RNA from 72 homogenized 90-day post-fertilization (dpf) zebrafish intestines was isolated using TRIzol Regent following manufacturer's instructions. The OD_{260}/OD_{280} and OD_{260}/OD_{230} values in all groups ranged from 1.8 to 2.1 demonstrating a high purity of RNA. Gene primers were designed by primer 3 online (http://primer3.ut.ee/) and synthesized by Sangon Biotechnological Co. (Shanghai, China) (Table S1) with *elfa* as the endogenous reference. [36] All PCR reactions were performed using three biological replicates and each biological replicate included three technical replicates.

2.5. Biomarker detection, Oil red O staining and biochemical analyses of liver lipids

Twelve zebrafish per treatment were sampled in the morning (10 a.m.), approximately 17 h after their last meal and euthanized in ice at 90 days after treatment. Zebrafish were measured for body mass and length (distance from tip of snout to base of caudal fin). Then, the intestines were extracted for quantification of malonaldeyhde (MDA) concentrations, an index of lipid peroxidation, using test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Triglyceride (TG) and total cholesterol (TCH) concentrations were determined in liver of 90-dpf zebrafish. Each group was analyzed in triplicates, and each replicate included 4 zebrafish, thereby 12 zebrafish were included for each group. TG and TCH were measured by using enzymatic and colorimetric test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Frozen sections of the liver were stained using Oil Red O (ORO) [37].

2.6. Histopathological observation

Intestine, spleen, liver and kidney were dissected from adult zebrafish after exposure to different treatments from 4 hpf to 90 dpf. Haematoxylin and eosin (H&E) staining on isolated tissues was conducted using standard protocols [38]. Morphological changes and structural damage in tissues were observed and photographed using optical microscopy (DM2700M, Leica, Germany).

2.7. Immunofluorescence staining of liver and gut

Sliced liver and gut were cut into 10 μm sections, mounted on glass slides, and stored at −20 °C. Immunofluorescence (IF) staining was carried out according to Chatterjee and coworkers [39]. The primary antibody was anti-CD4 (rabbit; Abcam, Cambridge, UK) and lysosome-associated membrane protein 1 (LAMP1) (mouse; Cell Signaling Technology, Danvers, MA, USA), and the secondary antibody was FITC-labeled goat anti-rabbit and Fluor 594-conjugated goat anti-mouse (GeneTex, Irvine, CA, USA). Nuclei were counterstained with DAPI (4′, 6-diamidino- 2-phenylindole) (1:1000; Roche, Mannheim, Germany). Histological sections were imaged and photographs were recorded with a laser scanning confocal microscope (Nikon, Tokyo, Japan).

2.8. Adult zebrafish behavioral assessment

All of the adult zebrafish experiments were conducted after a 90-day pretreatment of each group. All tests were performed at 27.5 ± 1 °C during the light phase between 8 a.m. and 6 p.m., and the test tank was placed in a soundproof box to avoid external interferences. Videos were recorded by a camera, which was placed 0.6 m above the tank and connected to a computer. The automated analyses of traces were performed by EthoVision XT software (Noldus IT, Wageningen, Netherlands) and all test endpoints were recorded as mean ± SD (standard deviation).

2.8.1. T-maze test

Learning and memory tests were conducted in a T-maze [40]. TCS-exposed and non-exposed treatments were subjected to an exploratory assay in which zebrafish were put in the starting arm location of a T-maze: a shallow area with a 5 cm water depth composed of a start arm ('s' area), a right area ('r' area) and a 'wrong area' ('w' area) (Fig. 7D). Before the test, zebrafish were trained by feeding them food in the 's' area and tapping stimulation in the 'w' area for 7 d (15 min each time; 3 times per day). Then zebrafish were individually placed in the 's' area, the baffle removed (red line of 's' area; Fig. 7) and motion recording begun (10 min for each zebrafish).

2.8.2. Bottom dwelling test

Adult zebrafish were individually placed in a 1.5-L transparent tank (15 × 28 × 23 × 7 cm; height × top-length × bottom–length × width; Aquatic Habitats, Apopka, FL, USA) to assess anxiety-like behaviors [41]. The tank was filled with water, and was divided into two equal virtual horizontal portions. After a 5-min adaptation, zebrafish swimming behavior was video-recorded for 10 min.

2.8.3. Social interaction test

Individual adult zebrafish were placed in a testing chamber that was divided into two regions (social and non-social areas) with the zebrafish free to swim between both areas. A transparent plastic tank containing 72 adult zebrafish was placed next to the social area (Fig. S3D). Once the test zebrafish were placed in the testing chamber, they could see each other if they swam to the social area. After a 15-min adaptation period, zebrafish movement was monitored for 10 min.

2.9. Statistical analysis

Each experimental group included three biological replicates, and each biological replicate was comprised of three technological replicates to assess accuracy and reproducibility. Each biological replicate included 12 zebrafish (female:male = 1:1) and in total 72 zebrafish (6 × 12) were used in each experiment. One-way analysis of variance (ANOVA) was used to analyze the effects of TCS-exposure and probiotics-feeding on zebrafish abnormalities and bioinformatics sequencing data. Dunnett's test was used to determine differences among control and experimental groups. All data were expressed as the mean ± SD (standard deviation). Data were graphed and analyzed by GraphPadPrism 7.0 (GraphPad Prism Software, San Diego, CA, USA). Significant differences were distinguished at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

3. Results

3.1. Analysis of Illumina MiSeq sequencing data for zebrafish

3.1.1. Multivariate statistical analysis of zebrafish intestinal microbiota

The specific 16S v4-v5 411bp (515–926 bp) fragments were all amplified in the intestinal microbial genome DNA of the six treatments using the two-step PCR amplicon method, suggesting appropriate library construction (Figs. S2A–B). In addition, the complete genome of *Lactobacillus plantarum* ST-III was sequenced and identified before it was used; and the sequencing results showed > 99% similarity to *Lactobacillus plantarum* ST-III sequences (Fig. S2C). A series of inhibition zone tests were performed to investigate the bacteriostatic effects of TCS on *Lactobacillus plantarum* ST-III, G^--*Escherichia coli* and G^+-*Staphylococus aureus*. The 125 and 200 μg/L TCS treatments had no inhibitory effect on *Lactobacillus plantarum* ST-III (Figs. S1D–a); however, G^+-*Staphylococus aureus* (Figs. S1D–b) and G^--*Escherichia coli* (Figs. S1D–c) showed a concentration-dependent inhibitory effect. In order to analyze the effects of chronic TCS exposure on zebrafish intestinal microflora communities and the effects of the *Lactobacillus* diet, we performed high throughput sequencing of the bacterial 16S rRNA V4-V5 region in 90-dpf zebrafish. Table 1 summarizes the sequence coverage, observed OTUs (Operational Taxonomic Units), diversity and species richness as estimated using Shannon, Simpson, Chao1 and Ace indices. Good's coverage indices of the six treatments were all larger than 0.999, indicating high integrity of sampling and sequencing. Among the six treatments, TCS-exposure led to the lowest Chao1 and Ace values (310 and 305), suggesting that TCS affected zebrafish intestinal flora structure and reduced biodiversity. In contrast, the Chao1 and Ace indices (384 and 383) for *Lactobacillus plantarum* ST-III-feeding groups (LAB) were significantly greater ($p < 0.05$) than those of control and TCS groups. Additionally, there was a slight increase from 305 to 310 to 314–315 in TL groups. Addition of *Lactobacilli* led to a significant increase ($p < 0.05$) in OTU richness compared to the control, but a slight decrease in response to TCS exposure (Table 1). Both *Lactobacillus plantarum* ST-III- (LAB) and BSH-feeding (BSH) groups had higher OTU values compared to the control group. Moreover, there were no significant differences in Shannon and Simpson indices among the six treatments ($p > 0.05$) (Fig. S4). These results demonstrate that zebrafish intestinal microbial community was affected following chronic TCS exposure; the species of intestinal microbial community of zebrafish was significantly changed, and the probiotic diet increased diversity of the intestinal flora, thus recovering a constant homeostasis of the intestinal flora.

