

Research article



Unraveling the mechanism of brain damage in *Carassius auratus* by polypropylene microplastics and oxytetracycline via the brain-gut-microbiota axis

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ABSTRACT

Microplastics (MPs) and antibiotic residues are widespread coexistence and pose a greater threat in aquatic environments, but the neurotoxic effects of co-exposure on aquatic animals remain poorly understood. This study investigated the mechanisms of MPs and oxytetracycline (OTC) on the brain-gut-microbiota axis of *Carassius auratus* at environmentally realistic levels. The carrier effect of MPs resulted in OTC accumulation levels of 221.84 ng/g and 2524.61 ng/g in the brain and intestine of the combined exposure, respectively, and aggravated infectious inflammation in the brain and intestinal dissolution and necrosis. MPs and OTC raised the abundance of Actinobacteriota, and markedly reduced the content of acetic acid and propionic acid by 41.86 % and 75.52 %, respectively. Moreover, combined exposure had a synergistic effect, with inhibition rates of 52.57 % and 61.99 % for AChE and BChE, respectively, further leading to a decrease in 5-hydroxytryptamine and dopamine levels. Transcriptomics further revealed that the toxic effects of co-exposure might induce nervous system disorders through synaptic vesicle recycle and GABAergic synaptic pathway, which were influenced by gut microbiota and neuroactive molecules. In summary, MPs and antibiotics trigger various physiological changes by disrupting the fish brain-gut-microbiota axis, which provides scientific information to understand the co-exposure risks of MPs and associated contaminants in aquatic ecosystems.

1. Introduction

The outbreak of coronavirus 2 known as COVID-19 has led to an increase in the use of disposable masks (Peng et al., 2021). These face masks are made from polypropylene, and their continued usage contributes to microplastic pollution in aquatic environments (Jimoh et al., 2023). Over the past few years, microplastics (MPs) have been regularly detected in oceans, rivers, lakes, groundwater and even polar glaciers (Kannankai et al., 2022), (Wong et al., 2020), (Zhang et al., 2021a). Currently, research on the toxicity of MPs in aquatic animals has mainly focused on immunotoxicity, metabolic disorders, gut damage, and reproductive toxicity, while fewer studies have reported MPs-induced neurotoxicity (D'Costa, 2022), (Elizalde-Velázquez and Gómez-Oliván, 2021). MPs exposure can lead to neurotoxicity, abnormal behavior and depression in aquatic organisms (Yin et al., 2021). MPs can either accumulate in the brain and inhibit acetylcholinesterase (AChE) activity or penetrate the skin and enter muscle tissue, causing nerve fiber

atrophy (Yang et al., 2020). However, the mechanism by which MPs cause neurotoxicity in aquatic organisms remains unclear.

Antibiotics have been detected in surface water, groundwater and sediments, and are widely used to prevent or treat bacterial diseases (Wang et al., 2023), (Danner et al., 2019). Antibiotics have been found to have oxidative stress, lipid and glucose metabolism disorders, neurotoxicity, and genotoxic effects on fish (Li et al., 2023), (Kovalakova et al., 2020). Oxytetracycline (OTC) is extensively used in the aquaculture and livestock industries for its high efficiency and low cost (Consumption (ESVAC) and of, 2020). Environmental levels of OTC in surface water were found to be 340 ng/L in the United States, and 19.2 ng/L in Italy, while the highest concentration detected in China was up to 361.11 µg/L (Almeida et al., 2019), (Li et al., 2008). The inefficient elimination of OTC from wastewater treatment plants allows them to invade the human body through food or drinking water and has been detected in the urine of pregnant women and children (Yu et al., 2023). A recent study found that 30–90 mg/L OTC increased AChE and

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butyrylcholinesterase activities of *Rhinella arenarum* (Lourido et al., 2022). Exposure to 100 µg/L OTC caused a decrease in mRNA levels of the neurotransmitter 5-HT receptor in juvenile zebrafish, which also demonstrated that OTC potentially affects neurobehavior (Li et al., 2020).

MPs and antibiotics are widely distributed in the aquatic environment and coexist over long periods. MPs interact with antibiotics through adsorption and desorption in natural waters, thereby modifying their toxicity, bioaccumulation, transport and degradation processes (Rafa et al., 2024). Previous studies found that MPs and antibiotics can influence the abundance and composition of gut microbiota (Li et al., 2023), (Zhang et al., 2022). Alterations in the gut microbiota not only affect gut function, but also affect neurotransmitter synthesis/metabolism, and produce neuroactive substances that affect brain function, thus influencing the host's locomotor behavior (Huang et al., 2024), (Kristie and Hsiao, 2021). Besides, the metabolites (e.g., short-chain fatty acids (SCFAs)) of gut microbiota play a pivotal part in gut-brain communication. SCFAs may directly alter neurotransmission, neurotrophic factors, and serotonin biosynthesis by crossing the blood-brain barrier (Morais et al., 2021). Tian et al. (2023) found that environmentally relevant levels of enrofloxacin led to disruption of the brain-gut-microbiota axis thereby triggering anxiety-like behavior in zebrafish. MPs reduced the number of *Fusobacteriia* and *Clostridia* that may secrete neurotransmitters in discus fish (*Sympodus aequifasciatus*) (Huang et al., 2022). Gut microbiota disruption triggered by the effects of contaminants on associated neurotransmitters may be a potential cause of behavioral abnormalities in organisms. However, it is unclear how MPs and antibiotics induce changes in nervous system function through the gut microbiota.

Crucian carp (*Carassius auratus*) is an essential freshwater economic fish and one of China's leading edible fish species, which is used as a bio-indicator to assess the influence of environmental pollutants (Filice et al., 2022). Herein, crucian carp were exposed to environmentally relevant concentrations of MPs and OTC (alone or in combination) for 21 days, and their neurotoxic responses and neurotransmitter levels, as well as brain transcriptomes and gut microbiota and their metabolites, SCFAs, were analyzed to explore the neurotoxic mechanisms of MPs and OTC in fish via the brain-gut-microbiota axis. Our study findings provide new insights and important support for future ecological health risk assessment of the combined pollution of emerging contaminants.

2. Materials and methods

2.1. Source of chemicals

OTC (purity ≥98 %, CAS: 79-57-2) was purchased from Sigma-Aldrich LLC (St. Louis, MO, USA). Referring to our previous studies (Zhang et al., 2024), irregularly shaped polypropylene microparticles were prepared in the laboratory using an electric grinder and sieving, with a volume median diameter of 6.37 µm. Particle size, surface structure, and shape of the prepared MPs were observed using a scanning electric microscope (Hitachi Regulus 8100, Japan), Fourier transform infrared spectroscopy (Thermo Scientific Nicolet iS5, USA), and laser particle size analyzer (Malvern Mastersizer, 2000; UK) (further information in Fig. S1). All chemicals in our study are of analytical grade in purity.

