Original Research

Combined effect of microplastic and triphenyltin: Insights from the gut-brain axis

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ARTICLE INFO

ABSTRACT

Microplastics (MPs), an emerging group of pollutants, not only have direct toxic effects on aquatic organisms but also cause combined toxicity by absorbing other pollutants. Triphenyltin (TPT), one of the most widely used organotin compounds, has adverse effects on aquatic organisms. However, little is known about the combined toxicity of MPs and TPT to aquatic organisms. To investigate the individual and combined toxicity of MPs and TPT, we selected the common carp (*Cyprinus carpio*) for a 42-day exposure experiment. Based on the environmental concentrations in a heavily polluted area, the experimental concentrations of MPs and TPT were set at 0.5 mg L^{-1} and 1 μg L^{-1}, respectively. The effects of MPs combined with TPT on the carp gut−brain axis were evaluated by detecting gut physiology and biochemical parameters, gut microbial 16S rRNA, and brain transcriptome sequencing. Our results suggest that a single TPT caused lipid metabolism disorder and a single MP induced immunosuppression in carp. When MPs were combined with TPT, the involvement of TPT amplified the immunotoxic effect induced by MPs. In this study, we also explored the gut−brain axis relationship of carp immunosuppression, providing new insights for assessing the combined toxicity of MPs and TPT. At the same time, our study provides a theoretical basis for evaluating the coexistence risk of MPs and TPT in the aquatic environment.

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1. Introduction

Plastic pollution has been declared by the United Nations Environment Programme as one of the most serious environmental problems of our time, and plastic debris has become a globally recognized emerging environmental problem [1]. Owing to unavoidable release, plastics, especially the microplastics (MPs; plastic particles smaller than 5 mm) [2], are detected in oceans, estuaries, fresh water, and sediments [3,4] and have even been found in remote regions of the Arctic and Antarctic [5,6]. Microplastics come in various shapes, including spherical, fibrous, fragmented, and thin-film shapes [7,8]. In addition to the breakdown of larger plastics, microplastics in the water come from "primary

microplastics," which are cosmetic beads, plastic pellets, or fibers specifically designed and produced for use in abrasive cleaning products such as face washes and toothpastes [9]. Spherical MPs have been found in Granada, Spain, the Northeast Atlantic Ocean, and Odisha Beach, India [10]. With the continuous breakdown of plastic waste and the increase in primary MP production, the residual amount of spherical MPs in the water is increasing rapidly. Therefore, spherical primary MP particles were selected as the research topic in this study.

Numerous studies have found that MPs can have a negative effect on aquatic biota. For example, MP exposure can affect the growth and reproduction of organisms [11,12], inhibit metabolism [13], induce immunotoxicity, lead to neurotoxicity [14], and disorder the endocrine [15]. The intestine is the main digestion, absorption, and immune organ, and plays an important role in maintaining the life activities of organisms [16]. Therefore, the intestine is a potential target organ of MPs and thus worthy of research. In recent years, many studies have proved that MPs can

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cause an imbalance of the intestinal flora and increase the proportion of harmful bacteria. Meanwhile, the intestinal barrier function will also be affected by MPs; that is, the structure of intestinal villi and mucus layer will change [17,18]. The gut–brain axis is the mutual information exchange system between the brain and gut, consisting of the neuroendocrine, vagal, and immune pathways, and plays a central role in host development, metabolism, and physiology [19]. Brain tissue, being an important link in the "gut–brain axis", is the tissue in which environmental pollutants mainly accumulate [20,21], and MPs are likely to cause damage to biological brain tissue through the blood–brain barrier. As an emerging pollutant, MPs not only cause direct toxicity to organisms through the food chain [22], but owing to their hydrophobic surface and high surface area, they can also adsorb and interact with other environmental pollutants, leading to combined toxicity [23].

Organotin compounds (OTCs) are widely used in industrial, agricultural, and aquaculture areas owing to their excellent general properties, but this has resulted in severe water pollution [24,25]. In recent years, triphenyltin (TPT) pollution has become a topic of concern. For example, the highest concentration of TPT in the waters of Saudi Arabia and the port of Cape Town of South Africa reached 1.9 and $23008.0 \pm 0.03 \times 10^{-3}$ µg L^{-1}, respectively [26]. Sewage wastewater is a major source of MPs and OTCs, so there is a high risk of coexistence with these two pollutants [27]. It was reported that MPs and nutritional status can mitigate TBT toxicity to the marine rotifer *Brachionus koreanus* during dietary exposure, but TBT-induced toxicity and its legacy effects are unavoidable [28]. Additionally, in a study of algae exposure to MPs combined with TPT, it was found that MPs damaged the structure of chlorella cells, thereby increasing the absorption of TPT by cells and amplifying the toxic effect [29]. In contrast, it is not only difficult for the MPs to destroy the dense silica shell of diatoms, but they can also adsorb TPT in water, reducing the TPT concentration in the water as well as the toxic effect on diatoms [30].

Currently, there are few reports on the combined toxicity of MPs and OTCs to aquatic fish despite the high risk of their coexistence. More importantly, MPs can be used as carriers of contaminants, such as polysulfates and metals, to reach the intestinal tract of fish through involuntary ingestion [31–33]. Therefore, it is necessary to study the combined effects of MPs and TPT on fish. The purpose of this study was to comprehensively explore the aquatic ecological risks of the coexistence of MPs and TPT by investigating the combined toxicity of MPs and TPT to common carp (*Cyprinus carpio*). Carp is often used as a biological indicator of polluted aquatic ecosystems, and it is also an important aquaculture species in many countries. In this study, the carp were exposed to TPT, MPs, and TPT combined with MPs for 42 days. Considering the possible adsorption effect of MPs on TPT, we hypothesized that their combined toxicity to carp might be lower than the individual toxicity of TPT, but greater than that of MPs alone.

To test this hypothesis, we investigated changes in intestinal flora, analyzed the results of brain tissue transcriptome sequencing, observed the intestinal glycoprotein secretion, and detected the biochemical parameters and the expression of genes involved in immunity and lipid metabolism. Additionally, we explored the effect of combined toxicity of MPs and TPT on the carp gut–brain axis in combination with the microbe–host relationship and provide a theoretical basis for evaluating the risk of combined toxicity caused by the coexistence of MPs and TPT.

2. Materials and methods

2.1. Chemicals and test fish

Polystyrene MP beads (diameter: 200 nm) were purchased from Wuxi Regal Biotechnology Co., Ltd. (Wuxi, China). The morphology of the beads was confirmed by scanning electron microscope TESCAN VEGA3 (Brno, Czech Republic) (Fig. S1). The final concentration of the MP-treated group was 0.5 mg L^{-1} (600 µL of the MP suspension at a concentration of 0.025 g mL^{-1} was added to 30 L of water). Considering the size-dependent toxicity of MPs and uncertainty as to whether plastic microspheres can penetrate various barriers in the organism, we chose submicron plastic microspheres with a particle size of 200 nm. The MP concentration of the MP exposure group was set with reference to previous studies [34,35].

Triphenyltin chloride (purity >96%, CAS: 639-58-7) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). In accordance with a previous study [36], a TPT chloride stock solution was prepared to a concentration of 300 ng $µL^{-1}$ in DMSO, and the final concentration of DMSO in the exposure group was less than 0.001%. The final concentration of TPT in the treatment group was set to 1 µg L^{-1}, based on the highest actual environmental concentration of TPT in the waters of Saudi Arabia and Cape Town of South Africa, which can reach up to µg L^{-1} [26,37].