To evaluate the sequencing data suitability, rarefaction curves were produced from archaeal sequences (Fig. 1A). All curves asymptotically approached a similar value, suggesting that the sequencing data were comprehensive. Further, the results imply that the majority of the microbial phylotypes from intestinal samples were identified and confirmed by rank abundance curves (Fig. 1B) because a wide OTU Rank was achieved for all six treatment samples.

3.1.2. Effects of probiotic diet and TCS-exposure on zebrafish intestinal microbiota communities

According to the weighted Unifrac distance, microbiota structure and diversity in the TCS group were significantly different from the control and other groups (Fig. 1C): LAB > control > TL and BSH > TB > TCS. An unweighted UniFrac tree revealed several distinct clusters based on bacterial community membership (Fig. S5B). The TCS and BSH libraries clustered together and were separated from other group libraries, demonstrating that the structure and diversity of zebrafish intestinal tract microbiota under TCS exposure were prominently different from probiotic diet and control groups. These results indicate that TCS exposure significantly affected zebrafish intestinal tract microbiota. Further, a diet of *Lactobacillus plantarum* ST-III improved gut bacterial communities, and consequently the TL group clustered with the LAB and control groups.

Taxonomic assignment revealed that 6 known bacterial phyla and 14 microbial classes (total percentage > 99.5%) were representative at the 97% sequence identity level (Fig. 1D). At the phylum level, zebrafish gut microbiota in the six groups were dominated by *Proteobacteria* (mean ± SEM relative abundance, 32.7 ± 0.1%), *Bacteroidetes* (30.1 ± 0.1%) and *Firmicutes* (23.48 ± 0.1%); other representative phyla were *Fusobacteria* (10.7 ± 0.1%) with minor amounts of *Actinobacteria* and *Planctomycetes*. TCS exposure led to a significantly higher abundance (55.8%) of *Proteobacteria* (Table S2). Upon addition of *Lactobacillus plantarum* ST-III (TL group) and BSH (TB group), the composition of phyla tended to be similar to those of the control and LAB treatments.

To gain better insight into differences in intestinal tract microbiota among the six treatments, heatmaps were generated to include the 50 most abundant OTUs based on the logarithmic value of transformed

Table 1
Summary of the Illumina MiSeq sequence data and the alpha diversity index.

Treatments	Valid sequence	Item [1]				Coverage	OUT
		Chao	Ace	Shannon	Simpson		
Control	45717	328^{a}	328^{a}	3.05	0.1428	0.999737	325^{a}
TCS	35007	310^{a}	305^{a}	3.96	0.0404	0.999428	294^{a}
BSH	26514	352^{acd}	352^{acd}	3.84	0.059	0.999321	345^{abc}
LAB	39434	384^{cd}	383^{cd}	3.62	0.1028	0.999696	380^{cd}
TL	42336	315^{ae}	314^{ae}	2.83	0.1341	0.999386	303^{ae}
TB	48448	374^{acd}	365^{acd}	4.40	0.0245	0.999711	361^{acd}

Note:
(1) Data are the means of three replicates.
(2) Data with different letters in a vertical row indicate a significant difference ($p < 0.05$) assessed with an analysis of variance followed by the Post-hoc Tukey test for multiple comparisons. Simpson, Simpson index; ACE, ACE index; Chao, Chao1 index.
(3) Good's coverage at a distance of 0.03.

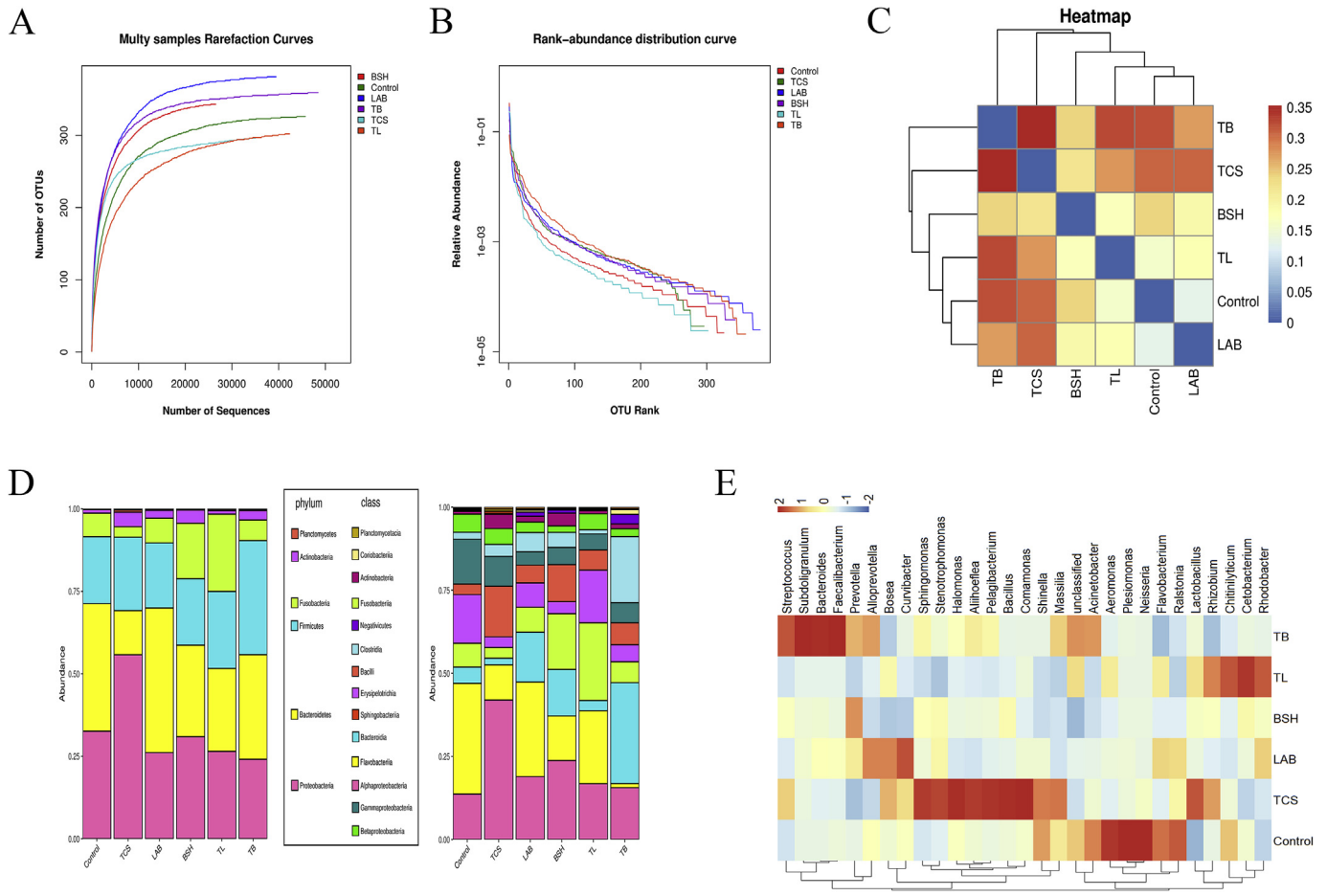

Fig. 1. Diversity and richness of the gut microbiota in zebrafish.
Note: (1) A-B, Rarefaction curve and rank abundance curves for six experimental treatments; C, Heatmap of weighted Unifrac distance for six experimental treatments (color block represents distance values. Blue represents a shorter distance between the samples as well as higher similarity while red represents a greater distance between the samples as well as lower similarity); D, Relative abundance (percentage of sequences) of gut microbiota at phyla and class levels for the six experimental treatments; F, Heatmap for genus level of six experimental treatments; (2) For color interpretation in this figure legend, the reader is referred to the web version of this article.