2.2. Animals and experimental scheme

A total of 200 crucian carp (body length, 12.1 ± 1.5 cm; weight, 25.4 ± 4.8 g) were equally divided into two 350 L tanks. The water in the tanks was dechlorinated aerated water (temperature: 23 ± 1 °C, pH: 7.0–7.5, dissolved oxygen >6 mg/L) and was domesticated for more than two weeks in a 12 h light/12 h dark circadian cycle. These fish were randomly assigned to blank controls, solvent controls (0.1 % ethanol), MPs single exposed group, OTC single exposed group, and a combined

exposed group of MPs and OTC. It was reported that an exceedingly high OTC concentration of 361.11 µg/L was found in the surface water of China (Almeida et al., 2019), (Yu et al., 2022). The emission concentrations of microplastics discharged from a sewage plant ranged from 28 to 169 µg/L (Yan et al., 2019). Fish were subject to the combined effects of multiple exposure pathways, and the concentrations of OTC and MPs were selected as 200 µg/L and 100 µg/L, respectively. Three parallel fish tanks were set up for each experimental group and control group. Fish were fed a commercial diet once a day at a rate of 1.5–2.0 % of the fish's body weight. The exposure lasted for 21 days and 3/4 exposure solutions were updated daily to maintain a stable exposure concentration. Fish were euthanized by 100 mg/L MS222 at the end. Brain, gut and intestinal contents were collected separately from 2, 3, and 5 fish randomly selected from each tank for OTC bioaccumulation analysis, biomarker activity and neurotransmitter content determination, as well as eukaryotic sibling transcriptomics sequencing, 16S rRNA gene sequencing and SCFAs determination, respectively. All experiments were approved by the Animal Protection Committee of Hohai University.

2.3. Biochemical analysis

Enrichment and extraction of OTC from crucian carp intestinal and brain tissues using a combination of ultrasonic extraction and solid-phase extraction methods. OTC was quantified using ultra-high-performance liquid chromatography-tandem mass spectrometry (Agilent, Waldbronn, Germany). Brain and intestinal tissues were immersed in a 4 % paraformaldehyde solution and fixed for more than 24 h to make tissue sections. Preparation of 10 % tissue homogenates in an ice-water bath. Total antioxidant capacity (T-AOC) levels, AChE and butyrylcholinesterase (BChE) activities were measured with commercial kits (Jiancheng Biotech, Nanjing, China). The levels of serotonin (5-HT), gamma-aminobutyric acid (GABA), dopamine (DA) and acetylcholine (ACh) were determined using an enzyme-linked immunosorbent assay kit and double-antibody sandwich assay. The manufacturer's manuals were adhered to in all the experimental methods and formulas. Total protein concentration was determined according to the Bradford (1976). The detailed experimental protocol is stated in the Supplementary Materials.

2.4. Gut microbiota analysis and intestinal SCFAs assay

To determine changes in gut microbial community and structure, gut contents of fish were extracted with E.Z.N.A.® Soil DNA Kits (Omega Bio-Tek, Norcross, GA, U.S.) after 21 days of exposure. PCR amplification using primers 338F (5'-ACTCCTACGGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGGTWTCTAAT-3') was followed by DNA library construction and sequencing on the Illumina MiSeq system. Then, for quantification of SCFAs, 20 mg of crucian carp fecal samples were weighed from each group of samples in 2 mL grinding tubes for cryomilling, sonication, centrifugation and extraction of the supernatant with n-butanol solvent. Agilent 8890B-7000D GC/MS gas chromatograph-mass spectrometry (Agilent Technologies Inc. CA, USA) was used for SCFA quantitative analysis. The default parameters of MassHunter quantitative software (Agilent, U.S.A., ver. 10.0.707.0) were used for the automatic identification and integration of each ionic fragment of SCFAs with the aid of manual checking. Standard curves were constructed and SCFA levels were calculated. More experimental protocols are stated in the Supplementary Materials.

2.5. Brain transcriptome analysis

Total RNA from fish brains in different treatment groups was extracted using TRIzol® reagent following the manufacturer's guidelines. RNA concentration, purity, integrity and integrity number were determined by Nanodrop (2000), agar gel electrophoresis and Agilent

2100, respectively. PCR amplification was performed with 15 PCR cycles using Phusion DNA polymerase. After quantification by TBS380, double-ended RNA-seq sequencing libraries were sequenced using an Illumina HiSeq xten/NovaSeq 6000 sequencer (2×150 bp read length). Differentially expressed genes (DEGs) were calculated using transcripts per million reads. Differential genes were considered significantly expressed when the Q value of differential analysis was ≤ 0.05 , $| \log_{2}FC | > 1$ or when the Q value was ≤ 0.05 (DESeq2 or EdgeR) and Q value was ≤ 0.001 (DESeq2/DEGseq/EdgeR). Functional enrichment analysis was performed by the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. More detailed steps are included in the Supplementary Materials.

2.6. Statistical analysis

All data were expressed as the mean plus or minus the standard error. Differences between treatments were analyzed using one-way ANOVA and Tukey's multiple comparison test and were regarded as statistically significant at $p < 0.05$. Statistical analysis was performed using SPSS Statistics 22.0 (Chicago, IL, USA).

3. Results and discussions

3.1. MPs exacerbated OTC bioconcentration and pathological damage

Due to their small size, large surface area and hydrophobicity, MPs

tend to adsorb a variety of chemicals, which affects bioaccumulation (Santos et al., 2021). In the present study, OTC was not detected in the tissues of solvent controls. The bioconcentrations of OTC in the brain and gut of fish in the mixed treatment were significantly higher than those in the OTC alone treatment, and the levels of OTC in the gut were significantly higher than those in the brain, whether alone or in combination. (Fig. 1A). The results showed that the carrier effect of MPs promotes OTC bioaccumulation in fish. This is similar to previous research results that polystyrene MPs markedly raised the bioconcentration of OTC, florfenicol and SMX in *Mytilus coruscus* (Han et al., 2021). MPs promoted the enrichment of propranolol in the brain tissue of red tilapia (Zhou et al., 2020). Tetracycline antibiotics are metabolised in the liver by the cytochrome P450 enzyme system and then excreted in bile (Remmer, 1970). Due to the enterohepatic circulation, they can be reabsorbed in the intestines. MPs promoted ROX bioaccumulation in different tissues of red tilapia and followed the same trend as ROX exposure alone (gut > liver > brain > gill) (Zhang et al., 2019). This is mainly because the intestine is the largest digestive organ in fish and can come into direct contact with contaminants through ingestion.