The juvenile carp (*Cyprinus carpio*) (16.70 ± 4.72 g, 11.67 ± 1.20 cm) came from Xinda Fish Farm (Tianjin, China). The relative fatness K (K = W(g)/L(cm)^3 × 100) is shown in Table S1. Prior to the exposure experiments, carp were temporarily adapted in the laboratory for one week. The fish were randomly divided into four groups (three tanks per group), and six healthy carp were stocked in each glass tank with 30 L of water. During this period, they were fed commercial bait (Xinda, China) twice a day (9:00 a.m., 15:00 p.m.). The water was changed every two days to remove residual bait and metabolic waste. The temporary holding aquarium was a temperature-controlled circulatory system (pH 7.6 ± 0.2, 23 ± 1 °C), and several aerated stones were set to ensure sufficient oxygen in the water. All experimental operations on carp in this study were approved by the Local Animal Ethics Committee.

2.2. Experimental design and sampling

The exposure experiment was carried out after the carp were temporarily adapted for a week. The control, 1 µg L^{-1} TPT, 0.5 mg L^{-1} MPs, and 1 µg L^{-1} TPT + 0.5 mg L^{-1} MPs (TPT_MP) treatment groups were set up based on the actual environmental concentration of the pollutants, considering the most severe cases of pollution. The exposure group was set with the conditions of the respite period. During the exposure, the water quality parameters and feeding were consistent with the adaptation period. We replaced 2/3 of the water and added the pollutant stock solution to ensure a stable concentration every two days. All solution samples were analyzed, and the measured concentration of TPT (0.93 ± 0.10 µg L^{-1}, corresponding to 1.0 µg L^{-1}) was within 20% of the nominal concentration, which meets the OECD guidelines (OECD Guideline for Testing of Chemicals No. 204, "Fish, Prolonged Toxicity Test").

After 42 days of continuous exposure, six fish (two fish from each tank in each group) were randomly selected from each group for sampling. The carp were not fed for 24 h prior to sampling. After the carp were anesthetized with MS222 (20 µg L^{-1}, tricaine mesylate), their body weight and body length were measured. The abdomen of the carp was cut open, the liver was quickly separated, and the intestinal tissue was taken out. Then, the carp cranium was cut open, and the brain tissue was carefully removed with forceps. Some intestinal tissues were fixed in 10% formalin solution for observation of tissue structure and the remaining intestinal tissues and brain tissues were snap-frozen in liquid nitrogen and stored at −80 °C for the detection of biochemical indicators, RT-qPCR, and brain transcriptome sequencing. Additionally, six fish were

randomly selected from each group (two fish from each tank in each group) for intestinal content sampling. Intestinal contents were removed after feeding for 6 h for 16S rRNA sequencing analysis of intestinal flora.

2.3. Histopathological examination

After a series of treatments, intestinal tissue fixed in 10% formalin was embedded in paraffin, cut into 4 μm-thick sections, and then stained with periodic acid−Schiff (PAS). The total area and density of glycoproteins in the carp intestinal tissue were detected and analyzed using Image Pro Plus 5.1 (MEDIA CYBERNETICS, Rockville, MD, USA).

2.4. Gut microbiota analysis

Three stool samples were randomly selected in each group to detect the intestinal bacterial community. According to the manufacturer's instructions, DNA was extracted from stool samples using a QIAamp DNA Stool Mini Kit (Qiagen 51504). The hypervariable V3−V4 region of the 16S-rDNA gene was amplified by PCR using primers. According to the standard protocol, the purified amplicons were pooled in the form of equimolar and paired-end sequencing (2 × 250) on the Illumina platform by Gene Denovo Biotechnology Co. (Guangzhou, China). Details steps and reagents used are presented in the supporting information (S1).

2.5. Biochemical parameter measurement

Intestinal tissues were taken out on a cold ice plate and then homogenized in ice-cold saline by sonication. Physiological saline was added in proportion (weight (g):volume (mL) = 1:9). The homogenate was centrifuged at 4 °C and 2000 g for 10 min, and the supernatant was taken and stored at −80 °C for analysis. Enzymes related to intestinal immunity include lysozyme (LZM), complement C3 and immunoglobulin (IgM), malondialdehyde (MDA), and digestive parameters of the intestine including total cholesterol (TCHO) and triglycerides (TG) were detected in carp. ELISA kits for detecting C3 (ELISA, CD90026) and IgM (ELISA, CD90081) were produced by Wuhan Chundu Biotechnology Co., Ltd. (Wuhan, China). The assay kits of LZM (turbidimetry, A050-1-1), MDA (TBA method, A003-1-2), TCHO (single-reagent GPO-PAP method, A111-1-1), TG (single-reagent GPO-PAP method, A110-1-1), and total protein (TP) (Coomassie brilliant blue method, A045-2-2) were purchased from Nanjing Jiancheng Bioengineering Institute and used according to the manufacturer's protocols (S2). All biochemical indexes were detected by Tecan enzyme marker (Männedorf, Switzerland).

2.6. Brain transcriptome analysis

To extract the total RNA of the sample, rRNA was removed using a conventional kit, and oligo (dT) magnetic beads were used for mRNA enrichment. We further reverse-transcribed the enriched mRNA to form a double-stranded cDNA, repaired both ends of the cDNA, added linkers, and amplified by PCR to construct a computerized library. To ensure the quality of sequencing, the purity of RNA was detected by agarose gel electrophoresis and a NanoPhotometer spectrophotometer (Hangzhou, China). The sequencing was completed using the Illumina Novaseq6000 of Gene Denovo Biotechnology Co. (Guangzhou, China). The detailed steps and reagents used are shown in the supplementary information (S3). The methods of RNA extraction and RT-qPCR were the same as those in previous studies [38]. The detailed steps are included in the supporting information (S4).

2.7. Statistical analysis

SPSS version 25 (SPSS Inc, Chicago, IL, USA) was used for all statistical analysis. The data are expressed as mean ± SD. All data were checked for normality of distribution and variance homogeneity using the Kolmogorov−Smirnoff test and Levene's test. Statistical significance was determined using the one-way ANOVA and post hoc Tukey−Kramer test. When the normality of variance was not apparent, equivalent non-parametric equivalent tests were used (Wilcoxon, post hoc Steel−Dwass test). For all analyses, there was a significant difference when $p < 0.05$. The Pearson correlation analysis was used to assess the correlation between variables.

Correlation analysis of gut biochemical parameters with genes in the brain was performed. Then, using O2PLS analysis and Spearman correlation analysis, the degree of correlation between microorganisms and transcriptome data was determined based on the correlation coefficient "r" and significance "p" ($|r| > 0.6$; $p < 0.05$).

3. Results

### 3.1. Changes in intestinal tissue glycoproteins

Mucus can surround bacteria and screen for metabolites or toxins to restrict their entry into epithelial tissues, and there are many molecules in mucus that can play an immunomodulatory role. Glycoproteins are the main component of intestinal mucus secreted by goblet cells. As shown in Fig. 1, the changes in the total area of glycoproteins and the relative density of glycoproteins were studied in the intestine of all test groups. Compared with the control group, the total area of glycoproteins in the TPT_MP group increased significantly ($p < 0.05$), and the relative density of glycoproteins in the TPT and TPT_MP treatment groups increased significantly ($p < 0.05$).