sequence abundance, which was determined to the genus level at the 97% similarity level (Fig. 1E). The 50 dominant OTUs differed slightly between the control and LAB groups, while the TCS group was considerably different from the other groups. The level of relatedness was closest for the LAB and control groups, followed by the TL and TB groups (Fig. S4B). This indicates that addition of *Lactobacillus plantarum* ST-III and BSH resulted in a microbial flora species distinction from the TCS-exposure treatment (TCS group).

3.2. Body characteristic index and biomarker detection

The effects of *Lactobacillus* or BSH additions on toxicity alleviation due to TCS exposure were evaluated from the body mass index (BMI) and organ weights (Fig. 2A). Body length and width of zebrafish were significantly longer ($p < 0.01$) in TCS and BSH groups than in the control (Fig. 2B and C). Compared to the control group, the BMI was slightly increased in the TCS group, but significantly decreased ($p < 0.01$) in other treatment groups (Fig. 2D). In contrast to the TCS group, the BMI was significantly lower ($p < 0.01$) in the TL group, but no prominent differences were observed between the BSH and TB groups (Fig. 2E), implying that the biological activity of *Lactobacillus* was higher than BSH. Except for a slight increase of liver and intestine weights in the TCS group, no significant differences in organ weight were observed for the other treatments.

Malonaldeyhde (MDA) is often measured as a biomarker of lipid peroxidation [42]. After exposure to some chemicals, imbalance of intestinal flora, metabolism of fatty acids and abnormal oxidation increase the accumulation of mitochondrial reactive oxygen species (ROS), leading to oxidative stress [43,44]. Compared to the control, MDA concentrations in the intestinal tract were significantly increased in TCS, BSH and TB groups ($p < 0.001$) (Fig. 2F). They were significantly lower in LAB and TL groups than in TCS groups, while no significant differences were observed for BSH and TB groups compared to the TCS group. Overall, these results demonstrated that *Lactobacillus* addition reduced damage from lipid peroxidation in the intestinal tract.

3.3. Effects of TCS exposure and probiotics diet on lipid metabolism in intestine and liver

ORO staining observations on 90-dpf zebrafish liver sections were conducted for the six treatment groups. Liver tissues (Fig. 3G-L) in the TCS group exhibited more lipid droplets than other groups (Fig. 3H); meanwhile, the probiotics diet prominently decreased the number of lipid droplets (Fig. 3K). Additionally, TCS exposure induced severe histopathological changes and damage to liver tissue. Under 400× magnification, the liver structure in the control and LAB groups was complete and clear, and the shape of hepatocytes was regular and arranged in tubules (Fig. 3A and D). In contrast, the TCS group liver structure demonstrated fibrosis, increased lipid droplet numbers and greater distribution area, larger cell gap and scattered cell distribution

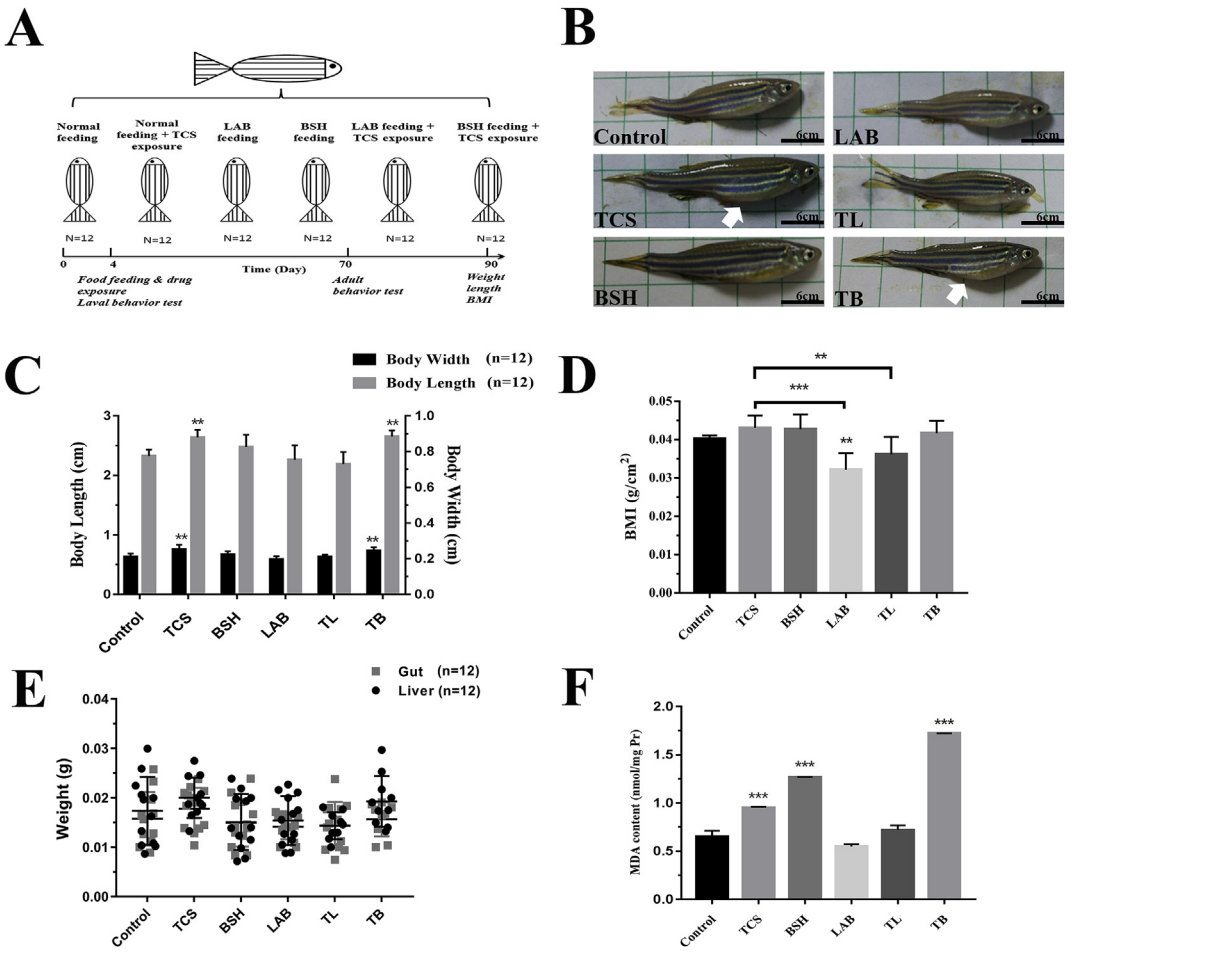

Fig. 2. Body mass index (BMI) and biomarkers (MDA) detection for zebrafish from the six experimental treatments.
Note: (1) A, Schematic overview of 12-week feeding protocol and phenotyping of zebrafish; B, Six experimental groups of zebrafish in the same tank at the 90-dpf stage, scale bars = 6 cm; C, Standard length and width of zebrafish in 90 dpf; D, Body mass index (BMI) of six experimental treatments; E, Liver and intestine weights for the six experimental treatments; F, MDA (nmol/mg Pr) concentration; (2) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; Twelve fish per treatment (n = 12, 4 fish per replicate aquarium).