After 21 days of exposure, histopathological sections of the brain and intestines in each treatment are shown in Fig. 1B. No significant abnormalities were observed in either the brain or intestinal sections of the solvent control group. In solvent controls, no significant abnormalities were observed in the sections of either the brain or the intestine. Intramedullary edema and a few hairy cell astrocytomas in brain cells

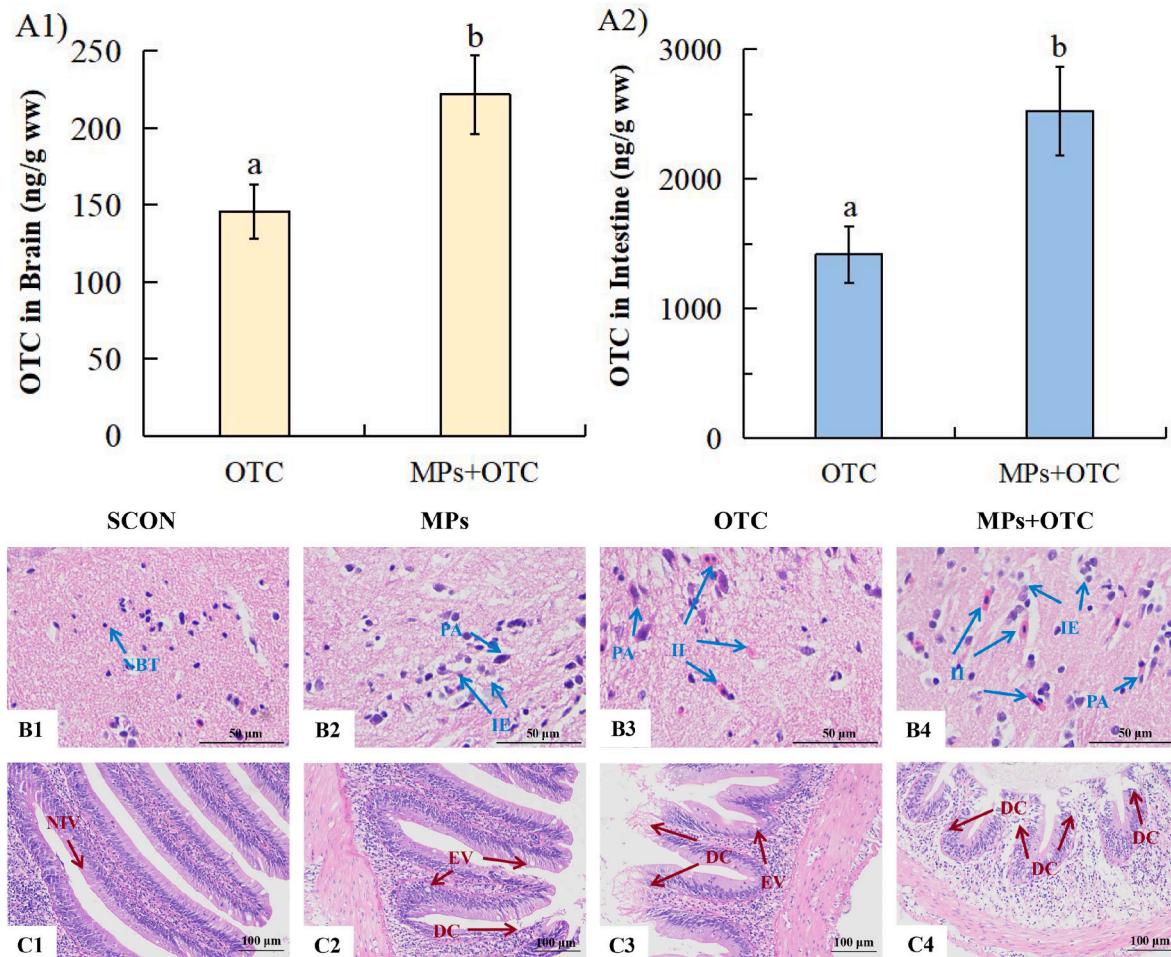


Fig. 1. Bioconcentration of OTC and histological sections in the brain (A1; B1-B4) and intestine (A2; C1-C4). Brain: normal brain tissue (NBT), infectious inflammation (II), intramedullary edema (IE) and hairy cell astrocytoma (PA). Gut: normal intestinal villi (NIV), vacuolization of enterocytes (EV), damaged cilia (DC) and lymphocytic infiltration (LI). Different letters show statistically significant differences ($p < 0.05$).

and vacuolization in intestinal cells were observed in the MPs' single treatment. OTC alone exposure resulted in infectious inflammation and hairy cell astrocytomas in the brain, as well as vacuolization and cilia damage of intestinal cells. Infectious inflammation of the brain, and lysis and necrosis of intestinal villus were more severe in the combined treatment. It showed that MPs and OTC single/combined exposures caused varying degrees of pathological damage to fish, with the combined exposure resulting in more severe histopathological damage. Yu et al. (2023) have found that OTC markedly decreased the number of midbrain neurons in zebrafish. After 28 days of exposure to MPs, the crucian carp brain appeared markedly congested and cavitated, with increased cellular interstitial space around the neuroglial cells (Shi et al., 2021). The first defense strategy of organisms against the effects of MPs is an increase in the number of cuprocytes and loosening of connective tissue in the intestine, followed by vacuolization of the intestine and the appearance of shedding of microvilli (Rochman et al., 2014), and the severity of damage increases with MPs concentrations (Pedà et al., 2016). The physical abrasive effects of MPs led to a significant increase in enterocyte and chromaffin cell damage, and severe leukocyte infiltration and hyperemia were observed in intertidal fish (Jabeen et al., 2018). Hence, the release of chemical additives/adsorbed coexisting contaminants in MPs and their physical abrasive effects are the main causes of abnormal intestinal morphology.

3.2. Effects of MPs and OTC on gut microbiota and its metabolites SCFAs

Gut microbiota is essential for host growth, development and immune function (Rawls et al., 2006). Gut microbiota imbalance can lead to reduced host resistance to pathogenic infections and chronic diseases (Fackelmann and Sommer, 2019). Previous studies have found that a single exposure to MPs or OTC can disrupt gut microbiota balance in zebrafish, rainbow trout, and marine medaka, thereby causing host intestinal inflammation and oxidative stress, and triggering metabolic dysfunction (Yu et al., 2022), (Wan et al., 2019), (Zhang et al., 2021b). In the present study, Fusobacteriota, Bacteroidota, Proteobacteria and Firmicutes were absolutely dominant microflora at the phylum level (Fig. S2A). The populations of Fusobacteriota were elevated in the OTC single exposure, and the populations of Proteobacteria in the MPs single exposure were markedly higher than those in solvent controls. The number of Proteobacteria and Actinobacteriota was obviously increased in the combined treatment. Zhang et al. (2021b) also found that Fusobacteriota, Firmicutes and Verrucomicrobia were the main gut microbiota affected by MPs in marine medaka. Polythene MPs caused an increase in the number of Firmicutes and Verrucomicrobia in the gut of tilapia, along with a reduction in Verrucomicrobia, and were associated with decreased growth rate and increased food conversion rate (Lu et al., 2022). Fusobacteriota can cause bacteremia when infected (Amess et al., 2007). An increased number of Proteobacteria is one of the hallmarks of dysbiosis (Shin et al., 2015). Actinobacteriota reduce the severity of colitis by releasing enterocyte vesicles with anti-inflammatory effects (Kang et al., 2013). At the genus level, *Cetobacterium* had the highest abundance in the gut microbiota and was more abundant in the OTC single treatment than in the other treatments. The abundance of *Acinetobacter* in the MPs single treatment, as well as *Pseudorhodobacter* and *Leifsonia* in the combined exposure, was significantly higher than those in the other groups (Fig. S2B). *Cetobacterium*, a fish probiotic, produces butyrate, acetate, and vitamin B12 that improve fish health (Xie et al., 2022). *Leifsonia* is a pathogenic microorganism associated with plague disease (Brumley et al., 2006). Consequently, MPs and OTC altered the gut microbial structure of crucian carp, and combined exposure increased the abundance of pathogenic microorganisms and triggered more severe intestinal inflammation (Fig. 1C).