3.2. Imbalance of intestinal microbiota

The top three phyla were Fusobacteria, Proteobacteria, and Bacteroidetes. For the genus level, the top three genera, *Cetobacterium*, *Bacteroides*, and *Aeromonas*, were found in the intestinal flora of carp (Fig. 2a and b). PCoA (principal co-ordinates analysis) showed differences in the structure of carp intestinal flora in different treatment groups (Fig. 2c). The analysis of the indicator species revealed there were no significant differences at the phylum level.

At the genus level, the abundance of *Aeromonas* flora in MP was significantly downregulated compared with the TPT_MP group. There were no significant differences between the other treatment groups and the control group (Fig. 2d). The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways enriched in all groups are classified into six categories at the KEGG level 1: "Metabolism", "Genetic Information Processing", "Cellular Processing", "Environmental Information Processing", "Organismal System", and "Human Diseases" (Fig. S2). Additionally, the network complexity of the gut bacterial community, the number of vertices, the number of edges, the average degree, and the average clustering coefficient were different in different exposure groups. Moreover, the combined exposure TPT_MP had the highest network complexity, which is likely because of the combined action of the two pollutants (Fig. 2e). In KEGG level 3, the functional abundance of lipid metabolism of the flora in the TPT_MP group such as "Fatty acid degradation", "Steroid biosynthesis", and "Fatty acid biosynthesis" was higher than that of the control group, while the immune function abundance "NOD-like receptor signaling pathway" was lower than that of the control (Fig. 2f).
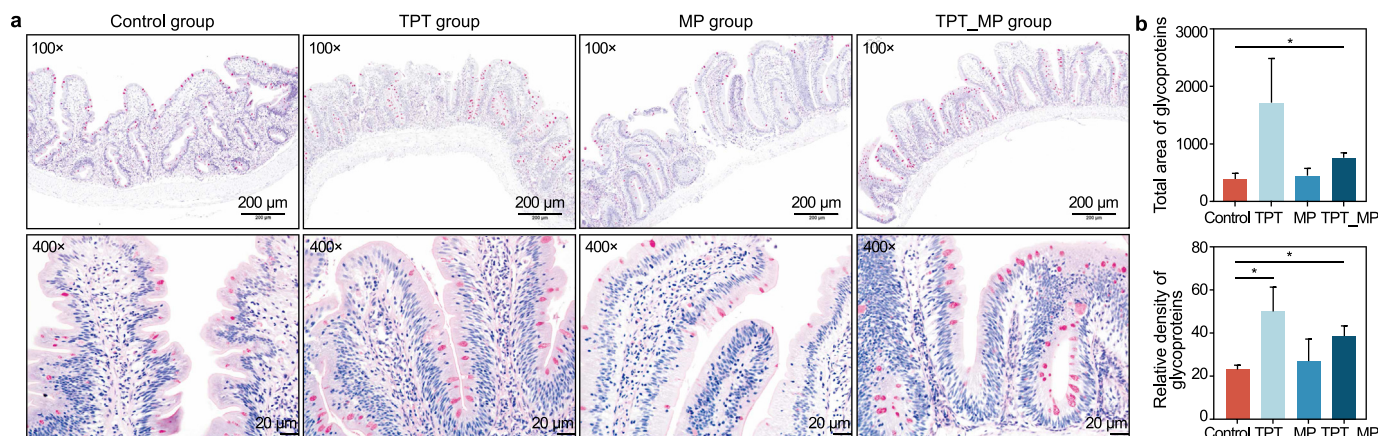
Fig. 1. Periodic acid−Schiff (PAS) staining analysis of carp intestine tissue after TPT, MP, and TPT_MP exposure. **a**, Magnification is 100 × and 400 × . **b**, The total area and relative density of glycoproteins in the carp intestine are also presented. The presented values are the means ± SEM ($n = 6$).

3.3. Intestinal biochemical parameters and immune genes

As shown in Fig. 3a and Table 1, after 42 days of exposure, the levels of TG and TCHO in the TPT_MP group ($p < 0.05$) were significantly higher than the control group. The level of MDA was significantly lower in the MP group ($p < 0.05$) compared with the control group, and the level in TPT ($p < 0.05$) was significantly higher than that in the control. Compared with the control, the IgM level in the TPT group, the IgM and LZM levels in the MP group, and the levels of three immune parameters (IgM, LZM, and C3) in the TPT_MP group were significantly increased ($p < 0.05$).

The RT-qPCR results of carp intestinal immunity-related genes are shown in Fig. 3b and Table 1. Compared with the control group, *IL-6* in the TPT group was significantly decreased ($p < 0.01$), and *IL-1* and *IL-6* in the MP group were significantly decreased ($p < 0.01$). In the TPT_MP group, *IL-6* and *IL-10* were significantly decreased ($p < 0.05$).

3.4. Brain transcriptome sequencing

To further study the single/combined toxicity of TPT and MPs to carp brain tissue, we performed transcriptome analysis based on the FPKM (fragments per kilobase per million mapped reads) value of the detected gene. According to the PCA results, the carp brain transcriptome samples are highly correlated (Fig. 4a), and the correlations of repeated samples within the same treatment group were all higher than 0.99 (Fig. S5). The upregulated and downregulated genes of each group are shown in Fig. 4b (fold change ≥1.5 or ≤ −1.5, and p-value ≤0.05). Compared with the control, the differentially expressed genes (DEGs) in the TPT group (upregulated: 698; downregulated: 712), MP group (upregulated: 592; downregulated: 717), and TPT_MP group (upregulated: 673; downregulated: 969) are presented (Fig. 4b). In total, 171 DEGs were found across the three groups (Fig. 4c). Subsequently, to further determine the function of DEGs and distinguish the differences among the treatment groups, KEGG analysis was performed, and the 15 top pathways in each group are shown in Fig. 4d. Clearly, TPT has a significant effect on "Fat digestion and absorption" and the "PPAR signaling pathway". Moreover, MP affects immune-related pathways. "Intestinal immune network for IgA production", "Toll-like receptor signaling pathway", "Th17 cell differentiation", "NF-kappa B signaling pathway" are significantly enriched. Some pathways related to immunity such as the "Intestinal immune network for IgA production", "Cytokine−cytokine receptor interaction" and other pathways, and the "JAK-STAT signaling pathway" in the TPT_MP group are also significantly enriched.

To explore the toxic effects of TPT, MP, and TPT_MP on carp, a weighted co-expression network analysis (WGCNA) considering gut immune parameter traits was studied. Results were validated by RT-qPCR and by exploring gene relationships in pathways. The results of WGCNA showed that genes with FPKM values > 5 were divided into 21 modules. According to the correlation analysis between intestinal immune parameters and the top 20 modules, LZM was significantly negatively correlated with module antique white (r = −0.72, $p < 0.01$) (Fig. 5a). The KEGG result of genes in the module antique white is involved in the immune-related NF-kappa B signaling pathway and insulin signaling pathway (Fig. 5b). The gene regulatory network map shows the association of the top ten pairs of transcription factors in the module antique white (Fig. 5c). To verify the KEGG pathway, we selected immune-related genes (*TNF-α, PI3K, STAT, JAK, IKβ-α*) and genes related to fat metabolism (*SCP-X, PAGR,* and *CPT-2*) and used RT-qPCR to check their relative expression. The trend of RT-qPCR results for eight genes is consistent with the transcriptome, confirming the reliability of the transcriptome results (Fig. S3 and Fig. 5d). The gene relationship between KEGG-enriched immune pathways and lipid metabolism pathways is shown in Fig. 5e. In terms of changes in DEGs, TPT induced an increase in the levels of lipid metabolism-related genes, and MPs induced a decrease in the levels of immune-related genes. Additionally, the differential gene expression changes were significant in the TPT_MP group. Thus, the co-exposure of TPT with MPs amplified the single toxic effects of pollutants.

