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Microplastic-induced changes in the rainbow trout gut: Insights from transcriptomic, proteomic, and microbial approaches

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Abstract: This study examined the intestinal effects of polyethylene (PE; size 46.6 ± 11.3 µm) and polystyrene (PS; size 52.5 ± 11.5 µm) microparticles at three different concentrations (0.5%, 2%, and 5%) on juvenile rainbow trout (*Oncorhynchus mykiss*) using an integrative approach combining transcriptomics, proteomics, and microbiome analysis. After a six-week experiment, molecular analyses identified concentration-dependent transcriptional responses, including downregulation of genes involved in ion exchange (*slc9a1b*) and appetite regulation (*ghrl*), alongside upregulation of immune- and stress-related genes (*il10*, *tfa*), particularly at higher concentrations. Proteomic profiling showed a more pronounced effect of PS compared to PE, including suppression of digestive enzymes, disruption of lipid and energy metabolism, and activation of proteins associated with oxidative stress and immune responses. Microbiome analysis confirmed plastic-induced dysbiosis, characterised by reduced microbial diversity, depletion of beneficial taxa (e.g., *Bacteroidota*, *Actinobacteriota*), and shifts in short-chain fatty acid–producing bacteria. By integrating multiple levels of biological organisation, this study provides new insights into how microplastics interfere with intestinal physiology, beyond acute toxicity. The findings emphasise the relevance of polymer type and exposure concentration in shaping biological responses and highlight the vulnerability of freshwater species to environmental microplastics.

Keywords: gene expression; microbiome; polyethylene; polystyrene; Salmonidae; sequencing

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Plastic pollution is a major issue of the modern age. Although the number of studies investigating its effects on aquatic organisms is increasing, our understanding of its toxicity remains limited and requires further investigation (Matavos-Aramyan 2024; Ghobish et al. 2025). Aquatic biota represent one of the most vulnerable components of the biosphere to plastic pollution, as plastic waste predominantly ends up in aquatic environments, and its production continues to rise annually (Walker and Fequet 2023).

Microplastics, defined as particles ranging from 1 to 5 μm in size (Hodkovicova et al. 2022), have been shown to affect fish behaviour (Wang et al. 2022a; Subaramaniyam et al. 2023; Li et al. 2024), growth, nutrition and the gut microbiome (Naidoo and Glassom 2019; Wang et al. 2024), and reproduction (Wang et al. 2019; Wang et al. 2024). They also disrupt immune, antioxidant, neural and endocrine functions (Hodkovicova et al. 2021; Barboza et al. 2023; Hollerova et al. 2023; Li et al. 2024; Wang et al. 2024), and lead to the depletion of energy reserves (Alberghini et al. 2022; Subaramaniyam et al. 2023). Since microplastic transfer through the food chain has been confirmed, potential risks to human health should also be taken into consideration (Alberghini et al. 2022).

Recent studies have confirmed that the host gut microbiota is closely linked to metabolism, immune function, energy storage, and the overall health status of fish (Morshed and Lee 2023; Kanika et al. 2025). The gut microenvironment is a delicate ecosystem that varies among fish species and is composed mainly of different bacterial phyla, predominantly Proteobacteria, Fusobacteria, and Firmicutes (Kanika et al. 2025). This balance can be easily disrupted by various factors, including changes in diet, environmental conditions, stress, pharmaceuticals, and xenobiotics (Kanika et al. 2025; Tolas et al. 2025).

Some authors have suggested that microplastics may induce gut dysbiosis and inflammation (Xie et al. 2021; Ma et al. 2025). However, knowledge about the effects of different types of microplastics on gut homeostasis across fish species remains limited and requires further investigation, particularly in freshwater species.

In addition, microplastics have been reported to exhibit a high affinity for lipids, potentially disrupting lipid metabolism in fish organisms (Del Piano et al. 2024). As they do not serve as a source of energy, their presence may lead to energetic and nutrient deficiencies (Qiao et al. 2019a). These disruptions may alter the physiology of the intestinal epithelium and, together with microbial dysbiosis, can impair nutrient absorption, intestinal permeability, hormone and insulin metabolism, and immune cell production (Qiao et al. 2019a; Del Piano et al. 2024; Lee et al. 2024; Yan et al. 2025).

Our primary hypothesis was that polyethylene (PE) and polystyrene (PS) microplastics – the most abundant plastic types found in surface waters (Yang et al. 2023) – may induce concentration-dependent alterations in the gut system of *Oncorhynchus mykiss*. To test this hypothesis, we employed microbiome, transcriptomic, and proteomic analyses – robust approaches for elucidating the impact of xenobiotics on biological pathways (Mishra et al. 2021; Zheng et al. 2024). However, to date, only a limited number of studies have examined the effects of microplastics at the proteomic and microbiome levels, particularly in freshwater fish species.

MATERIAL AND METHODS

Ethical statement

The studies were carried out in accordance with institutional guidelines and national legislation, Act No. 246/1992 Coll., on the Protection of Animals against Cruelty, as amended. The experiments were implemented with the approval of the Ethics Committee of Mendel University in Brno (Czech Republic) (No. 15371/2019, 7062/2020-2).

Experimental design

The data for this study were obtained from two separate experiments conducted under identical conditions. The design of these experiments has already been published in our previous studies on rainbow trout (Hodkovicova et al. 2021; Hollerova et al. 2023).

In brief, both experiments were conducted according to a modified guideline of the Organisation for Economic Co-operation and Development for the testing of chemicals No. 215 (Fish Juvenile Growth Test; OECD 2000), using the same model organism – juvenile rainbow trout (*O. mykiss*,

https://doi.org/10.17221/85/2025-VETMED

n = 160 per experiment in total). Both studies were performed in duplicate.