Moreover, amino acid transport and metabolism and energy production and conversion were the main functions of fish gut microbiota, as predicted by the function of PICRUSt software (Fig. S3B). SCFAs are a collection of saturated fatty acids containing less than six carbon

molecules, including acetic, propionic, butyric, valeric, and hexanoic acids. Among them, acetate, propionate and butyrate are the most abundant SCFAs, which are beneficial to the body's energy balance, metabolism and maintenance of intestinal health. Lack of SCFAs may affect the pathogenesis of various diseases, such as immune, metabolic and neurological diseases (Bolognini et al., 2021). The present study showed that the acetic acid content in all treatment groups significantly decreased; the contents of propionic and isohexenoic acids were markedly reduced in single OTC or mixed exposure with MPs; OTC single exposure obviously diminished butyric acid compared to the solvent control (Fig. 2A–C). In contrast, isobutyric, valeric, and hexanoic acids were not significantly different across treatments. These results showed that OTC had a marked inhibitory effect on most SCFAs, which was exacerbated by the carrier effect of MPs. Extracellular SCFAs act through the G protein-coupled free fatty acid receptor 2 (FFA2) and FFA3, which are key regulators of intestinal inflammation and epithelial barrier function (Tan et al., 2014). Propionic acid is the most potent agonist for FFA3 and FFA2. Acetic acid is more selective for FFA2, whereas butyric acid is more active for FFA3 (Le Poul et al., 2003). Acetic acid and butyric acid are mainly involved in lipid biosynthesis (He et al., 2020). Butyric acid is also a major source of energy for intestinal epithelial cells and induces the differentiation of regulatory T cells to control inflammation (Schulthess et al., 2019). Vancomycin and metronidazole triggered ecological dysregulation of gut microbiota and exacerbated inflammation by suppressing SCFA levels (Gao et al., 2019). Yan et al. (2022) also found that combined exposure to MPs and phthalates markedly reduced the levels of acetic acid, butyric acid, and total SCFAs in the mucosal simulator system of the human gut microbial ecosystem, which in turn caused a decrease in the metabolic activity of gut microbiota, and demonstrated that the abundance of *Acidaminococcus* and *Morganella* was correlated with the exposure concentration of phthalates and the content of SCFAs.

The network analysis showed that the concentrations of acetic, propionic, butyric and isocaproic acids were obviously positively related to the number of *Bacteroides*, *Acinetobacter* and *Proteocatella*, and negatively related to the number of *Rhodobacter*, *Phreatobacter*, *Roseomonas* and *Gemmobacter*; Isovaleric acid was significantly negatively correlated with *Leifsonia*, *Bosea*, *Vibrio* and *Shewanella* (Fig. 2D). Propionic acid is produced by *Bacteroides* that decompose carbohydrates and has anti-inflammatory properties. *Akkermansia*, *Lactobacillus*, *Lactocaseibacillus*, *Blautia*, *Bacteroides*, *Roseburia* and *Prevotella* are microflora producing SCFAs in the gut and exert a significant influence on host physiology (He et al., 2020).

3.3. MPs and OTC-induced oxidative damage and neurotoxicity, and correlations with SCFAs

In the antioxidant system, T-AOC consists of both enzymatic and non-enzymatic systems and can be used as a measure of the total antioxidant level consisting of various antioxidant substances and antioxidant enzymes (Farhat et al., 2018). All biomarker responses were not significantly different between blank and solvent controls, so different exposure groups were compared with the solvent controls. MPs/OTC single exposure caused an obvious increase in T-AOC in the brain and intestine, as well as the combined exposure elevated intestinal T-AOC levels (Fig. 3A), suggesting activation of antioxidant defenses by MPs and OTC. A previous study revealed that MPs alone/co-exposed with Cd significantly enhanced T-AOC in eye worms (*Euglena gracilis*) (Wan et al., 2019). Tetracycline caused an increase in glutathione peroxidase activity and T-AOC in the gills of tilapia (*Oreochromis niloticus*), which may be an adaptive response of organisms to exogenous substances and help to prevent the adverse effects caused by the increased reactive oxygen content (Xu et al., 2022). In this study, the combined exposure of MPs and OTC had a higher induction effect on T-AOC than the single exposure, probably because MPs exacerbated OTC bioconcentration in the intestine. A previous study also demonstrated that MPs enhanced