3.5. Correlation analysis between intestine and brain in carp

In this study, biochemical parameters of gut immunity and lipid metabolism were correlated with brain tissue immunity and lipid metabolism genes (Fig. 6a and b), with the aim of investigating the effects of TPT, MP, and TPT_MP exposure on the gut−brain axis. Gut immune parameters (LZM, IgM, and C3) were significantly negatively correlated with immune genes (*TNF, STAT* and *Ikβ-α*) in brain tissue. To study the relationship between the abundance of carp intestinal flora and the expression of brain immunity and lipid metabolism genes, a cluster heat map was presented using Spearman correlation analysis. In our results, the level of bacterial genus abundance has little correlation with lipid metabolism in the brain. However, the correlation between immune-related pathways in the brain and the intestinal flora is significant, mainly at the
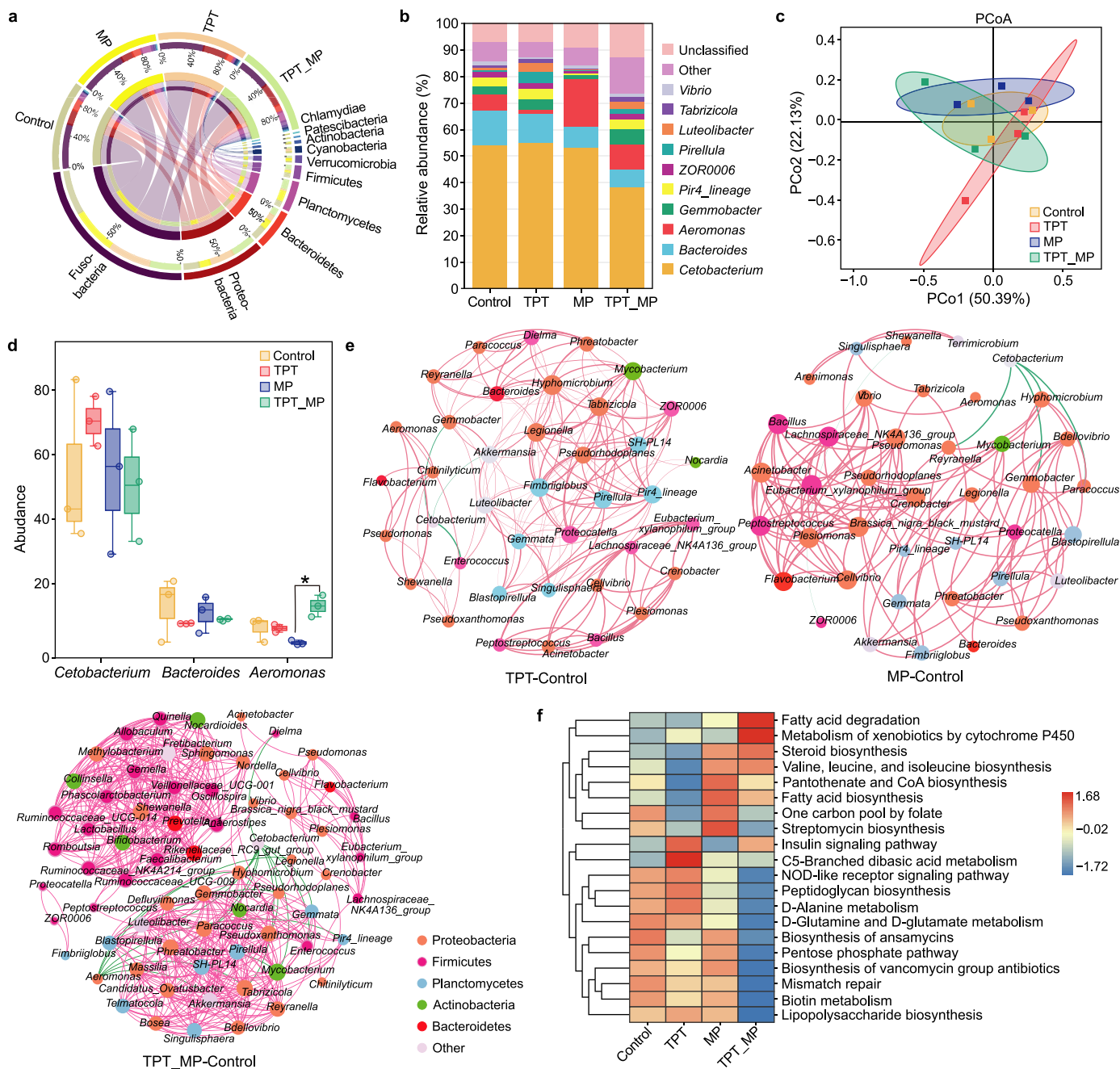
Fig. 2. The species composition and indicator species analysis of carp gut microbes after exposure to TPT, MP, and TPT_MP. **a**, The composition of microbial species at the phylum level. **b**, The composition of microbial species at the genus level. **c**, Principal component (PCoA) analysis of OUT (Operational Taxonomic Unit) level. **d**, Indicator species at the genus level. *$p < 0.05$ indicates significant difference. **e**, Co-occurring network of the gut bacterial community based on correlation analysis. **f**, KEGG level three functional abundance cluster analysis.

genera *Pir4_lineage*, *Singulisphaera*, *Gemmata*, *Pirellula*, and *Blasto-pirellula*. These genera belong to the phylum Planctomycetes. The genes in the "NOD-like receptor signaling pathway" were mainly positively correlated with the above five bacterial genera of the phylum Phytophthora. Some genes of the "NF-kappa B signaling pathway" and "Th17 cell differentiation" were positively correlated with intestinal flora, whereas some genes were negatively correlated (Fig. 6c). The network interaction diagram of brain genes and the top 20 intestinal flora at the genus abundance are shown in Fig. 6d. These genes were also enriched in immune-related pathways through KEGG (Fig. S4). In conclusion, the abundance of bacteria in the intestine of carp was significantly correlated with genes related to brain immunity.