(Fig. 3B). For the same TCS concentration (200 μg/L), liver structure injury and lipid accumulation were lower in probiotics diet groups (TL and LAB groups) as compared to BSH diet group (Fig. 3D and E). Triglyceride (TG) and total cholesterol (TCH) levels in zebrafish liver were significantly higher in TCS groups than the control ($p < 0.01$). Additionally, TG and TCH levels were significantly decreased ($p < 0.01$ and $p < 0.001$) in LAB and TL groups as compared to the TCS group (Fig. 3M and N).

3.4. qRT-PCR analyses of immune-related genes in adult zebrafish intestines

To investigate mechanisms by which probiotics and BSH mediate cellular and molecular responses, six immune-related genes were selected for *q*RT-PCR analyses on the basis of previous studies [45]. In zebrafish intestines, the pro-inflammatory cytokines nuclear factor-κB (*NF-κB*, a ubiquitous transcription factor), interleukin 1 beta (*IL-1β*, a pro-inflammatory cytokine) and tumor necrosis factor alpha (*TNF-α*, a multifunctional pro-inflammatory cytokine) were up-regulated in the TCS group (Fig. 4A–C). This demonstrates that TCS exposure caused inflammatory damage to zebrafish intestinal mucosa. In contrast, expression of the three pro-inflammatory factors was slightly down-regulated in LAB and BSH groups. Compared to the control group, *lysozyme* levels (catalyzing hydrolysis of certain mucopolysaccharides in bacterial cell walls) in mRNA were significantly up-regulated ($p < 0.001$) in TCS and TB groups (Fig. 4E), but down-regulated ($p < 0.05$) for probiotics treatments (TL and LAB groups). Up-regulation of *lysozyme* represents an aggravation of inflammation, which the BSH diet did not alleviate. Transcription levels of toll-like receptor 4 alpha (*TLR4α*, recognized as LPS, mannan, and glycoinositolphospholipids) and interleukin 10 (*IL-10*, a potent suppressant of macrophage functions) showed no obvious changes among treatment groups (Fig. 4D and F), with the exception of a significant down-regulation ($p < 0.01$) of *TLR4α* and up-regulation of IL-10 ($p < 0.01$) in the BSH group. The significant down-regulation of *TLR4α* and up-regulation of *IL-10* indicate an enhanced immune inhibitory effect in the BSH group. Chronic TCS exposure stimulated an immune response in zebrafish intestines resulting in mild injury to the intestinal mucosa immune system. The *Lactobacillus* diet effectively alleviated the inflammatory effect, however, the BSH diet did not attenuate this effect.

3.5. Chronic TCS-exposure damage to intestinal mucosa and immune organs of zebrafish

The intestinal lamina propria is the largest immune area of the intestinal mucosal immune system hosting a considerable quantity of T lymphocytes that play a critical role in immune surveillance of the epithelium [46]. Disturbance of T cell homeostasis can induce chronic intestinal inflammation that resembles human inflammatory bowel disease [47]. Significant increases in $CD4^+$ T cells were detected in TCS and TB groups (Fig. 5B and F). Meanwhile, the number of $CD4^+$ T cell

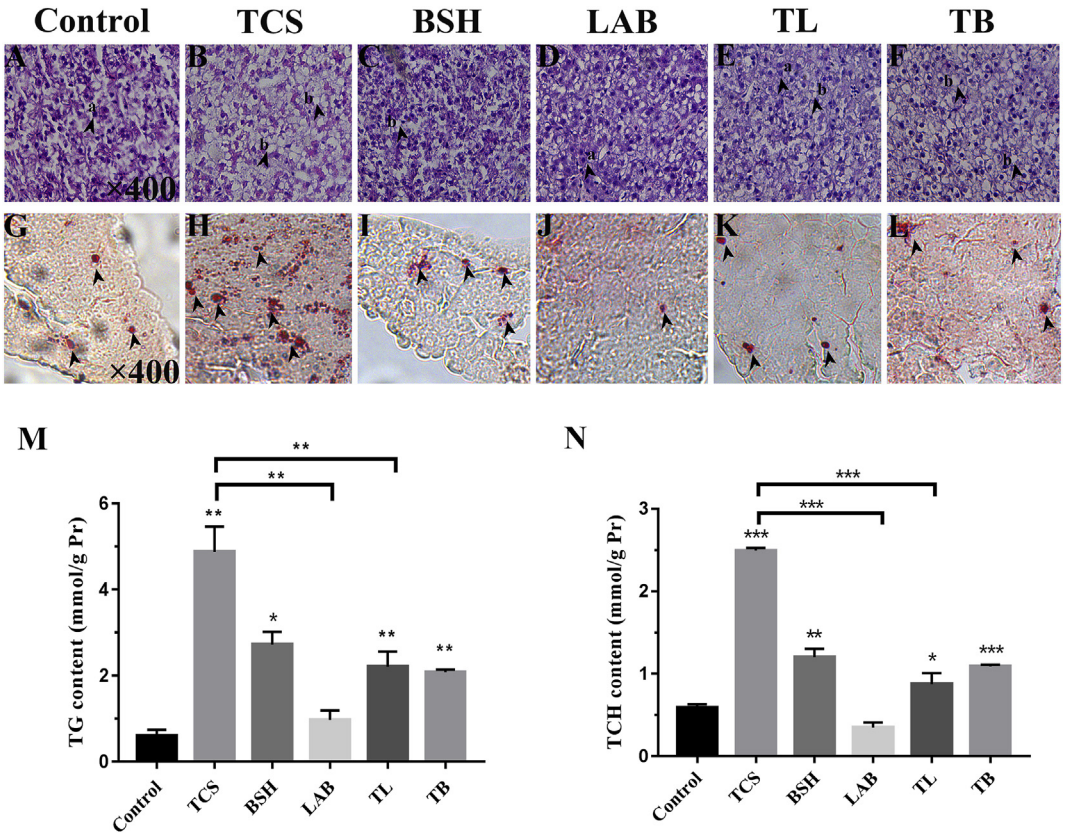

Fig. 3. Oil red O staining, histopathological observation and biochemical analyses of liver lipids.
Note: (1) A - F, HE dyeing of zebrafish liver from six experimental treatments, "black arrow" shows the decrease in nucleus number and steatosis; (2) "a arrow", normal hepatocyte; "b arrow", streatosis; (3) G - L, Histopathological Oil red O analysis of zebrafish liver after 90 days of TCS exposure and probiotic diet, "black arrow" shows lipid droplets; (4) M and N, TG and TCH levels in six experimental treatments; (5) *", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; Twelve fish per treatment (n = 12, 4 fish per replicate aquarium). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lymphocytes in the lamina propria of the duodenal mucosa was lower in LAB and TL groups (Fig. 5D and E) than TCS and BSH groups. This finding is in agreement with the expression trend for the six inflammatory genes in the intestinal mucosa as described above.