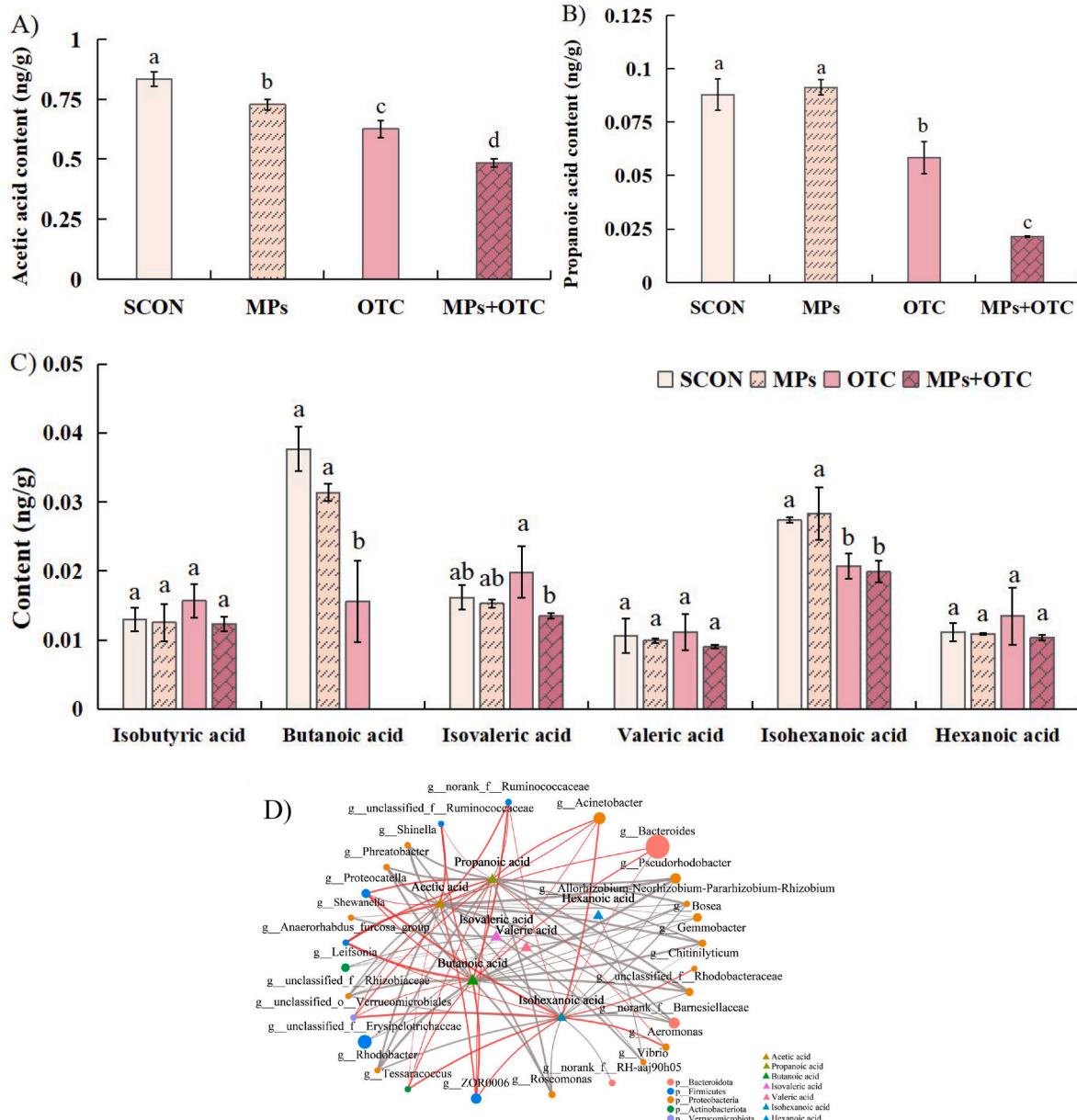


Fig. 2. Contents of acetic acid (A), propionic acid (B), and isobutenoic, butyric, isovaleric, valeric, isocaprylic, and caproic acids (C) of gut microbiota in different treatment groups. Different letters indicate statistically significant differences between treatments in each SCFA ($p < 0.05$). Network analysis between SCFAs and top 30 genera (D). Red and gray edges represent positive and negative correlations, respectively ($p < 0.05$). The thickness of lines indicates the magnitude of the correlation coefficient.

tetracycline-induced oxidative damage in the liver and intestine of *Ctenopharyngodon idella* (Liu et al., 2022).

AChE and BChE are two cholinesterases that play a key role in neurotransmission. AChE terminates ACh activity at the postsynaptic membrane of neuromuscular junctions, whereas BChE hydrolyzes ACh as well as other endogenous and exogenous esters. In comparison with solvent controls, OTC single exposure markedly inhibited AChE and BChE activities in the brain, and MPs single exposure significantly inhibited AChE activity in the brain ($p < 0.05$). The inhibitory effect of combined exposure was stronger, with inhibition rates of AChE and BChE reaching 52.57 % and 61.99 %, respectively (Fig. 3B and C). These results suggested that MPs and OTC produced neurotoxicity in fish, and the mixed treatment had a synergistic effect, causing more severe impairment of nervous system function. Wen et al. (2018) found that MPs caused an obvious suppression of AChE activity in discus fish (*Syphodus aequifasciatus*). Prolonged exposure to OTC resulted in a

significant decrease in AChE activities and metabolic disturbances in rainbow trout (*Oncorhynchus mykiss*) eyes (Rodrigues et al., 2018). MPs increased the bioavailability of bisphenol A in adult zebrafish and induced neurotoxicity (Chen et al., 2017). Cr(VI) did not alter AChE activity in goby but co-exposure with MPs led to an obvious reduction of 67 % in the predation rate, and significant suppression of AChE activity by 31 % (Luís et al., 2015). Inhibition of AChE activity by more than 30 % in fish may lead to the accumulation of ACh, causing overstimulation of postsynaptic receptors and consequent disruption of neurotransmission (Vieira et al., 2009).

Neurotransmitters are major endogenous metabolites in neurotransmission, which play an important role in psychomotor control, motor coordination and gastrointestinal homeostasis (Ma et al., 2021). Compared to solvent controls, MPs single exposure significantly increased GABA levels; OTC single exposure raised ACh and suppressed 5-HT levels; and their co-exposure markedly elevated ACh and GABA

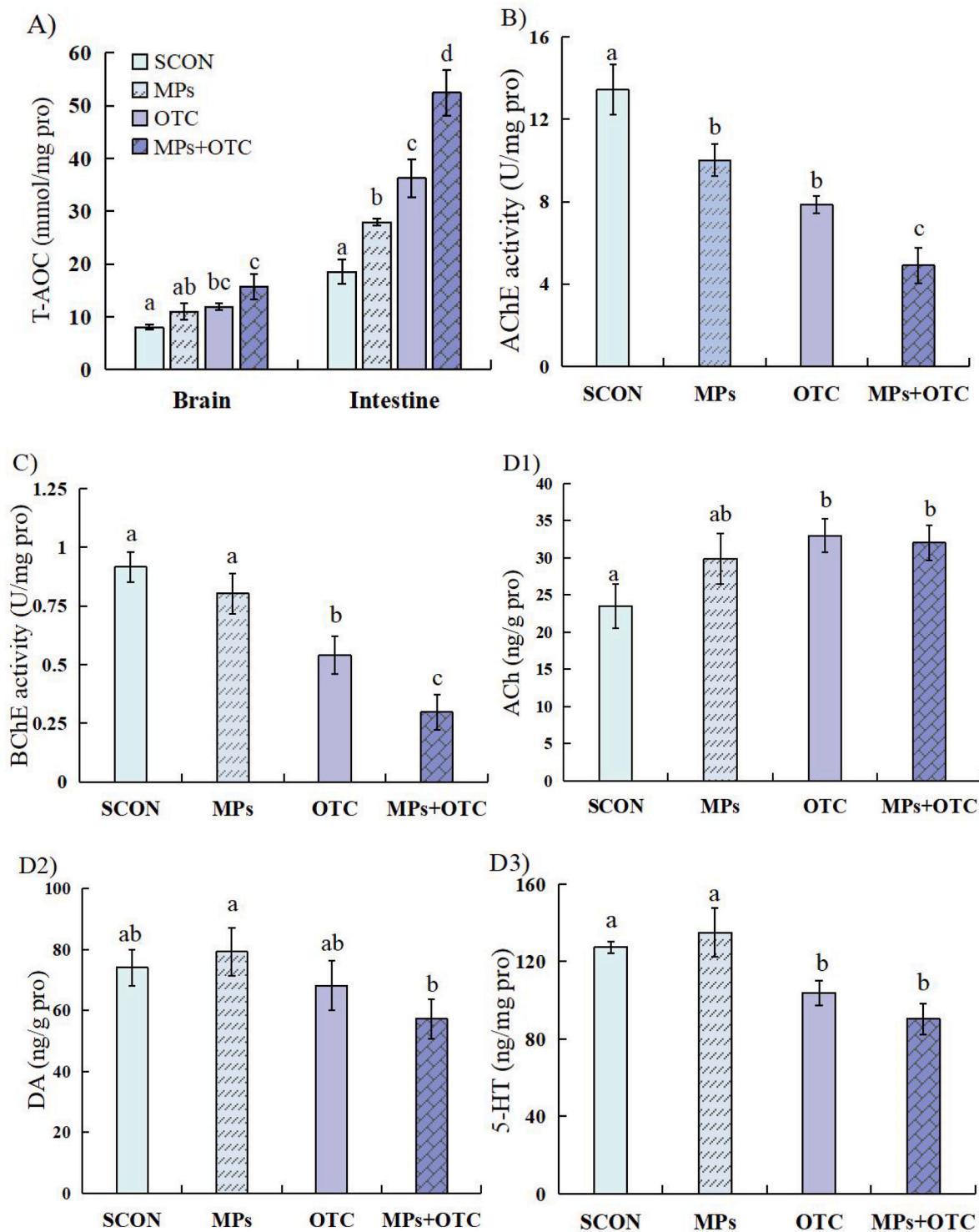


Fig. 3. Changes in T-AOC (A) in the brain and intestine, and AChE (B) and BChE activities (C) as well as ACh (D1), DA (D2), 5-HT (D3) and GABA (D4) concentrations in the brain after MPs and OTC exposure. Different letters show statistically significant differences ($p < 0.05$). Spearman correlation between SCFAs and brain biomarkers (E). “**” indicates $p < 0.05$, “***” indicates $p < 0.01$.