In both experiments, fish were fed similar commercial feed pellets (EFICO Enviro 921 Advance 3 mm, Biomar, Denmark) in a total amount corresponding to 1.5% of their wet body weight, divided into three portions per day. The feed composition was as follows: 42–45% crude protein, 24–27% crude lipid, 13.5–19.5% carbohydrates, 0.8–2.3% crude cellulose, 4.5–6.5% ash, and 0.9% total phosphorus.

Each experiment lasted six weeks, preceded by a two-week acclimatisation period. In both experiments, identical microplastic exposure concentrations were tested by supplementing the feed with PE or PS microparticles at doses of 0.5%, 2%, and 5%, with results compared to a control group. PE microparticles were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA) and PS microparticles from EPRUI Biotech Co., Ltd. (Beijing, P.R. China). The sizes of the tested polymers were 46.6 ± 11.3 µm for PE and 52.5 ± 11.5 µm for PS. The properties of the microparticles, including protocols for feed preparation and quality evaluation, are described in detail in our previous studies (Hodkovicova et al. 2021; Hollerova et al. 2023).

Throughout both experiments, water quality and fish health were regularly monitored, with no deviations observed. After six weeks, sampling was conducted. Intestinal tissue and content were collected specifically for this study and used for gene expression, proteomic, and gut microbiome analyses.

Gene expression analysis

For gene expression analysis using quantitative real-time polymerase chain reaction (qRT-PCR), samples from the cranial part of the intestine were collected during autopsy (*n* = 8 per concentration and polymer type, i.e., PE and PS). The samples were immediately preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA), then homogenised in TRI Reagent RT (Molecular Research Center, Cincinnati, OH, USA), and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

The extracted RNA was reverse transcribed into messenger RNA (mRNA) using the LunaScript RT SuperMix Kit (New England BioLabs Inc., Ipswich, MA, USA), following the manufacturer's protocol. For complementary DNA (cDNA) synthesis, the reaction was incubated at 25 °C for 2 min to allow primer annealing, followed by 55 °C for 10 min, and then inactivated at 95 °C for 1 minute.

One part of the transcriptomic analysis focused on detecting potential immunological changes through the expression of cytokine-related genes. Primer sequences for interleukin 1 beta (*il1β*), 2*β* (*il2β*), 8 (*il8*), and 10 (*il10*), as well as tumour necrosis factor alpha (*tnfα*), were adapted from Perez-Sanchez et al. (2011). Another part of the analysis targeted genes involved in intestinal function. These primer sequences were designed using the NCBI Primer-BLAST tool (available at ncbi.nlm.nih.gov/tools/primer-blast/). The genes of interest included: flap endonuclease 1 (*fen1*), ghrelin/obestatin prepropeptide (*ghrl*), interferon gamma (*ifng*), intelectin (*ilect*), nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor, *nr3c1*), PCNA-associated factor (*paf*), solute carrier family 9 member A1b (*slc9a1b*), and transferrin-a (*tfa*).

To determine the most suitable reference gene for normalisation, 60S ribosomal protein L31 (*60S*) and beta-actin (*β-actin*) were tested using NormFinder software (Moma, Aarhus, Denmark; Andersen et al. 2004). Both candidate reference genes were adapted from Perez-Sanchez et al. (2011), and *60S* was selected as the most stable gene for normalisation. Primer sequences are listed in Electronic supplementary material (ESM) Table S1.

Gene expression analysis was performed for all samples in triplicate using the LightCycler 480 system (Roche, Basel, Switzerland) and the QuantiTect SYBR Green PCR Kit (Qiagen, Germany). The cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 seconds. Melting curve analysis was conducted between 60–95 °C. The resulting data were analysed using the LightCycler 480 SW 1.5 software (Roche, Basel, Switzerland), based on threshold cycle (Ct) values and the comparative ΔΔCt method. The relative expression of each gene of interest (GOI) was calculated using the formula: $[1/(2^{Ct^{GOI}})]/[1/(2^{Ct^{60S}})]$. Normalised gene expression is presented as mean ± standard deviation (SD) relative to the control group.