Under 200× magnification, the small intestine structure in the control, LAB, and TL groups was complete and clear, and its peripheral muscle layer was relatively intact. We observed normal mucosal fold tissue (MF) and a proper quantity of goblet cells (GC) in control and LAB groups (Fig. 5I–a,d). In particular, the intestinal mucosa in the LAB group was thick and intact. In contrast, rupture and dissolution phenomena were observed in the TCS group along with intestinal wall thinning and destruction of intestinal epithelia (Fig. 5I–b,f, black arrows), swelling of columnar epithelial cells of intestinal villi, and a sharp increase in goblet cell number in intestinal villi. Additionally, the number and height of intestinal villi were slightly decreased in the TL group compared to the TCS group (Fig. 5 I-e).

Kidney (pro- and meso-nephros) and spleen are the major lymphoid organs of teleost [48]. No histological impairments were observed in spleen of control zebrafish; organelles of lymphocyte were evenly distributed throughout the cytoplasm, and chromatin was dispersed throughout the nucleus (Fig. 5S–a). The LAB, BSH and TL groups showed no obvious damage (Fig. 5S–c,d,e), while considerable lesions were observed in spleen of the TCS group (cell shrinking, widening of intercellular space and a few edematous mitochondria) (Fig. 5S–b). In contrast to the TCS group, an alleviated pathological lesion was observed in the TB group (Fig. 5S–f). In zebrafish kidneys of the TCS and BSH groups (Fig. 5 K-b,c; black arrows), glomeruli showed slight shrinkage, and the areas between renal tubules and glomeruli appeared less tightly arranged compared to those of control and LAB groups (Fig. 5 K-a,d). Normal proximal tubules (PT) and distal tubules (DT) were observed in the control, LAB and TL groups (Fig. 5K–a,d,e). The kidney tissue structure in the LAB group was well protected displaying only mild interstitial edema and low tubular epithelial vacuolation and detachment when compared with the TCS group (Fig. 5 K-e).

3.6. Chronic TCS exposure induces inflammation in liver

Increasing evidence has linked NAFLD progression to nonalcoholic steatohepatitis (NASH), which is posited to involve inflammasome activation [49,50]. Our previous research confirmed that acute TCS exposure causes fat accumulation in larvae zebrafish liver [51]. To determine whether or not chronic TCS exposure induced inflammation in liver, we conducted a series of immunofluorescence experiments using zebrafish liver sections labeled $CD4^+$ and LAMP1.

Immunofluorescence analysis demonstrated a prominent increase of $CD4^+$ T cell lymphocytes in the TCS group (Fig. 6H), which showed $CD4^+$ T cells clustered around a granuloma-like structure. Compared to the TCS group, lower $CD4^+$ cell numbers were observed in probiotics diet groups (LAB and TL; Fig. 6J and K). Loss of hepatocytes and the presence of single hepatocytes with apoptotic morphology (pyknotic nucleus, rounded cytoplasmic margins) were frequently observed within inflammatory cell aggregates. We labeled these apoptotic cells using LAMP1, which is a major protein component of lysosome and primarily located on the lysosomal membrane [52]. In the TCS and BSH groups, a number of inflammatory cells displayed mononuclear morphology, were positive for LAMP1, and were co-localized with CD4

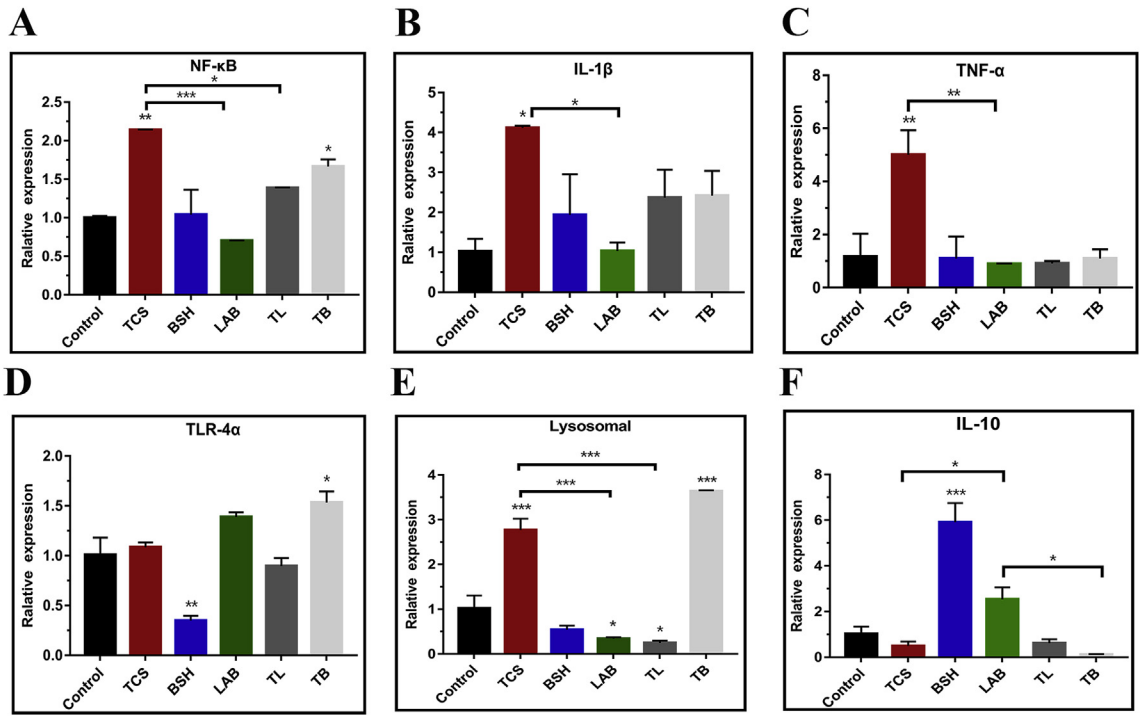

Fig. 4. Differential expression of the immune-related genes by *q*RT-PCR.
Note: (1) A - F, Expressions of immune-related genes of *NF-κB* activating protein-like, and tumor necrosis factor (*TNF-α*), *lysosomal*, toll-like receptor 4 alpha (*TLR4α*), interleukin (*IL*)-*1β, IL-10*; (2) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; Each experiment was repeated at least three times.

antigen (Fig. 6N,R). The addition of *Lactobacillus* resulted in a significant decrease in the number of inflammatory cells in LAB and TL groups (Fig. 6P and Q). These results suggest that chronic TCS exposure induced inflammation in zebrafish liver and a probiotics diet was effective in preventing inflammatory effects, but the alleviated inflammatory effect was not apparent in the BSH group (Fig. 6O).

3.7. Behavioral effects of adult zebrafish resulting from TCS exposure and probiotics diet

Previous zebrafish studies demonstrated that chronic TCS exposure led to lipid metabolism disorders [31], intestinal flora imbalance [53] and behavioral abnormality [22]. Probiotics is a biological therapy widely used in the treatment of various diseases. Therefore, we investigated the effects of a probiotics diet on a series of zebrafish behavioral effects.