levels while suppressing 5-HT levels ($p < 0.05$, Fig. 3D). Moreover, the combined exposure obviously elevated GABA level compared to the OTC single exposure, and decreased DA and 5-HT levels compared to the MPs single exposure. After ACh is synthesized by choline-acetyltransferase and released into the synaptic gap, it is rapidly degraded to acetic acid and water by AChE and BChE (Dal Forno et al., 2013), which can cause activation of the cholinergic signaling pathway which in turn leads to

neuronal hyperexcitability and behavioral responses. The activity of AChE and BChE was markedly decreased in the combined exposure of this study, resulting in a significant increase in ACh levels. In addition, GABA reduces central nervous system excitability, which is an inhibitory neurotransmitter (Horzmann and Freeman, 2016). GABA transmission plays a major role in the regulation of stress-induced behavioral sequelae, whereas 5-HT has been implicated in the regulation of several

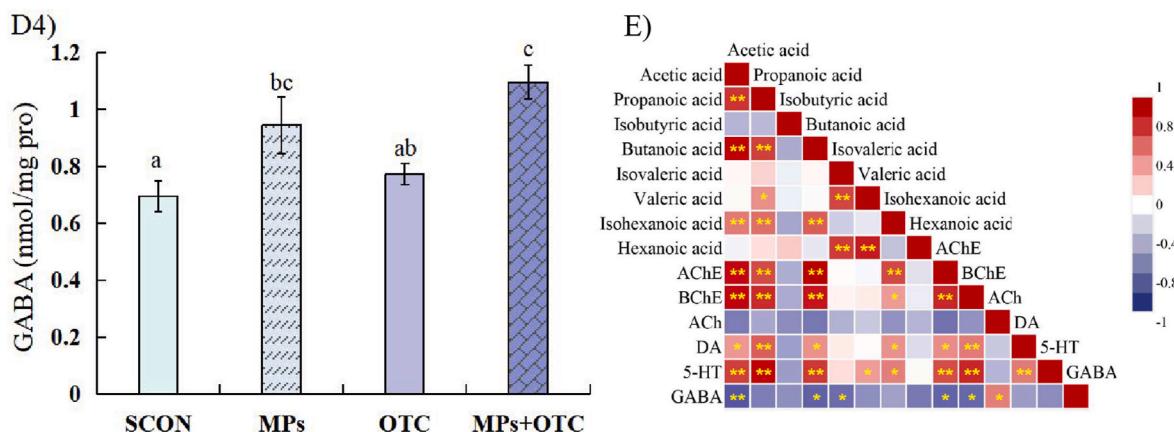


Fig. 3. (continued).

developmental, behavioral, and physiological processes (Manzanares et al., 2005). This result was similar to that of Huang et al. (2022), which showed that MPs contributed to the elevation of GABA levels in the brain of discus fish. However, OTC significantly reduced monoamine

neurotransmitter (5-HT, DA, norepinephrine) levels in zebrafish (Qiu et al., 2021). The carrier effect of MPs on OTC exacerbated the inhibition of DA and 5-HT. Hence, MPs and OTC may mediate neurobehavior in crucian carp through different mechanisms, and different types of

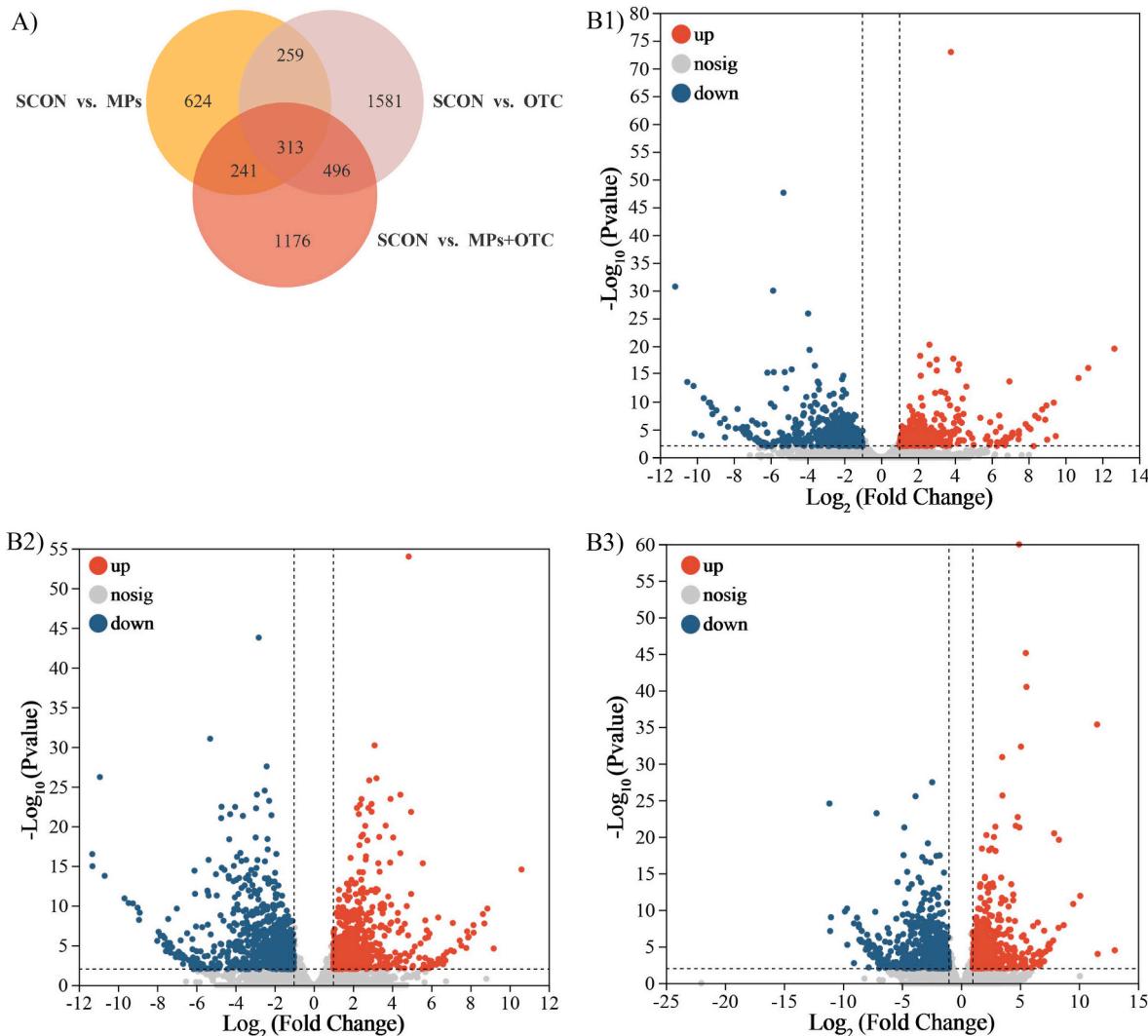


Fig. 4. Venn diagram describing the overlapping DEGs between different exposure groups and solvent controls (A). Volcano plot of DEGs between (B1) SCON vs. MPs, (B2) SCON vs. OTC and (B3) SCON vs. MPs + OTC. Scattered points represent different genes, and the gray, red and blue dots indicate genes without significantly, significantly upregulated and significantly downregulated, respectively.

neurotransmitter biomarkers may allow a more systematic assessment of the neurotoxicity of different pollutants in fish.