4. Discussion

4.1. Effect on the intestinal barrier

Intestinal health has an important effect on the growth of fish [39,40]. The intestinal barrier occupies an irreplaceable position in life and health. It responds to various stimuli and can effectively prevent the body from being harmed by endogenous
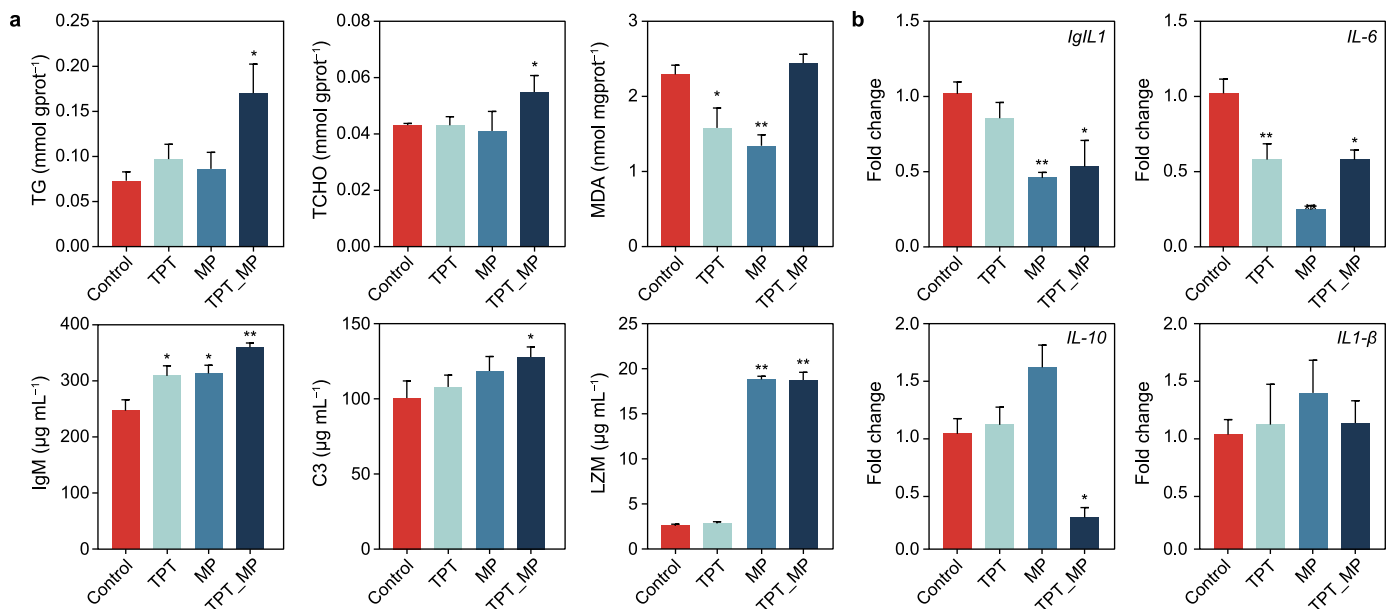
Fig. 3. Analysis of physiological parameters and immune genes of carp intestine tissue exposed to TPT, MP, and TPT_MP. **a**, Intestinal biochemical parameters. **b**, Real Time -qPCR results of gut immune genes. The presented values are the means ± SEM ($n = 6$). *$p < 0.05$, **$p < 0.01$, indicate significant differences from the control.

Table 1
One-way ANOVA of variance for the effects of TPT, MP, and TPT_MP on parameters measured in fish after chronic exposure.

| Source	Control	F	TPT		MP		TPT_MP	
	Mean ± SD		Mean ± SD	P	Mean ± SD	p	Mean ± SD	p
TG	0.0733 ± 0.0170	3.705	0.0971 ± 0.0286	0.9334	0.08633 ± 0.0318	0.9950	0.1703 ± 0.0559	0.0259
TCHO	0.04318 ± 0.0010	1.484	0.0431 ± 0.0053+4	0.9999	0.0411 ± 0.0120	0.9994	0.0549 ± 0.0101	0.0406
MDA	2.2926 ± 0.2156	11.71	1.5816 ± 0.4588	0.0268	1.3434 ± 0.2530	0.0039	2.4449 ± 0.2019	0.2252
IgM	247.1250 ± 33.4833	4.882	309.2925 ± 30.8460	0.0386	31304545 ± 25.2121	0.0259	360.3239 ± 13.1050	0.0058
C3	100.5405 ± 19.6611	2.096	107.8831 ± 13.8420	0.9851	118.5372 ± 16.7225	0.6280	127.8055 ± 11.7382	0.03518
LZM	2.5694 ± 0.3182	266.7	2.8472 ± 0.3182	0.9980	18.8194 ± 0.6365	<0.0001	18.7500 ± 1.5023	<0.0001
IgIL1	1.0175 ± 0.1948	5.699	0.8562 ± 0.2557	0.7340	0.4598 ± 0.0863	0.0097	0.5338 ± 0.4280	0.0272
IL-6	1.0206 ± 0.2340	15.45	0.5800 ± 0.2367	0.0078	0.2475 ± 0.0642	<0.0001	0.5798 ± 0.1115	0.0230
IL-10	1.0438 ± 0.3232	10.13	1.1232 ± 0.3721	0.9815	1.6213 ± 0.4741	0.0613	0.3172 ± 0.1734	0.0309
IL1-β	1.0382 ± 0.3180	0.3798	1.1235 ± 0.8571	0.9951	1.3940 ± 0.6911	0.7549	1.1376 ± 0.4312	0.9933

microorganisms and their toxins [41]. Mucus is the first physical defense in the barrier. The structure of intestinal flora is affected by various factors [42,43], and its structural changes may mediate many diseases [44,45]. Mucus can surround bacteria and screen for metabolites or toxins to limit their entry into epithelial tissues. Moreover, there are many molecules in mucus that can have immunomodulatory effects [47]. Glycoprotein is the main component of intestinal mucus secreted by goblet cells. Recent studies have demonstrated that MPs, metals, and their combinations can increase the expression of the intestinal mucin gene in common carp [48], which is consistent with the results of this study. We found that mucus glycoprotein secreted by the intestine in the combined exposure group increased significantly. It may be that TPT_MP was toxic to the gut and protected against foreign TPT_MP by stimulating and regulating the secretion of mucus glycoproteins to promote intestinal barrier function. Based on changes in glycoprotein density and area, it was further demonstrated that TPT played an important role in the combined exposure of TPT_MP.

To further determine the toxicity of TPT combined with MPs, 16S rRNA sequencing of the carp intestinal flora was performed. At the genus level, in previous studies, MPs greatly altered the intestinal flora of aquatic organisms [49,50]. In our study, there was no significant difference between the treatment group with MPs and the

control group (Fig. 2d). This may be because of the inconsistency of the plastic, exposure time, and the species of the tested animals, which contribute differently to the imbalance of intestinal flora [51]. Intestinal function is predicted by PICRUST (Fig. 2f). In KEGG level 3, the changes in the TPT_MP group were the most obvious, and the immune function abundance of intestinal flora decreased compared with the control group. It may be that the combination of TPT and MPs amplifies MP-induced immunotoxicity.