3.7.1. T-maze experiment

Learning and memory tests were conducted in a T-maze [54]. After food bait and tapping stimulus training, zebrafish in the control and LAB groups almost immediately swam to the right area (Fig. 7-D, 'r' area) and remained there, which demonstrated that zebrafish had a strong memory and recognition capacity compared to the other groups. TL treatment resulted in an increased number of transitions to the right side compared to the TCS group (Fig. 7C), suggesting that a probiotics diet partially alleviated memory disorders resulting from TCS exposure. In the TCS group, mean velocity and distance traveled were significantly enhanced ($p < 0.001$ or $p < 0.01$), but the time spent in the right side was significantly reduced ($p < 0.05$), indicating that zebrafish were less capable of identifying bait and tapping stimulation. Zebrafish in the BSH and TB groups demonstrated similar responses, implying no obvious improvement in discernment and memory capacities (Fig. 7D).

3.7.2. Bottom dwelling test (BWT)

The BWT is used to measure changes in zebrafish habitat preference. Zebrafish from the control group preferred swimming in the bottom of the tank (Fig. 8D) while those from TCS, BSH and TB groups showed no preference. Zebrafish swimming velocity and activity significantly increased ($p < 0.001$) when receiving a probiotics diet (LAB and TL groups; Fig. 8A and B). A lower preference for the bottom of the tank was displayed by the BSH and TB groups compared to control group ($p < 0.001$) (Fig. 8C), suggesting that zebrafish suffered an anxiety-like state when exposed to TCS.

3.7.3. Social interaction

Adult zebrafish are social animals like humans and other teleost [55]. To analyze the effects of a probiotics diet on the social activities of zebrafish, a series of social parameters were evaluated (Fig. S3D). Among the six treatment groups, the longest swim distance was found for the TCS group, which was significantly higher than the control group ($p < 0.05$), but the swim distance within the social area was similar to the control group (Fig. S3C). Compared to the control group, mean swim velocity increased by 10% ($p < 0.001$) for the BSH group and decreased for the TB group ($p < 0.05$), suggesting that the BSH diet did not overcome the mild anxiety-like state in zebrafish. No significant changes in mean swimming distance were observed in the social area among the treatment groups (Figs. S3C–E).

4. Discussion

The role of the microbiome in influencing numerous aspects of metabolic, immunologic and neurological functions has attracted much recent attention [56,57], with a number of studies showing that some metabolic diseases, inflammatory responses and neurodevelopmental disorders have a crucial linkage with mammalian gut microbial community [58–60]. The diversity of intestinal flora is a product of long-term interactions between diet and environment [61]. Many animal studies have demonstrated that TCS poses potential risks as an

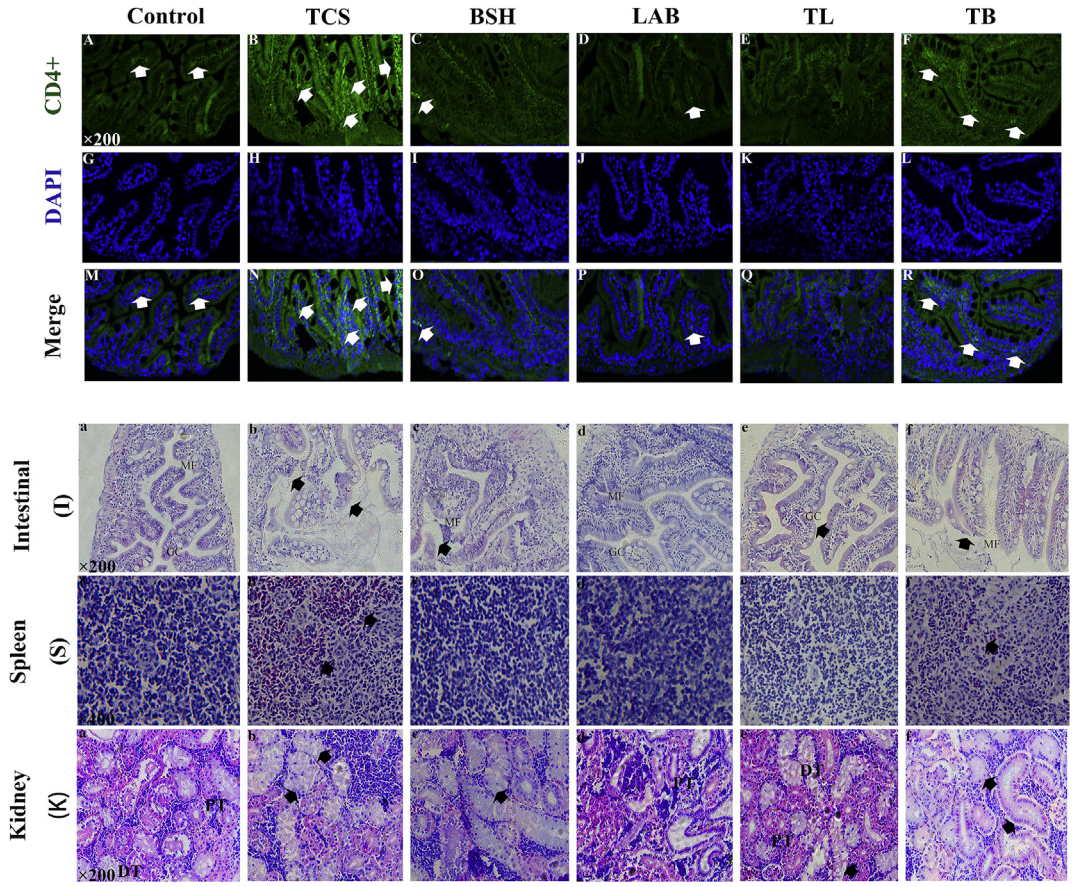

Fig. 5. Gut histopathology of zebrafish and immunofluorescence of intestinal mucosa detection.
Note: (1) A - R, Confocal image of intestinal sections of zebrafish from six experimental treatments, DAPI (blue), $CD4^+$ T cell (green), "white arrow" shows increased CD4 positive antigen; (2) Capital letters in Fig. 5I, S, 5K, I: intestines, S: spleen and K: kidney; (2) a-f, Control, TCS, BSH, LAB, TL and TB treatment groups; (3) MF, mucosal fold; GC, goblet cells; PT, proximal tubules; DT, distal tubules; (4) "black arrow" in Fig. 5I–b, I-f shows destruction of intestinal epithelia in TCS and TB treatments; "black arrow" in Fig. 5S–b, S-f shows cell shrinking, intercellular space widening and mitochondrion edema in TCS and TB treatments; "black arrow" in Fig. 5 K-b, K-c, K-f shows structural damage of glomerulus and renal tubules: the glomeruli showed slight shrinkage and the areas between renal tubules and glomeruli appeared less tightly arranged in TCS, BSH and TB groups.