Additionally, the correlation between gut microbial metabolites and neurochemical biomarker responses in the brain further unveiled Spearman correlation analysis (Fig. 3E). The levels of AChE, BChE, DA and 5-HT were significantly positively correlated with acetic acid, propionic acid, butyric acid and isocaproic acid. GABA levels were significantly negatively associated with acetic acid, butyric acid, and isovaleric acid. Wu et al. (2020) found a marked positive correlation between 5-HT in the hypothalamus and acetic and valeric acids in mouse feces. Acetic

acid crosses the blood-brain barrier and reduces appetite by activating neurons that drive satiety (Frost et al., 2014). Deficiency of SCFAs may cause host immune, metabolic and neurological disorders (Tan et al., 2014). The main effect of GABA is to reduce the intensity of stress by decreasing neuronal excitability throughout the nervous system (Rawls et al., 2006). Thus, SCFAs may mediate interactions between host brain function and gut microbiota.

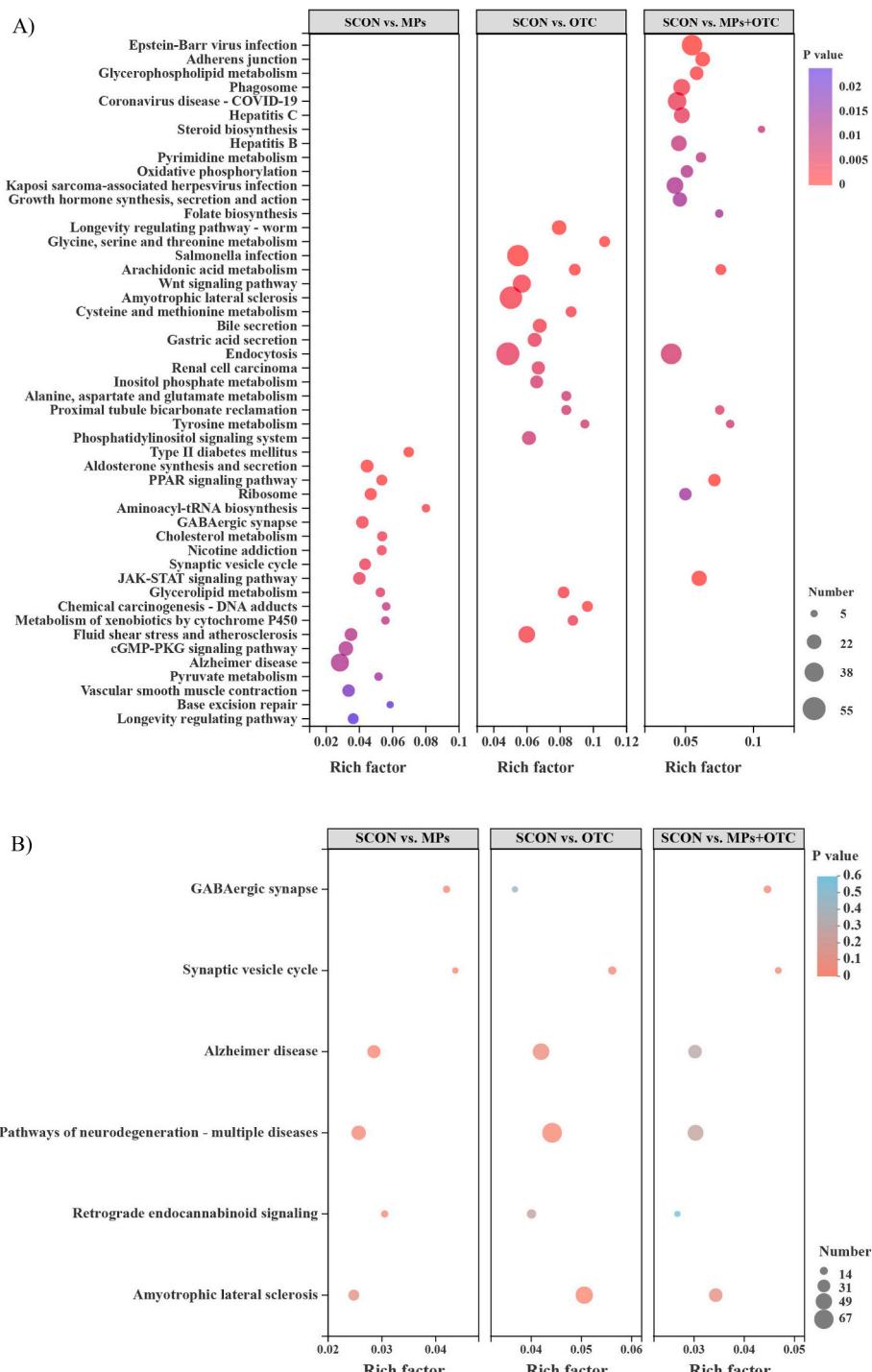


Fig. 5. KEGG pathway analysis of DEGs in three comparisons (SCON vs. MPs, SCON vs. OTC, and SCON vs. MPs + OTC) was demonstrated with a bubble diagram (A). DEGs enrichment pathways relevant to the neuro activity (B).

3.4. Transcriptomics-based toxicity mechanisms of MPs and OTC

The brain is the central organ that controls fish behavior and physiology (Liu et al., 2018). Transcriptomics plays an important role in the study of cellular phenotypes and functions, and can reflect at the molecular level the changes induced by exogenous substances in the organism. DEGs and significantly enriched pathways in different treatments were further explored by transcriptome analysis, which showed that more than 42, 390, 000 clean quality control data were obtained for all treatments, and then the data were subjected to transcriptome assembly on Trinity. The Q30 (the percentage of total bases with sequencing quality of 99 % or higher) and GC content (the percentage of total bases corresponding to the sum of G and C bases in the quality control data) in all treatments exceeded 92.81 % and 45.24 %, respectively (Table S1). The database and functionally annotated single genes of *Carassius auratus* were searched using BLAST2GO software analysis for GO 42079 (68.89 %), KEGG 40682 (66.60 %), COG 55575 (90.99 %) and NR 59276 (97.05 %). The Venn diagram clearly illustrated that 313 genes were co-expressed in the three exposure groups (Fig. 4A). Compared to the solvent control, 624, 1581 and 1176 DEGs were expressed in the MPs alone, OTC alone, and combined groups, respectively. The volcano plot revealed that 352, 874 and 611 genes were markedly up-regulated in the MPs alone, OTC alone, and combined groups, while 525, 913 and 802 genes were significantly down-regulated ($p < 0.01$, Fig. 4B).