In this study, MP and TPT_MP affected intestinal lipid metabolism and glycolipid digestion. Indeed, according to previous studies, MPs inhibit the amylase activity of Mytilus galloprovincialis [52]. In the TPT_MP group, TCHO increased significantly, and TG showed an upward trend, indicating that the combination of TPT and MPs would increase the accumulation of intestinal lipids in carp. This may be because of the disturbance in the intestinal circulation of bile acids, which affects the digestion and absorption of lipids, resulting in lipid metabolism disorders [53]. Moreover, the results of previous studies are consistent with the results of this study. Triphenyltin has the effect of inducing fat accumulation in fish, amphibians, and mammals [54,55]. Microplastics can also induce glucose and lipid metabolism disorders in organisms [56]. Currently, it is difficult to clarify the biochemical mechanism of biological oxidative stress induced by MP exposure, but MPs can
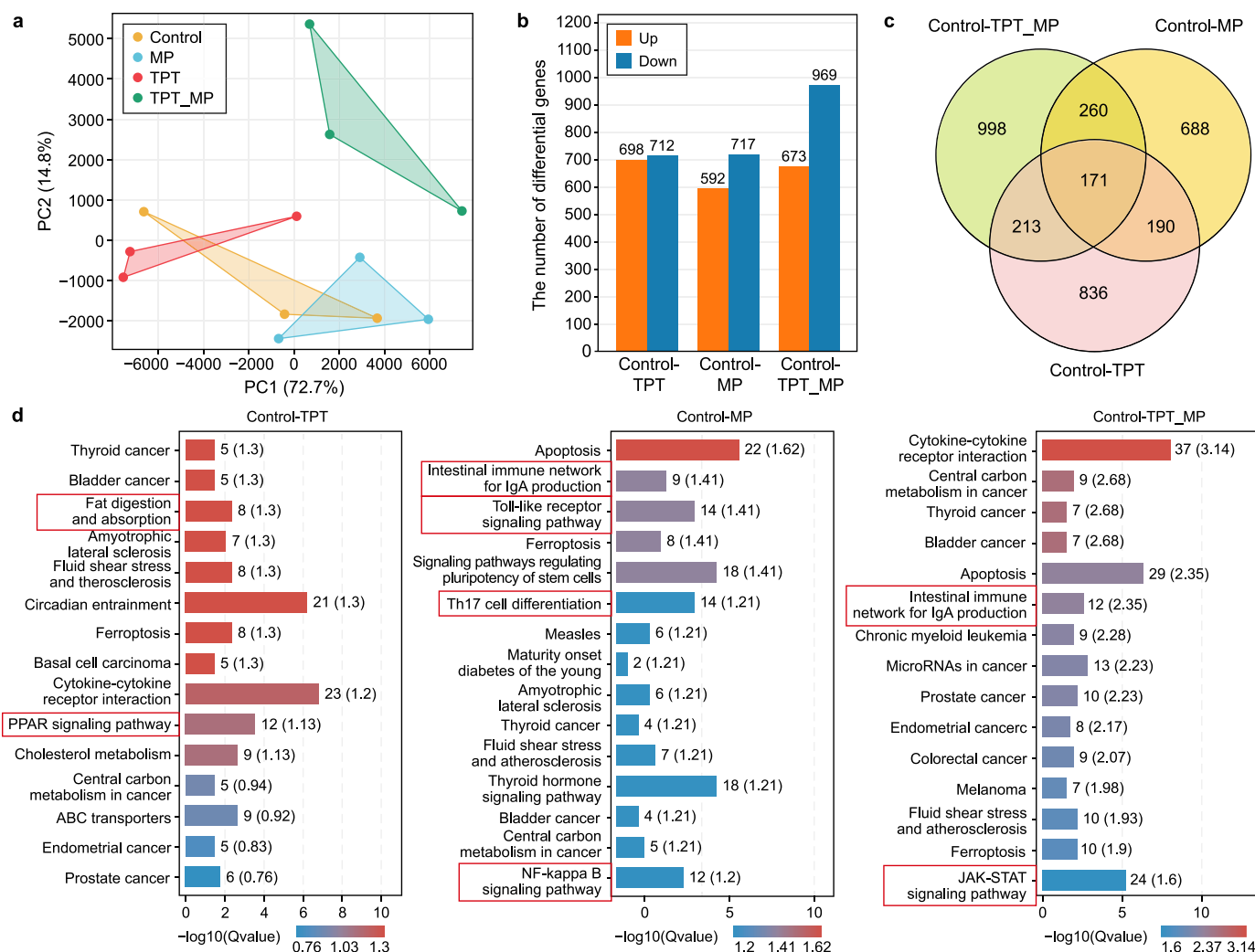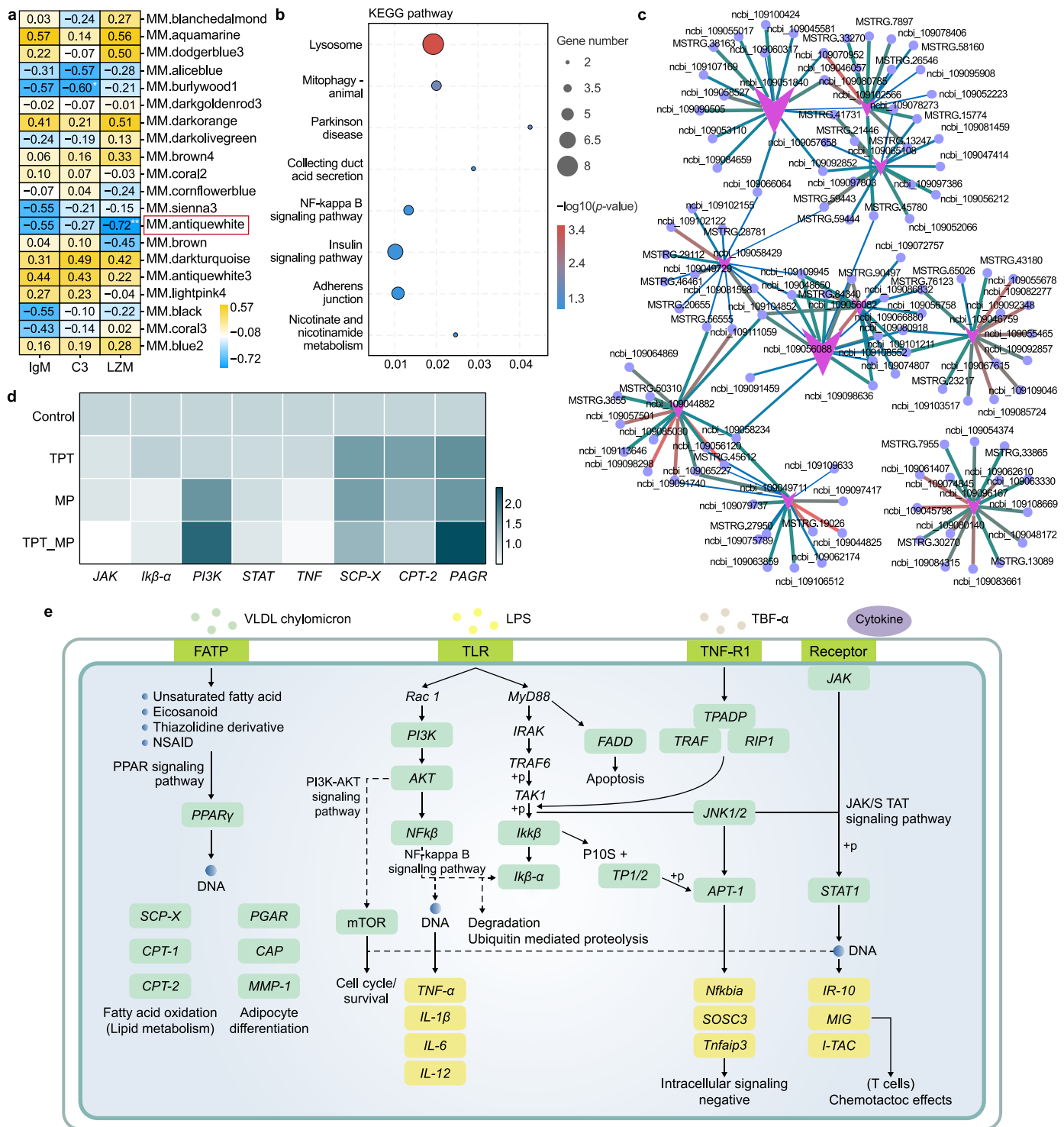
Fig. 4. Transcriptome analysis of brain tissue of carp exposed to TPT, MP, and TPT_MP. **a**, PCA analysis. **b**, Histogram of differentially expressed genes (DEGs). **c**, Venn diagram of differentially expressed genes (DEGs). **d**, The KEGG pathways that were significantly enriched in DEGs in carp brain tissue were exposed to TPT, MP, and TPT_MP ($p < 0.05$).