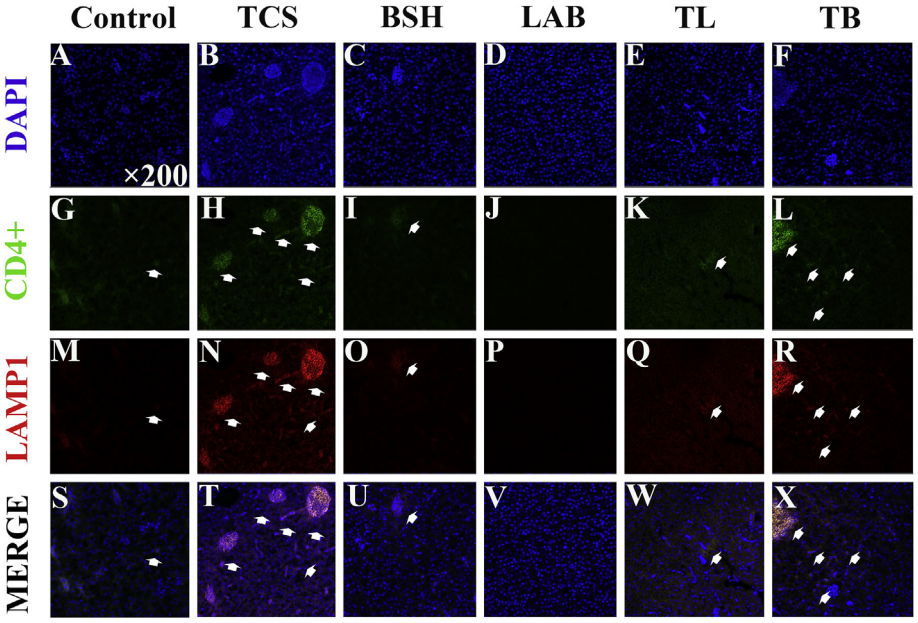

Fig. 6. Immunofluorescence of zebrafish liver.
Note: (1) A - X, Confocal image of liver sections from the six experimental treatments, DAPI (blue), $CD4^+$ T cell (green), LAMP1 (red); (2) "white arrow" shows increased $CD4^+$ and LAMP1 positive antigen.

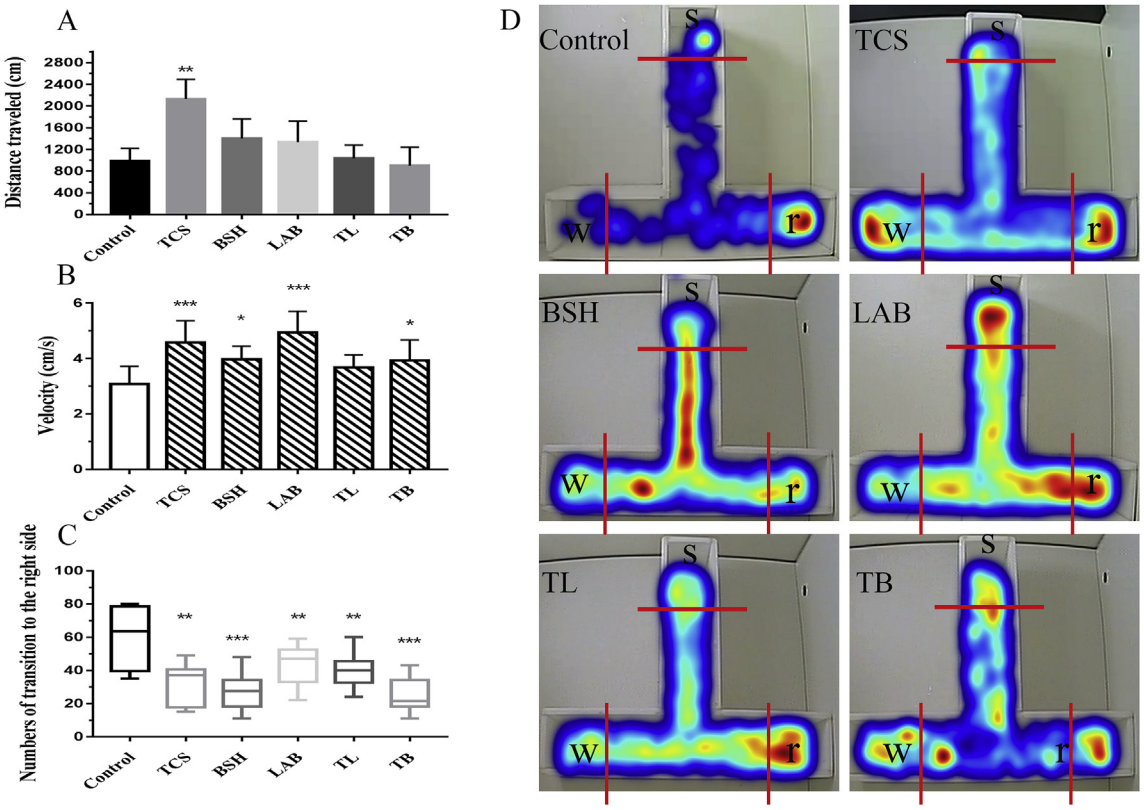

Fig. 7. Effects of six experimental treatments on 90-dpf adult zebrafish of T-maze test behavior.
Note: (1) A, Mean velocity; B, Distance traveled; C, Number of transitions to social area; D, Heat map of T-maze test, 's' arrow, start; 'r' arrow, bait zone (right side); 'w' arrow, stimulating zone (left side); (2) Blue to green = lower frequency, yellow and red = higher frequency (For interpretation color in this legend, the reader is referred to the web version of this article). (3) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; Twelve fish per treatment (n = 12, 4 fish per replicate aquarium).

endocrine disruptors altering host physiology [62], lipid metabolism [31] and abnormal behavior [22]. In this investigation, zebrafish were exposed to 200 μg/L TCS for three months with or without a diet containing *Lactobacillus plantarum* ST-III or BSH to construct a long-term drug-diet intervention pattern. Davis et al. (2016) reported that *L. plantarum* attenuated anxiety-related behavior and protected against stress-induced dysbiosis in adult zebrafish [63], consistent with the findings of this study. In T-maze and BDT experiments, a long-term probiotics diet alleviated the abnormal behavior caused by TCS exposure resulting in enhanced identification and memory ability along with anxiety relief.

This study demonstrated that the abundance of *Bacteroidetes* decreased due to TCS exposure, and their abundance was positively correlated with zebrafish BMI index (Fig. S5C). Further, ORO staining and TG and TCH quantification in zebrafish liver determined that long-term TCS exposure caused abnormal fat accumulation with a prominent decrease in lipid droplets resulting from a *Lactobacillus* diet (Fig. 3). These findings strongly suggest that a *Lactobacillus* diet can attenuate lipid metabolism disorder caused by TCS exposure.

Emerging evidence posits that intestinal microbiota and their products regulate host gene expression and affect systemic inflammation [64,65]. The gut-liver axis has been proposed as a key factor in the pathogenesis of NAFLD, as the passage of bacteria-derived products into the portal circulation could trigger innate immunity, which in turn may lead to liver inflammation [66]. In this study, accumulation of T lymphocytes and lysosomal antigens in the liver of TCS-exposed zebrafish indicated that TCS caused severe liver inflammation, while the number of positive T lymphocytes significantly decreased in the probiotics diet group. Further, the Illumina MiSeq sequencing results indicated an increase and recovery of intestinal microbial diversity in the TCS treated, probiotics diet group (TL group). Based on these results, we speculate that *Lactobacillus* may alleviate inflammatory injury by regulating the intestinal flora of zebrafish. Further study is warranted to illustrate how the intestinal microflora affect lipid deposition and to probe the relationship between *Lactobacillus* diet and changes in gut microflora.