To capture the specific distribution and key functional pathways of DEGs, functional annotation and enrichment analyses of different treatments were performed by GO and KEGG databases, respectively. GO functional annotations showed that cellular components, molecular functions and biological processes were the main enriched functional types (Fig. S4). Among them, the enriched abundance of genes related to metabolic processes, cellular processes, bioregulation, catalytic activities, binding, cellular parts, membrane parts and organelles was relatively high. The top 20 KEGG pathways significantly enriched for DEGs between treatments are shown in Fig. 5A, with the majority of DEGs-enriched KEGG pathways belonging to the Human Diseases, Metabolism, and Organismal Systems categories. With regard to lipid metabolism, the glycerolipid metabolic pathway was significantly enriched in all treatments ($p < 0.05$). Glycerolipids play a physiological role in membrane structure and are abundant cellular lipids (Van Meer et al., 2008), whose alterations may affect lipid metabolism and cell membrane stability (Jeppe et al., 2017). MPs single exposure significantly enriched the sphingolipid metabolic pathway in comparison with solvent controls ($p < 0.05$). Metabolomics results in zebrafish liver suggested that the main pathways altered by polystyrene MPs were lipid and energy metabolic pathways (Lu et al., 2016). Residual MPs in crayfish inhibited catabolic activities related to lipid storage (Welden and Cowie, 2016). The sphingolipid metabolic pathway regulates lipid and lipid peroxidation metabolism, which is also stimulated when organisms become inflamed and respond to oxidative stress (Xu and Yu, 2021). Arachidonic acid belongs to a group of polyunsaturated fatty acids released during phospholipid lipolysis. Obvious enrichment of the arachidonic acid pathway in the OTC alone or in combination with MPs treatments implied inhibition of phospholipid metabolic functions in fish (Liu et al., 2021). In terms of the endocrine system, the peroxisome proliferator-activated receptor (PPAR) signaling pathway was significantly enriched in the MPs single or combined treatments with OTC ($p < 0.05$), which was associated with the enhancement of T-AOC. PPAR, a nuclear transcription factor, regulates the homeostasis of lipid metabolism by modulating the expression of genes involved in fatty acid uptake, binding, oxidation, and lipid transport (Schoonjans et al., 1996). Activation of the PPAR signaling pathway during antioxidant processes in fish is a protective mechanism in organisms (Tseng et al., 2011). The tyrosine metabolic pathway was also significantly enriched in the combined treatment ($p < 0.05$), which may be due to the decrease in DA levels. Tyrosine is a precursor substance to DA, which is then taken up

through active transport mechanisms in the brain (Santos et al., 2023).

In addition, the enrichment of KEGG pathways associated with neurological and neurodegenerative diseases in the DEGs enrichment pathways between each treatment group and the solvent control group is shown in Fig. 5B. Compared with the control group, synaptic vesicle cycle pathway, GABAergic synaptic pathway and Alzheimer's disease pathway were enriched in single MPs treatment; while amyotrophic lateral sclerosis pathway, pathway of neurodegeneration and synaptic vesicle cycle pathway were markedly enriched in single OTC exposure. For the combined treatment, the synaptic vesicle cycle pathway and GABAergic synaptic pathway were enriched ($p < 0.05$). These results indicated that the presence of MPs stimulated the GABAergic synaptic pathway leading to elevated GABA levels (Fig. 3D4). A correlation analysis was carried out on 35 DEGs associated with the synaptic vesicle cycle pathway and the top 20 microbial genera (Fig. S5). The results demonstrated that the abundance of *Cetobacterium*, *Bacteroides*, *ZOR0006*, and *Bosea* significantly affected the enrichment of the synaptic vesicle cycle pathway ($p < 0.01$). The main function of the synaptic vesicle cycle pathway is to maintain basal neurotransmitter release and regulate synaptic vesicle levels (Südhof, 2004). Synaptic vesicles release neurotransmitters into the synaptic gap to act on postsynaptic receptors, thereby transferring information from presynaptic to postsynaptic. The integrity of the synaptic vesicle cycle is critical for neural signaling in the brain. Lei et al. (2018) showed that MPs caused significant damage to cholinergic and GABAergic neurons in *Caenorhabditis elegans*. MPs can cause various neurodegenerative diseases mainly by generating oxidative stress closely related to mitochondrial dysfunction and neurodegeneration or by altering gut microbiota (Rai). Moreover, the inhibitory effect of OTC on SCFAs might influence the development of neurodegenerative disorders. OTC influenced host gut microbiota, which further affected host neurobehavior through the microbiota-gut-brain axis (Almeida et al., 2019). Gut microbiota regulates 5-HT synthesis in the brain by directly modulating tryptophan availability (Agus et al., 2018). Studies have demonstrated that neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, and Huntington's chorea are associated with ecological dysregulation of gut microbiota (Tilocca et al., 2020), (Follmer, 2020). SCFAs are key transmitters involved in gut-brain communication and are neuroprotective in neurodegenerative diseases. In a mouse model suffering from Alzheimer's disease, acetic acid exerted anti-inflammatory effects through upregulation of FFAR3 and inhibition of the ERK/JNK/Nf- κ B intracellular signaling pathway (Liu et al., 2020). *Clostridium Butyricum*, which produces butyric acid, protects against microglia-mediated inflammation in mice with Alzheimer's disease (Sun et al., 2020). Therefore, MPs and OTC act on different functional pathways through changes in the gut microbiota and neuroactive molecules (SCFAs and neurotransmitters), which in turn cause neuroinflammation and various neurodegenerative diseases. These results provide an important theoretical basis for developing human health risk assessment strategies.

4. Conclusion

In summary, this study revealed that the carrier effect of MPs facilitated the accumulation of OTC in the brain and intestine, leading to severe pathological damage. Simultaneous exposure to MPs and OTC increased the number of pathogens in the gut microbiota and significantly reduced SCFA production. MPs and OTC activated antioxidant defenses and induced neurotoxicity and neurotransmitter disorders. Neurochemical biomarker responses correlated closely with variations in SCFAs, signifying a mediating effect of SCFAs within the brain-gut-microbiota axis. MPs and OTC have different mechanisms of toxic action at the transcriptional level. MPs stimulated GABAergic synaptic pathways, OTC induced neurodegenerative diseases, and the combined exposure resulted in a significant enrichment of synaptic vesicle cycle and GABAergic synaptic pathways. Overall, the work found the intrinsic

linkage mechanism between the brain, gut, and microbiota induced by MPs and antibiotics, which contributed to a deeper understanding of comprehensive toxicity mechanisms of complex pollutants, and provided new perspectives and important support for the establishment of ecological risk assessment indexes for emerging pollutants.

CRediT authorship contribution statement

Peng Zhang: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Guanghua Lu:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Leibo Zhang:** Validation, Investigation. **Zhenhua Yan:** Visualization, Validation. **Jiaqi Zhang:** Validation. **Keqiang Ding:** Investigation.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2025.126711>.

Data availability

Data will be made available on request.

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