https://doi.org/10.17221/85/2025-VETMED

Proteomic analysis

For the proteomic analysis, intestinal samples (n = 8 per concentration and polymer type, i.e., PE and PS) were processed from the homogenates for gene expression analysis. The bottom organic fraction from TRI Reagent separation was taken to a new tube without the precipitated DNA in the interphase. Three volumes of ice-cold acetone (MS grade; Sigma-Aldrich, St. Louis, MO, USA) were added and kept at –20 °C for 30 min until proteins were fully precipitated. Tubes were then centrifuged at 15 000 × g for 10 min at 4 °C. The protein pellet without supernatant was washed three times in 0.3 M guanidine hydrochloride in 95% ethanol (Penta, Prague, Czech Republic) and 2.5% glycerol (Carl Roth, Karlsruhe, Germany). The last washing step was done only in ethanol/glycerol solution. The protein pellet was resuspended in 8 M urea (Serva, Heidelberg, Germany), 0.1% SDS (Carl Roth, Karlsruhe, Germany), 0.2% Triton X-100 (Carl Roth, Karlsruhe, Germany) and 25 mM TEAB (triethylammonium bicarbonate; Sigma-Aldrich, St. Louis, MO, USA), vortexed and incubated for one hour at room temperature with constant shaking. After centrifugation at 20 000 × g for 10 min, the supernatant was taken, and protein concentration was measured using Pierce BCA Protein Kit (Thermo Fisher Scientific, Prague, Czech Republic). 50 µg of proteins were used for each sample preparation with the FASP (filter-aided sample preparation) method (Wisniewski et al. 2009). Each sample was washed six times with 8 M urea in Vivacon 500 centrifugal tubes (Sartorius Stedim, Göttingen, Germany) with a 10 000 MWCO membrane filter. Dithiothreitol (10 mM; Sigma-Aldrich, St. Louis, MO, USA) and iodoacetamide (50 mM; Serva, Heidelberg, Germany) in 25 mM TEAB buffer were used for reduction and alkylation, respectively. Proteins were then digested with trypsin (Thermo Fisher Scientific, Waltham, MA, USA) in a 1 : 50 ratio, for one hour at 37 °C and then overnight at 25 °C. After centrifugation, the eluate with digested peptides was evaporated (DNA120 SpeedVac; Thermo Savant, Holbrook, NY, USA), and the peptide pellet was resuspended in 0.1% aqueous formic acid (Sigma-Aldrich, St. Louis, MO, USA) which serves as a mobile phase for liquid chromatography (UltiMate 3000 RSLCnano; Thermo Scientific, Waltham, MA, USA). For separation and elution of peptides, a 2-hour gradient with an increasing concentration of acetonitrile (0.1% formic acid in acetonitrile; Sigma-Aldrich, St. Louis, MO, USA) at a flow rate of 300 nl/min was used. Peptides were separated on a 25 cm column (Acclaim PepMap RSLC C18, 2 µm, 100 A, 75 µm I.D.; Thermo Scientific, Prague, Czech Republic). uHPLC was connected to an EASY-spray ion source (Thermo Fisher Scientific, Waltham, MA, USA) and a Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). A survey scan over the m/z range 390–1 700 was used to identify protonated peptides with charge states of at least 2, which were automatically selected for data-dependent MS/MS analysis and fragmented by HCD dissociation. Ten fragment mass spectra after each full scan were recorded. Measured spectra were then searched using Proteome Discoverer (v2.4; Thermo Scientific, USA) with Sequest HT as a search algorithm. Oxidation of M, deamidation of N and Q as dynamic modifications, and carbamidomethylation of C as a static modification were used. Precursor and fragment mass tolerances were set up to 10 ppm and 0.02 Da, respectively. The Uniprot database for the *Oncorhynchus mykiss* strain (from 2021/03) was used in Sequest HT. Only peptides with a false discovery rate of less than 0.01 were considered well identified. The quantity of identified proteins is expressed by the area under the curve of the chromatographic peak detected by a mass spectrometer. Relative quantification is given by the mean of the three most abundant peptides for each identified protein. Normalisation was applied according to the total peptide amount.

Microbiome analysis

During autopsy, intestinal contents (n = 10 per concentration and polymer type, i.e., PE and PS) were collected into sterile 2 ml Eppendorf tubes, kept on ice throughout the dissection, and immediately frozen at –80 °C. In total, samples from eight individuals per concentration group were collected in duplicate.

Gut microbiota composition was analysed by next-generation sequencing of the V3/V4 variable regions of 16S rRNA genes, following the protocol described by Kubasova et al. (2017). Briefly, individual faecal samples were homogenised with zirconia/silica beads (0.1 mm diameter; Beckman Coulter, Brea, CA, USA) using a Precellys 24 tissue homogeniser

https://doi.org/10.17221/85/2025-VETMED

(Bertin Instruments, Montigny-le-Bretonneux, France). DNA was then extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

DNA concentration and purity were assessed spectrophotometrically using a NanoDrop 2000 device (Thermo Scientific, Waltham, MA, USA), based on 260/280 and 260/230 absorbance ratios. PCR amplification targeting the V3/V4 region of the eubacterial 16S rRNA gene, DNA clean-up, and MiSeq sequencing were performed as previously described (Kubasova et al. 2017).

Amplicon data were processed using QIIME 2 (v2021.2; Bolyen et al. 2019). Raw sequence data were demultiplexed, quality-filtered, and sequencing primers were removed using in-house scripts based on Je (Girardot et al. 2016) and fastp (Chen et al. 2018). Sequence denoising was performed using DADA2 (Callahan et al. 2016) via the q2-dada2 plugin. Taxonomic classification of amplicon sequence variants (ASVs) was conducted using the q2-feature-classifier (Bokulich et al. 2018) with the scikit-learn naïve Bayes classifier trained on the Silva 138 99% OTU full-length reference database (Quast et al. 2013).

Statistical analysis

All data are presented as mean ± SD. Statistical analyses were performed using Prism v8 software (GraphPad, San Diego, CA, USA). Normality of data was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Bartlett's test. For normally distributed data, comparisons were made using one-way ANOVA followed by Tukey's multiple comparison post hoc test. For non-normally distributed data, the Kruskal–Wallis test was applied.

Statistical comparisons were performed across all groups, with $P < 0.05$ considered statistically significant. In tables and figures, means denoted with different superscript letters (a,b,c) indicate statistically significant differences. Box plots display the median, first (Q1) and third (Q3) quartiles, with whiskers representing a maximum of 1.5 times the interquartile range; outliers are shown as individual points.

All proteomic data were analysed using Proteome Discoverer software (v2.4; Thermo Fisher Scientific, Waltham, MA, USA). Visualisations of principal component analysis and the heatmap were performed using R software with the R-package plugins Openxlsx (v4.2.4), Factoextra (v1.0.7), Gplots (v3.1.1) and Ggplot2 (v3.3.5). Statistical analysis of qualitative and quantitative proteomic data was performed in Proteome Discoverer, proteins were identified with a false discovery rate (FDR) of 0.01, the statistical significance of protein abundance ratios within groups was determined by an adjusted *P*-value of less than 0.05. Results that were not statistically significant are not discussed in detail.

RESULTS AND DISCUSSION

Morphology, behaviour and mortality

During both studies, morphological parameters, behaviour, and mortality were monitored. Regarding morphometric analysis, no significant differences were observed among individuals within each experiment. In the study involving PE microparticles, one fish from the 5% PE group was euthanised (day 33). Similarly, in the PS group, two individuals from the 5% concentration group were euthanised (day 34 and 36). In both cases, euthanasia was performed due to extensive necrotic skin lesions.