In this study, the zebrafish gut microbiota community varied substantially due to TCS exposure, and the *Lactobacillus* diet effectively reduced this imbalance. Our research found that the *Proteobacteria*, *Bacteroidetes* and *Firmicutes* at phylum level are the primary members of the gut microbiota among all the treatment groups. *Proteobacteria* abundance was considerably higher in the TCS group (55.8%) than the other treatment groups. We also observed that the intestinal mucosal injury was more severe in the TCS group than for other treatments. Significant up-regulation of the pro-inflammatory factors *NF-κB*, *IL-1β* and *TNF-α*, as well as increased CD4 positive antigens indicated that the zebrafish intestinal mucosa experienced a serious inflammatory reaction from TCS exposure. In contrast, expression of pro-inflammatory factors was significantly decreased and the number of CD4 antigen in the intestinal mucosa decreased with a *Lactobacillus* diet. Additionally, the MDA level was significantly increased in the TCS group, indicating serious lipid-peroxidation damage in zebrafish intestines. In contrast, the TL treatment experience attenuated damage to the intestinal mucosa and lipid peroxidation. Microbiota dynamics are important for the development and regulation of innate and adaptive immune systems [67]. He et al. (2016) established an IBD-like model for zebrafish larvae and found that gut microbiota disorders were mainly manifested by increased proportion of *Proteobacteria* (especially *Burkholderia*) and a decreased of *Firmicutes* (*Lactobacillus* group), which were significantly correlated with enterocolitis severity [68]. Recent studies suggest dysbiotic expansion of *Proteobacteria* was a potential diagnostic microbial

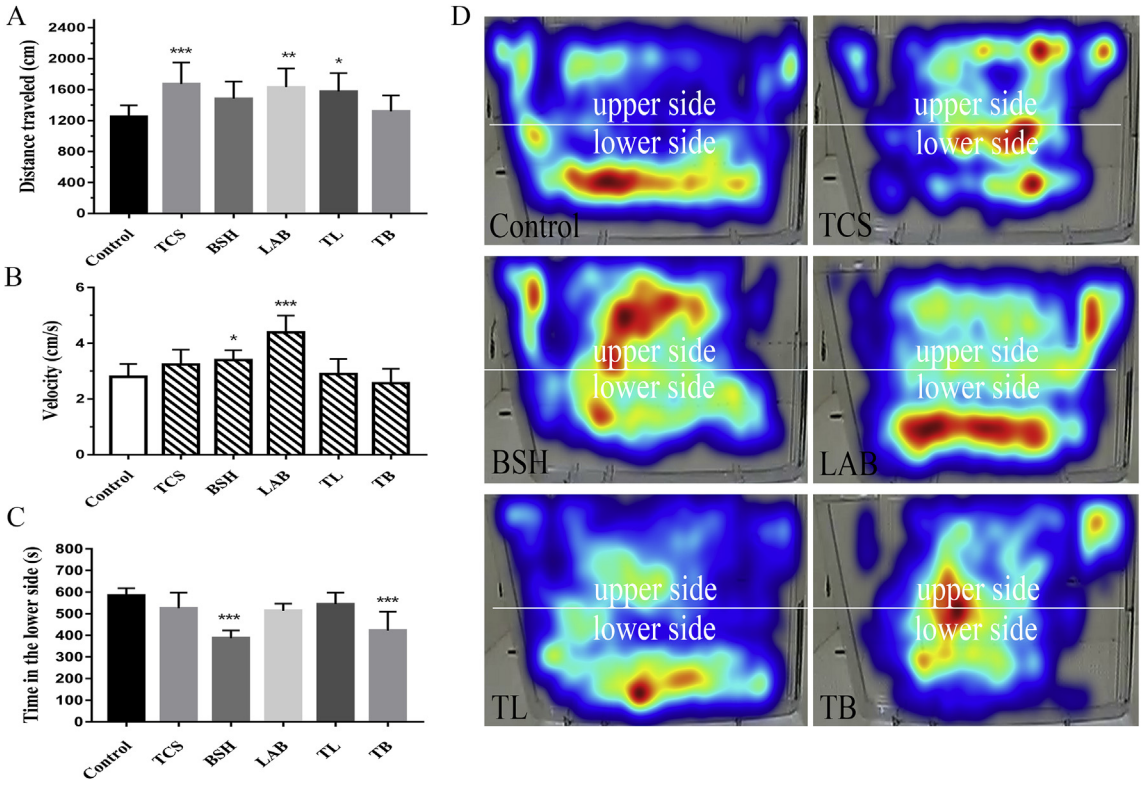

Fig. 8. Effects of six experimental treatments on 90-dpf adult zebrafish on swimming behavior.
Note: (1) A, Mean velocity; B, Distance traveled; C, Time in the lower tank; D, Heat map for tank test; (2) Blue to green = lower frequency, yellow and red = higher frequency (For interpretation of color in this figure legend, the reader is referred to the web version of this article). (3) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; Twelve fish per treatment (n = 12, 4 fish per replicate aquarium).

signature of epithelial dysfunction, and a sustained overabundance of *Proteobacteria* might contribute to dysbiotic of gut microbiota [69,70]. The colonic epithelium is hypoxic; however, TCS exposure may result in intestinal inflammation and an increase in epithelial oxygenation in the colon, thereby disrupting anaerobiosis to drive dysbiotic expansion of facultative anaerobic *Proteobacteria* through aerobic respiration [69]. We speculate that the TCS caused the injury of zebrafish intestinal mucosa, changed the anaerobic environment of the intestinal flora, and increased the proliferation of *Proteobacteria*, and in turn, aggravated inflammation and mucosal damage. Sequencing results showed that probiotics might mitigate this damage by means of indirect regulation of gut microbiota composition and diversity.

Increasing evidence indicates that probiotics can improve glucose control, lipid profiles, blood pressure [15,16], and relieve anxiety by modulating gut microbiota [71]. *Lactobacilli* play an important role in bile salt deconjugation *in vivo* [72], because they can potentially reduce serum cholesterol concentrations in humans [73]. To investigate the specific mechanism associated with probiotics, we selected *Lactobacillus* with high BSH activity as the experimental target, and also extracted crude BSH liquid by ultrasonic fracturing. Notably, *Lactobacillus* relieved damage from TCS exposure, however, the crude BSH liquid had no effect. We hypothesized that the cause of this phenomenon may be due to the loss of activity of the bile salt hydrolase during the extraction process.

5. Conclusions

Herein, we demonstrated that chronic TCS exposure affected liver fat metabolism, intestinal mucosal immunity and motor behavior of zebrafish, and dietary *Laetobacillus plantarum* ST-III alleviated these toxic effects. TCS exposure reduced diversity of intestinal flora, while probiotic diet recovered abundance of beneficial bacteria. TG and TCH content changes, as well as ORO and HE staining all identified that *Lactobacilli* can effectively relieve liver fat accumulation from TCS exposure. Meanwhile, the up-regulation of pro-inflammatory genes (*IL-1β*, *TNF-α*) and high MDA concentrations confirmed that TCS exposure resulted in severe inflammatory responses to intestinal mucosa, and *Lactobacillus*-feeding effectively alleviated this inflammatory effect. HE staining of immune organs and immunofluorescence of liver and gut indicated that chronic TCS exposure caused an obvious damage in immune system of zebrafish, but this injury was somewhat mitigated following probiotics feeding. Moreover, a series of anxiety-like abnormal behavior was attenuated by long-term probiotics feeding in T-maze, bottom dwelling and social tests. These findings inform assessment of health risk from TCS exposure and document the application value of probiotics in alleviating fish immune-related diseases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.007.

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