lead to the disturbance of oxidative stress parameters [57]. Huang et al. found that when guppies were exposed to MPs at concentrations of 100 and 1000 μg L^{-1} for 28 d, the increase in MDA content indicated that MPs did indeed cause oxidative stress and peroxidative damage [58]. However, in the present study, the results were reversed. Malondialdehyde is an indicator of lipid peroxidation. Levels of MDA depend on the presence of ROS and free radicals as well as substrates, unsaturated fatty acids [59]. Therefore, the decrease in MDA may reflect the decrease in highly unsaturated fatty acids (HUFAs) in intestinal fat. This further suggests that exposure to these toxic substances can affect lipid metabolism, possibly by altering the distribution of fatty acids and reducing the proportion of HUFAs, leading to a decrease in MDA. Additionally, fish can metabolize aldehydes, and the decrease in MDA may be related to the activation of hydrolytic enzymes [60].

Lysozyme, C3, and IgM play important immune roles in animals [61−64]. In this study, compared with the control group, TPT significantly increased IgM levels, and MPs significantly increased LZM and IgM levels. This may be because the carp intestines secrete immune factors to increase immune barrier function after exposure to pollutants [65]. Triphenyltin combined with MPs will significantly increase the levels of three immune parameters. The MP group also significantly increased the levels of IgM and LZM.

Moreover, the trend of the TPT combined exposure group is also higher than that of the other treatment groups. Interestingly, the immune parameters in the intestine are significantly increased after the combined exposure, and its immune function may be activated. Furthermore, we found that TPT_MP can inhibit the immune function of the intestine through PICRUST2 predicting the function of intestinal flora, causing it to be immunosuppressed. The reason for the conflicting results may be that the gene level is more sensitive to the corresponding pollutants than the biochemical physiological parameters, or that the damage caused by the inflammation leads to the decrease of the gene level. Additionally, at the gene level, MP and TPT_MP gradually suppressed the occurrence of immune responses. An anti-inflammatory cytokine, *IL-10*, inhibits the expression of pro-inflammatory factors and prevents the development of inflammation [66]. Based on the expression level of the anti-inflammatory factor *IL-10* gene, the single toxic effect of MPs is further amplified when combined with TP, and the occurrence of anti-inflammatory factors is inhibited. In conclusion, long-term exposure to MPs will produce immunotoxicity in the carp intestine and inhibit the occurrence of immune function at the gene level.

Overall, long-term exposure to TPT_MP induced an increase in the intestinal TG and TCHO levels of carp, leading to disturbances in

Fig. 5. Weighted correlation network analysis of brain transcriptome and expression of genes related to immunity and lipid metabolism. **a**, Correlation analysis between intestinal immune function and each module. **b**, The KEGG pathways ($p < 0.05$) in module antique white. **c**, Network regulation of genes in module antique white. **d**, mRNA levels of immune and lipid metabolism genes in brain transcriptome. **e**, Immune and lipid metabolism pathways.

lipid metabolism. Additionally, TPT_MP led to the disturbance of intestinal immune function in carp. The TPT_MP treatment induced an increase in intestinal mucus and increased the level of intestinal immune biochemical parameters such as LZM, IgM, and C3, while inhibiting the occurrence of immune function at the gene level. Moreover, the combined exposure of TPT and MPs amplified the

toxic effects of single pollutants on lipid metabolism and the MP-induced intestinal immune dysfunction.

4.2. Effect on the brain

High-throughput transcriptomics sequencing technology has
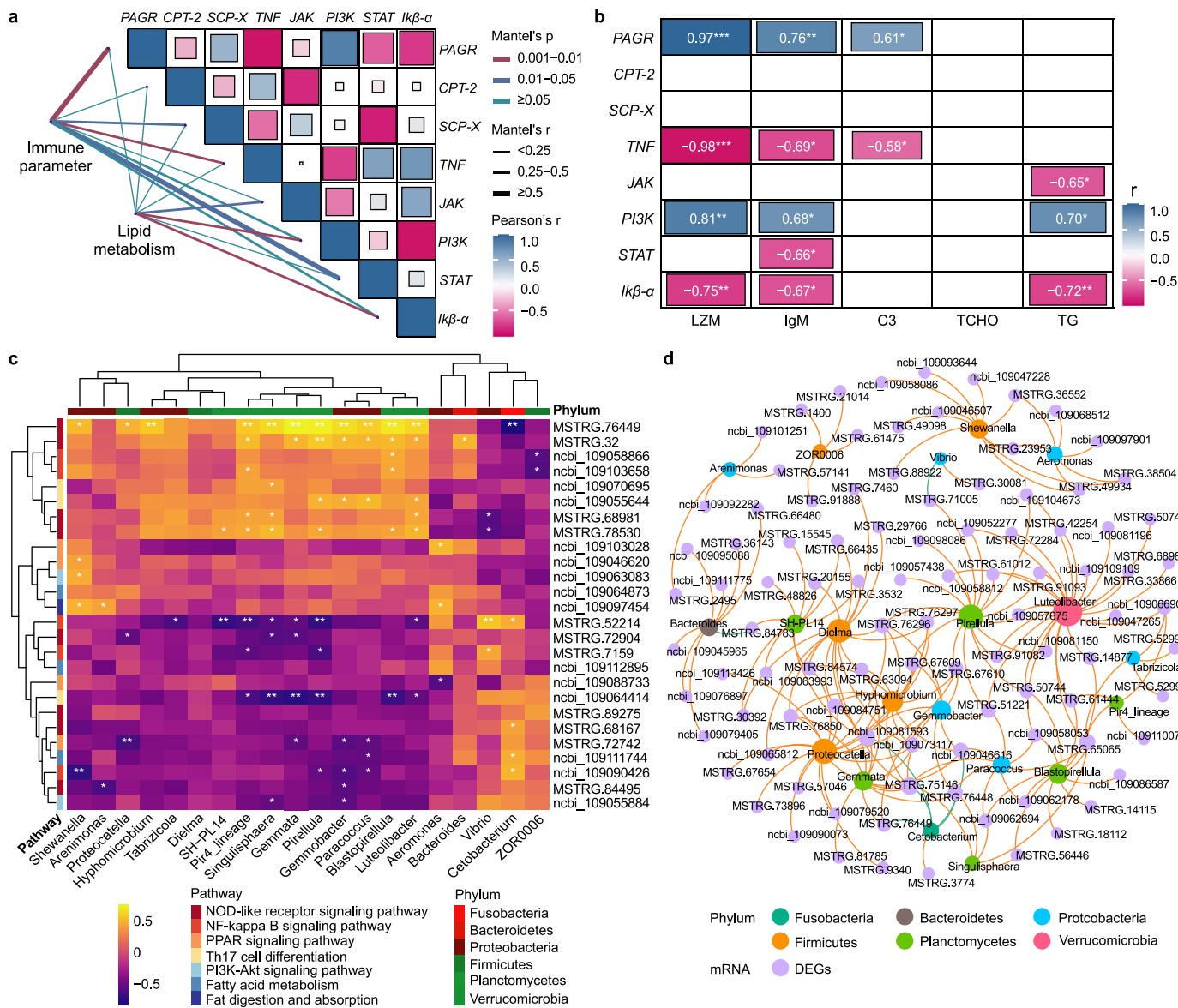
Fig. 6. Correlation analysis between intestine and brain tissue. **a**, Correlation heat map of immune and metabolic functions with differential genes. **b**, Correlation analysis between differential genes and biochemical parameters. **c**, Clustering heat map of genes related to immunity and lipid metabolism in brain tissues and the top 20 intestinal flora at the genus abundance. The color change from light yellow to dark blue indicates that the correlation changes from positive to negative. **d**, The interaction network between the top 20 intestinal flora at the genus abundance and brain DEGs. The gray nodes represent the DEGs of the brain, each node in the remaining colors represents a bacterial genus, and the node color represents the bacterial phyla. The orange line indicates that the correlation is positive, and the green line is negative.