In addition, reduced feeding time, increased aggression, and elevated activity levels were observed in the 5% concentration groups for both PE and PS during the entire experiment. The heightened activity and aggression were likely associated with the non-nutritional nature of microplastics added to the feed pellets. Furthermore, both aggressive behaviour and the adhesion of microplastics to the skin surface were considered contributing factors to the development of skin lesions, which ultimately led to the need for euthanasia.

These observations are in line with previous findings showing that microplastic exposure can alter behavioural patterns in fish, including increased activity and aggressive interactions. For example, increased locomotor activity has been associated with aggression (Critchell and Hoogenboom 2018). Although behavioural responses may vary between groups and exhibit high individual variability, such changes suggest that microplastic exposure can disrupt normal behavioural dynamics, including feeding-related activity and social interactions (Narwal et al. 2025).

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Gene expression

Microplastics have been shown to interfere with key physiological pathways, including metabolic and energetic processes (Limonta et al. 2019; Zhao et al. 2020), xenobiotic metabolism, immune function (Limonta et al. 2021; Banaei et al. 2022), and glucose and lipid regulation (Wan et al. 2019; Zhao et al. 2020), particularly at the transcriptomic level. Based on this evidence, we selected genes of interest that are involved in pH regulation, immune response, and oxidative stress, as these processes are closely interlinked and critical for intestinal homeostasis. Changes in their expression may thus reflect early molecular responses to microplastic-induced epithelial disruption and systemic stress.

Maintaining pH is essential for proper cellular function and survival, and one of the primary mechanisms involved in pH regulation is ion transport. Fish must prevent ion loss, and most species actively absorb ions through specialised chloride cells (Kumai and Perry 2012). One of the regulators of intracellular pH is the sodium–hydrogen exchanger (NHE), which is encoded by the SLC9 gene family (Anderegg et al. 2022). NHEs are responsible for H^+ efflux and the maintenance of intracellular pH; their primary role is to prevent intracellular acidosis by exporting H^+ ions (Yang et al. 2022). The SLC9 gene family also plays a role in maintaining overall cellular homeostasis and, when coupled with Cl^- and H_2O transport, contributes to cell volume regulation (Chen et al. 2023). Studies also suggest a potential role for NHEs in inflammation and cancer progression, with NHE upregulated in inflammatory conditions and tumours, possibly due to its role in pH regulation and cellular proliferation (Zhou et al. 2021).

In our study, a significant decrease in *slc9a1b* expression was observed in fish exposed to 5% PE ($P < 0.024$). This could result in impaired regulation of H^+/Na^+ exchange, leading to plasma acidification and, consequently, disrupted enzymatic activity, metabolism, and overall cellular function. Moreover, given the role of NHEs in pH regulation and the maintenance of intestinal microbiota, reduced expression may also contribute to microbial dysbiosis.

Interestingly, this downregulation was accompanied by a significant increase in the mRNA expression of *il10* and *tfa*. For *il10*, the increase was observed in the 2% and 5% PE exposure groups compared to the control ($P < 0.041$), and mRNA expression was also significantly upregulated at the 5% concentration ($P < 0.027$) of PS when compared to both the 0.5% and 2% concentrations. The upregulation of *il10*, an anti-inflammatory cytokine, may reflect a compensatory response to inflammatory or oxidative stress induced by microplastics. Similarly, the increase in *tfa* expression suggests a potential response to elevated oxidative stress, as transferrins are involved in iron sequestration and the mitigation of reactive oxygen species (ROS) via the Fenton reaction (Mokhtar et al. 2023). Transferrins are important acute-phase proteins capable of binding free iron ions; their function is essential for maintaining oxidative balance in the organism (Bayne and Gerwick 2001). In our study, *tfa* gene expression was significantly downregulated in the 0.5% PS group, whereas a significant upregulation was observed at the 5% PS concentration when compared to the 0.5% PS group ($P < 0.048$). This increase in *tfa* expression is likely a physiological response to oxidative stress induced in the intestinal tissue by PS microparticles. The induction of oxidative stress by PS in other organs – namely the liver, gills, and kidneys – has already been confirmed in this experiment and published by Hollerova et al. (2023).

These combined molecular changes in genes involved in pH balance, immunity, and oxidative stress may indicate a shift in the intestinal environment toward stress adaptation and inflammation control, possibly a consequence of microplastic-induced epithelial disruption and dysbiosis.

Some studies of microplastic exposure have observed changes in diet intake when organisms are exposed to microplastic pollution (Hodkovicova et al. 2021; Liang et al. 2023). The effect of plastic presence in the diet on feeding behaviour appears to be species-specific in fish, with both orexigenic and anorexigenic responses reported (Delgado et al. 2017; Basto-Silva et al. 2020). In vertebrates, ghrelin is a gut–brain hormone that plays a central role in the regulation of food intake and intestinal motility (Basto-Silva et al. 2020).

In our study, a significant decrease in *ghrl* expression was observed in fish exposed to 5% PE. Considering the findings described above – namely, signs of ongoing intestinal inflammation – it is likely that ghrelin production was also impaired. This reduction may contribute to altered food intake, an energy deficit, and overall stress

https://doi.org/10.17221/85/2025-VETMED

in the fish. In addition to its metabolic functions, ghrelin also plays a role in immune regulation by influencing the expression of cytokines such as *il1β*, *il10*, and *tnfα*, as demonstrated in hybrid tilapia (*Oreochromis* spp.) (Han et al. 2020). The same study also showed that ghrelin stimulation promoted the formation of ROS, suggesting its involvement in oxidative stress responses. The gene expression results for both PE and PS exposures are shown in Figure 1.

Proteomics

The overall changes in the intestinal protein expression profile, as measured by mass spectrometry, are presented in the form of scatter plots (Figure 2). Protein abundance in each sample from microplastic-exposed fish was compared to the control group, and the resulting ratios are shown as $\log_2$ fold changes. A $\log_2$ ratio between –1 and 1 indicates no biologically significant difference in protein abundance. In contrast, values above 1 or below –1 (corresponding to an abundance ratio >2 or <0.5, respectively) reflect biologically relevant changes.

When comparing the effects of the two polymer types, a greater number of significant changes in the intestinal proteome were observed following PS exposure compared to PE. While a concentration-dependent response was evident in fish exposed to PE, PS exposure caused pronounced alterations in protein expression even at the lowest tested concentration.

Principal component analysis (PCA) did not reveal significant differences between the control group and any of the PE-treated groups. In contrast, clear separation was observed in the 0.5% and especially the 5% PS groups. Although the 2% PS group did not show major divergence from the control, it still exhibited more pronounced proteomic changes than any of the PE concentrations (ESM Figure S1). A heatmap was used to visualise the proteomic data (Figure 3).

POLYETHYLENE

In general, PE exposure affected fewer proteins than PS. Among the proteins upregulated by PE, two uncharacterised proteins showed high sequence similarity to fatty acid-binding proteins (FABPs) previously identified in other fish species.

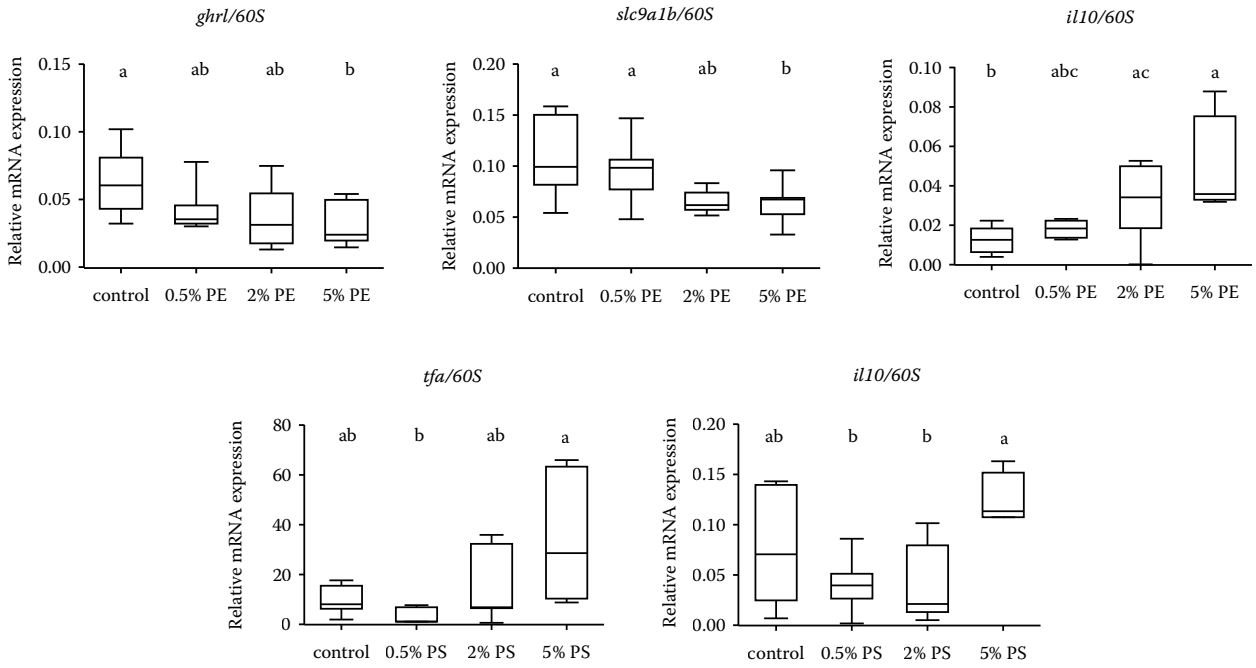

Figure 1. mRNA expression levels of *ghrl*, *slc9a1b*, *il10*, and *tfa* in the cranial part of the intestine of *O. mykiss* after six weeks' oral exposure to three different concentrations (0.5%, 2%, and 5%) of polyethylene (PE) or polystyrene (PS) microparticles

[a–c]Statistically significant differences are indicated by different superscript letters