become an effective tool to assess the potential toxicity mechanisms of environmental pollutants from the mRNA level [67]. In our results (Fig. 4), TPT led to disorders of lipid metabolism in carp. The digestion and absorption of fat and PPAR signaling pathway in the TPT group were significant. It has been reported that TPT induces lipid accumulation in rainbow trout [27]. Our research results also support this point. Many studies have reported that after MP exposure, the immune response of fish is significantly increased [68]. However, in the present experiment, the results were opposite, with MPs significantly suppressing immunity, which inhibits the toll-like receptor signaling pathway and NF-kappa B signaling pathway. Additionally, it was found that the absorption of MPs reduces the level of immune-functioning neutrophils in *Oreochromis niloticus* [69]. As foreign compounds, MPs could stimulate the immune response of fish or inhibit immune function by inducing immunotoxicity.

The expression of genes related to lipid metabolism and immunity was detected by RT-qPCR. The detected gene expression trend is consistent with the transcriptome trend, which proves the reliability of the transcriptome data. The immune system of fish is sensitive to chemicals, and changes in innate immunity can be used as an early indicator of the response to environmental stress factors [70,71]. Interestingly, the expression of related immune genes in the TPT_MP group was significantly lower than that in the control group; that is, immunosuppression occurred. However, most studies have shown that MPs induce the occurrence of immune function [72,73]. In our study, the expression of the immune factors TNF-α and IKβ-α and the upstream genes STAT and JAK in the MP group and TPT_MP treatment group decreased significantly. This may be because nanoscale plastic microspheres can enter the carp body through daily activities such as breathing and eating, and are likely to cross the blood−brain barrier, thereby causing damage to

the brain tissue [68,74]. This brain damage is likely to lead to a decrease in the ability to produce the inflammatory factors *TNF-α* and *IKβ-α*, leading to immunosuppression. Moreover, owing to the adsorption of MPs [75,76], TPT can use MPs as carriers to enter the body of carp and combine with MPs to amplify the toxicity of TPT to carp brain tissue, but the mechanism of its compound toxicity needs to be explored in the future.

Generally, we found that TPT can lead to lipid accumulation in carp brain tissue and MPs can lead to immunosuppression in carp brain tissue. The exposure of TPT combined with MPs also amplified the immunosuppression of MPs on brain tissue and increased the disturbance of TPT on carp brain tissue metabolism. This may be because of the relatively large surface area of nanoscale MPs that can adsorb TPT, increasing the risk of these two pollutants entering brain tissue and damaging it.

4.3. Effect on the gut−brain axis

Various reports have shown that TPT and MPs can cause brain and intestinal organ damage [36,77,78]. The intestine is also known as the "the brain in gut" [79,80]. It interacts with the brain through the vagus nerve to form the "gut−brain axis" and has significant biological functions [81].

As shown in Fig. 6, after TPT_MP exposure, intestinal immune function was suppressed through function prediction. Although the levels of LZM, IgM, and C3 increased (see Fig. 3), immunosuppression occurred at the gene level based on the decrease in expression of immune genes (*TNF*, *STAT*, and *Ikβ-α*) (Fig. 6). When the expression level of immune factors such as *TNF*, *IKβ-α*, and other genes in the brain tissue is reduced, immunosuppression will also occur. Similarly, compared with the control, the genes related to lipid metabolism and accumulation (*SCP-X*, *PAGR*, and *CPT-2*) were also increased in the brain tissue. This may be attributed to the role of the gut−brain axis.

New insights were provided from the perspective of the "gut−brain axis". That is, immune-related DEGs in carp brain tissue were significantly negatively correlated with gut immune parameters and significantly correlated with the dominant microbes in the gut microbiota. Flora of the genera *Pir4_lineage*, *Singulisphaera*, *Gemmata*, *Pirellula*, and *Blastopirellula* are associated with brain tissue immune dysfunction. For example, "NOD-like receptor signaling pathway", "NF-kappa B signaling pathway", and "Th17 cell differentiation pathway" were inhibited. Additionally, TCHO and TG levels were elevated in the gut in the TPT_MP group, and genes related to lipid metabolism and accumulation, such as *SCP-X*, *PAGR*, and *CPT-2*, were also increased in brain tissue compared with controls. This may be attributed to the role of the "gut−brain axis". In conclusion, when TPT is combined with MPs for long-term exposure, the involvement of TPT amplified the MP-mediated immunosuppressive effect. However, the regulation mechanism of the gut−brain axis is not yet fully understood, and current studies have mainly focused on mammals such as mice. The regulation of the gut−brain axis of lower organisms like fish still needs to be further explored.

In this study, intestinal flora phyla of Fusobacteria, Bacteroidetes, Proteobacteria, Firmicutes, Planctomycetes, and Verrucomicrobia pass through the "NOD-like receptor signaling pathway", "NF-kappa B signaling pathway", "PPAR signaling pathway", "Th17 cell differentiation", "PI3K-AKT signaling pathway", "Fatty acid metabolism", "Fatty acid metabolism", and "Fat digestion and absorption" feedback to brain immune and lipid metabolism changes. This resulted in lipid metabolism disorders and immune function disorders. The results of this study still require further verification and discussion.

5. Conclusion

Our results demonstrate that single exposure to TPT resulted in the disturbance of lipid metabolism in carp, while MPs suppressed immunity. Meanwhile, TPT_MP also amplified the immunosuppressive effect induced by a single MP. Additionally, the secretion of mucus granules in the intestinal tissue of carp, the detection results of immune parameters and immune genes, changes in the abundance of intestinal flora, and functional prediction results all proved that the combined exposure of TPT and MPs resulted in an immunosuppressive response. This study provides new insights from the perspective of the gut−brain axis and finds that immune-related DEGs are significantly associated with gut-dominant microbes and immune parameters, but the mechanisms require further investigation. Therefore, based on actual environmental conditions as well as a novel perspective, this study comprehensively evaluates the toxic effects of MPs and TPT and the existing ecological risks, while also identifying directions for future research.

CRediT author contribution statement

Siqi Zhang: Writing - Original draft preparation. **Ping Li:** Writing - Reviewing and Editing. **Shuwen He:** Indices Measurement. **Shaoying Xing:** Data analysis. **Zhihan Cao:** Fish Culture and sampling. **Xueli Zhao:** Methodology and Software. **Cuici Sun:** Data analysis. **Zhihua Li:** Conceptualization and Overall guidance.

Declaration of competing interest

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

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ese.2023.100266.

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