https://doi.org/10.17221/85/2025-VETMED

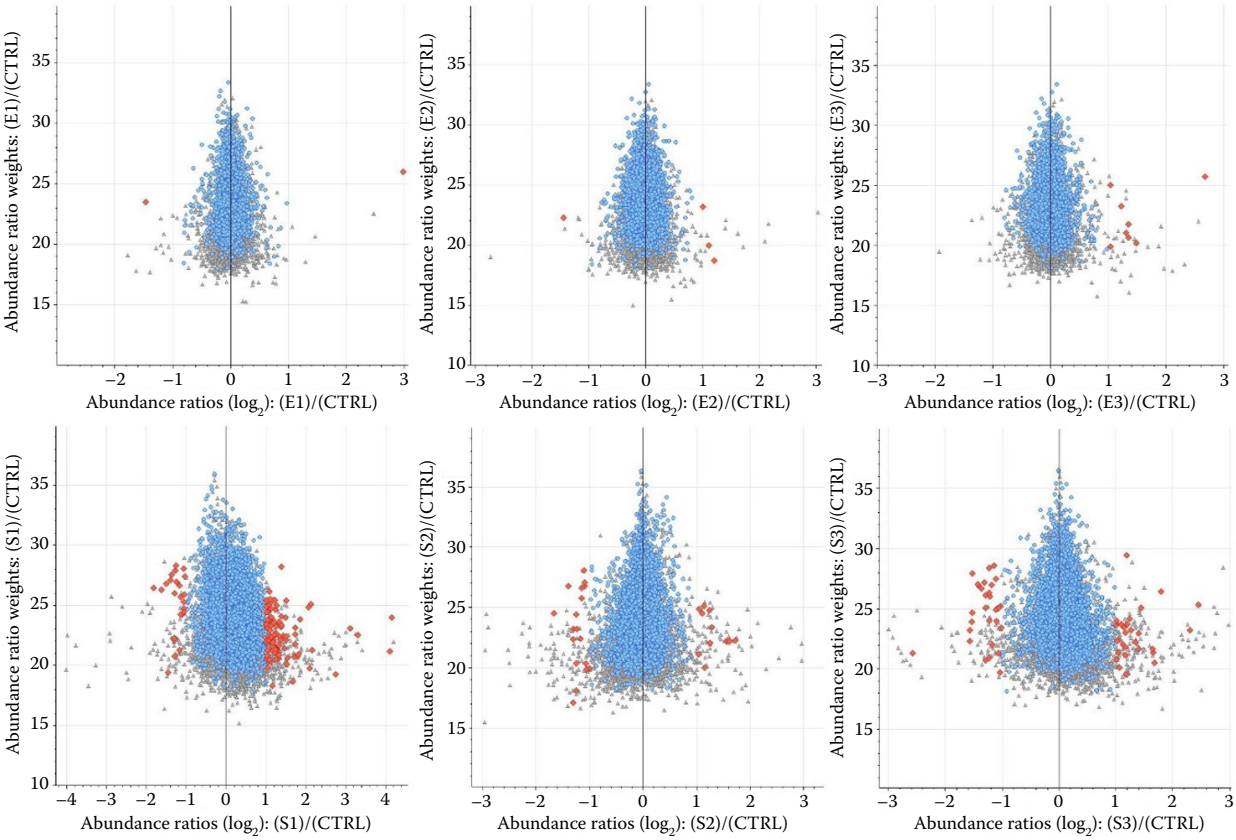

Figure 2. Scatter plots showing differentially expressed proteins in the intestinal tissue of *O. mykiss* after six weeks' oral exposure to three concentrations (0.5%, 2%, and 5%) of polyethylene (E1, E2 and E3, respectively) or polystyrene (S1, S2 and S3, respectively) microparticles relative to the control group (CTRL)

Each blue dot represents an individual protein and its abundance ratio compared to CTRL. Grey triangles represent filtered-out proteins. Red diamonds show proteins with significant changes in abundance ratio higher than 2 or lower than 0.5 (>1 or <–1 in $\log_2$) compared to the same protein of the control group

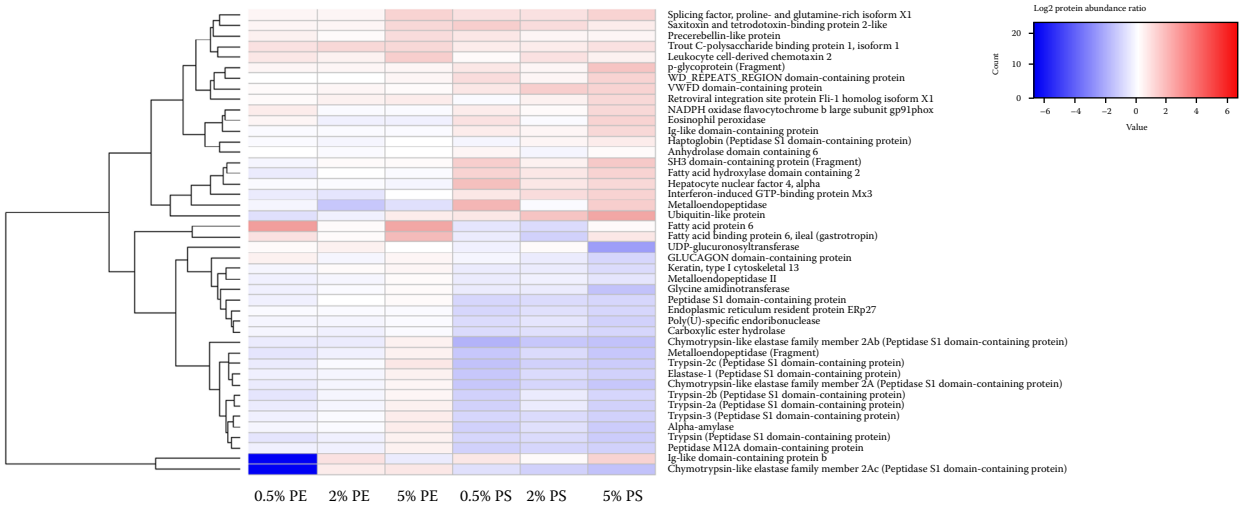

Figure 3. Heatmap of differentially expressed proteins in the intestinal tissue of *O. mykiss* after six weeks' oral exposure to three concentrations (0.5%, 2%, and 5%) of polyethylene (PE) or polystyrene (PS) microparticles

Rows represent individual proteins; columns represent treatment groups. Colour intensity reflects relative protein abundance, with red indicating upregulation and blue indicating downregulation

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FABPs are a class of proteins involved in the intracellular transport and metabolism of long-chain fatty acids and other hydrophobic ligands (Agellon 2024). The production of ROS triggered by fatty acids and their metabolites can be mitigated by their binding to FABPs, which serves a protective role.

PE exposure has been shown to increase ROS production; for example, elevated *fabp* gene expression was observed in the liver of zebrafish following exposure to 10–40 μm PE particles (Kurchaba et al. 2020). In our study, FABP expression was significantly upregulated in the 5% PE group, supporting the hypothesis that PE interferes with fatty acid metabolism and induces oxidative stress at higher concentrations. In addition, fatty acids have been recognised as regulators of mucin – the main component of intestinal mucus – which plays a key role in innate immunity and in protecting the intestinal wall (Sun et al. 2021). In contrast, larval zebrafish exposed to PS showed decreased *fabp6* mRNA levels (Wan et al. 2019). Similarly, in our study, PS exposure at 0.5% and 2% resulted in reduced FABP protein levels, consistent with previous findings.

Regarding the immune system, several proteins were slightly upregulated in the 5% PE group. Among them was leukocyte cell-derived chemotaxin 2 (LECT2), which is known to regulate *il1β* and *il10*, neutrophil chemotaxis, and the innate immune response (Li et al. 2021). Additionally, precerebellin-like protein (CBLNL) and trout C-polysaccharide binding protein 1 (TCBP1) are both recognised as acute-phase reactants in *O. mykiss* (Kumar et al. 2019, Syahputra et al. 2019). The upregulation of both proteins in the 5% PE group suggests a response to pathological processes and homeostatic disturbance, potentially caused by infection, inflammation, or tissue trauma (Kobis et al. 2017). Mechanical trauma caused by PE particles, leading to inflammatory processes in the intestinal tissue, is a plausible explanation (Hodkovicova et al. 2021). This is further supported by the upregulation of a cluster of peptidase S1 domain-containing proteins, as shown in the heatmap. These proteases are known to participate in inflammatory responses (Modica et al. 2015).

POLYSTYRENE

PS exposure resulted in a broader impact on digestive proteases compared to PE, particularly downregulating proteins with serine endopeptidase activity, such as trypsin- or chymotrypsin-like enzymes, which are crucial for protein digestion. This downregulation across all PS concentrations likely impairs intestinal digestion and reduces feed utilisation. The decreased activity of trypsin after exposure to PS was already documented for juvenile orange-spotted groupers (*Epinephelus coioides*) (Wang et al. 2022a). Notably, a peptidase (A0A060XWS0), possibly involved in feeding and starvation responses, was also suppressed in our study.

In compensation, a VWFD-domain protein – homologous to mucin-2 – was upregulated, suggesting increased mucus secretion by goblet cells in response to xenobiotic stress (Wang et al. 2024). Their upregulation in response to environmental concentrations of microplastics has already been observed, for example, in *Oryzias melastigma* exposed to PS (Chen et al. 2022). Additionally, certain hydrolases were affected, e.g., ABHD6, implicated in monoacylglycerol hydrolysis and gut motility (Pusch et al. 2022), was upregulated in the 5% PS group, while enzymes such as alpha-amylase and carboxylic ester hydrolase were downregulated, indicating potential disruptions in carbohydrate and lipid metabolism.

Metalloendopeptidases were generally downregulated across all tested PS concentrations, except for one (i.e., HCE23), with no clear concentration-dependent trend. These enzymes are involved in various functions, mainly tissue repair and immunity (Xie et al. 2023). Furthermore, certain fish pathogens (e.g., *Aeromonas*, *Vibrio*, *Yersinia*, *Pseudomonas*) secrete metalloendopeptidase exotoxins that are highly immunogenic and may have haemagglutinating properties (Mekasha and Linke 2021). Thus, the observed downregulation may impair host defence mechanisms against these pathogens. Supporting this, a study on Japanese flounder (*Paralichthys olivaceus*) revealed that metalloproteinases (a key subgroup of metalloendopeptidases) are involved in environmental stress responses and immune defence (Xie et al. 2023).

Immune-related proteins were also upregulated under PS exposure (notably at 5%), including Ig-like domain-containing proteins associated with antigen binding and complement activation (Mokhtar et al. 2023). Conversely, poly(U)-specific endoribonuclease was downregulated, and a peptidase S1 domain-containing protein – similar to trypsin and involved in cytokine regulation – was also reduced.

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Proteins associated with oxidative stress responses were upregulated, reflecting increased ROS production and xenobiotic efflux activities in line with previous findings of increased oxidative stress by PS exposure (Hollerova et al. 2023). However, UDP-glucuronyltransferase, which facilitates xenobiotic detoxification via glucuronidation, was significantly downregulated at 5% PS, mirroring reports in *Gobiocypris rarus* (Wang et al. 2022b). Additionally, an ER-resident protein (A0A060YT94) was downregulated, hinting at potential endoplasmic reticulum damage and subsequent metabolic dysregulation (Wang et al. 2021).

Finally, PS exposure decreased the expression of glycine amidinotransferase (GATM), important for creatine synthesis and energy storage, and appeared to suppress glucagon activity – possibly via microbiome-altered propionate levels. These changes collectively indicate impaired energy metabolism and altered feeding regulation. Regarding other proteins, PS appears to downregulate glucagon activity, probably *via* microbiome alterations. The short-chain fatty acid propionate, produced by *Bacteroidota*, stimulates the glucagon-related protein glucagon-like peptide 1 (de Souza-Silva et al. 2022). In fact, *Bacteroidota* decreased at all tested PS concentrations in our study, as discussed below, which correlates with the decreased glucagon activity at the protein level.

Microbiota characterisation

The gut does not function solely as a mechanical and chemical barrier. Together with its microbiome, it also plays a critical role in immune and energy system activation, nutrient absorption and source of vitamins, hormone and enzyme regulation, etc. (Chapagain et al. 2019; Varo et al. 2021; de Souza-Silva et al. 2022). The composition of the gut microbiota is a crucial indicator of an individual's health. Disruptions to its balance, i.e., dysbiosis, can lead to disease onset and metabolic imbalance. Numerous factors can alter microbiota structure, including diet composition, pharmaceuticals, environmental and other stressors (de Souza-Silva et al. 2022; Luan et al. 2023; Wu et al. 2025).

Among the xenobiotics capable of altering gut microbiota composition are microplastics. However, knowledge of their impact on fish remains limited, and the available studies are often inconsistent (Varo et al. 2021; de Souza-Silva et al. 2022). Similarly, in our study, results revealed that both PE and PS exposure altered the gut microbiota composition; however, in distinct ways. Nevertheless, in both cases, bacterial richness – both in terms of abundance and diversity – was higher in the control group. Results are given in Figure 4.

Reduced diversity of gut microbial phyla is a mark of dysbiosis and has been associated with increased disease susceptibility, as a less diverse microbiota provides limited competition against pathogens (Qiao et al. 2019a; Colin et al. 2022). The most abundant phyla in the intestinal microbiota of freshwater and marine fish are typically *Firmicutes* and *Proteobacteria*, considered core gut taxa. Together with *Actinobacteria*, *Bacteroidota*, *Fusobacteria*, and *Tenericutes*, they can account for up to 90% of gut microbial communities, although their proportions vary based on numerous internal and external factors (Butt and Volkoff 2019; Terova et al. 2019; Rimoldi et al. 2021).

The ratio of *Bacteroidota* to *Firmicutes* is particularly important, as both phyla produce short-chain fatty acids like butyrate and propionate, which are key energy sources and play roles in immune modulation (Butt and Volkoff 2019). Microplastics have been shown to increase *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia*, while decreasing *Bacteroidota* and *Actinobacteria* – a shift associated with metabolic disorders (de Souza-Silva et al. 2022). Butyrate, primarily produced by *Firmicutes*, supports colonocyte energy metabolism, has anti-inflammatory effects, and enhances insulin sensitivity and gut barrier function (Terova et al. 2019; de Souza-Silva et al. 2022). However, in our study, *Firmicutes* abundance declined with increasing PE concentrations and at 0.5% PS. We also observed a positive correlation between Firmicutes and Proteobacteria, with *Proteobacteria* increasing in a concentration-dependent manner in PE groups. These bacteria are linked to cancer, insulin resistance, and the production of lipopolysaccharides, which damage the mucosa and increase gut permeability (Qiao et al. 2019a; de Souza-Silva et al. 2022).

For PS exposure, bacterial diversity was again higher in control. At 0.5% and 5% PS, *Fusobacteriota* increased, while *Firmicutes* decreased, in line with previous zebrafish studies (Jin et al. 2018; Qiao et al. 2019b). *Fusobacteriota* promote mucus secretion and anti-inflammatory responses (Chapagain et al.

https://doi.org/10.17221/85/2025-VETMED

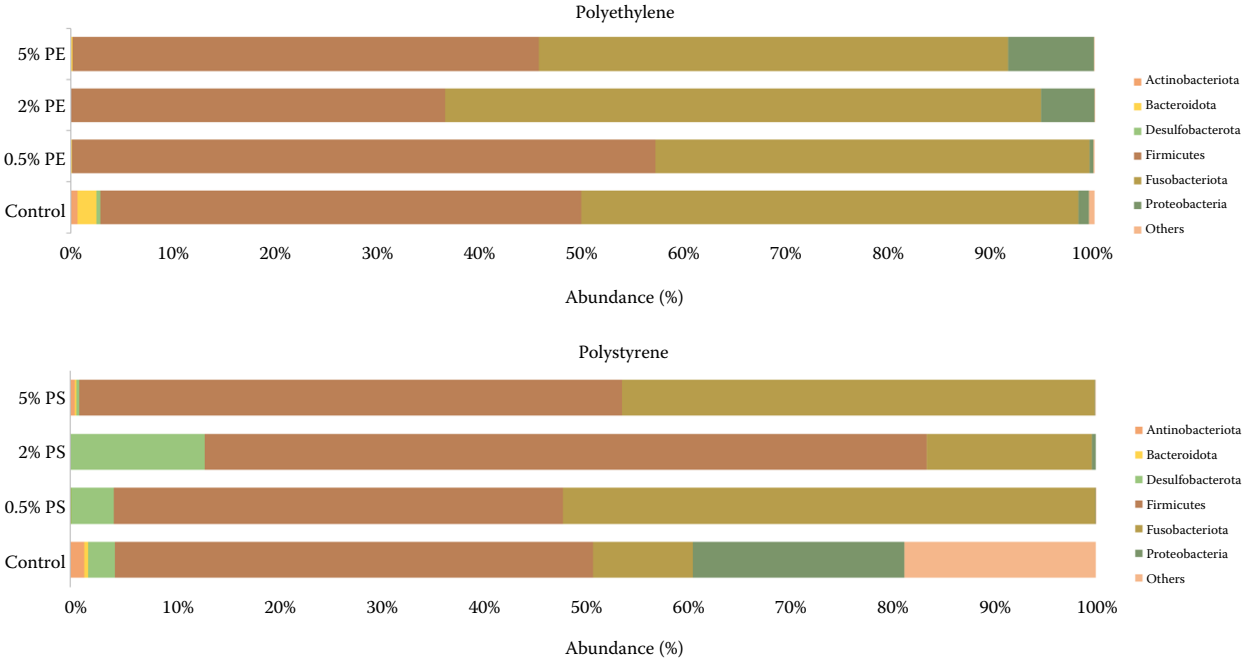

Figure 4. Relative abundance of dominant bacterial phyla in the intestinal microbiota of *O. mykiss* after six weeks' oral exposure to three different concentrations (0.5%, 2%, and 5%) of polyethylene (PE) or polystyrene (PS) microparticles Data are presented as the mean relative abundance (%) per group (n = 10)

2019) which is in line with the transcriptomic and proteomic results of our study.

Actinobacteriota were notably reduced in both PE and PS groups, compared to controls. As producers of antimicrobial, antitumor, and immunosuppressive compounds, their depletion may weaken gut defence (Salwan and Sharma 2020). Similar reductions have been reported in zebrafish and *Poecilia reticulata* exposed to PS (Qiao et al. 2019a; Huang et al. 2020).

To summarise, exposure to microplastics induced significant dysbiosis in the gut microbiota of fish, characterised by reduced bacterial diversity, a decline in beneficial phyla such as *Bacteroidota* and *Actinobacteriota*, and an increase in potentially pro-inflammatory taxa like *Proteobacteria*. The observed microbial shifts may therefore impair nutrient absorption, compromise gut barrier function, and promote intestinal inflammation. The microbiome changes are associated with immune responses and the subsequent predominant abundance of pathogen species, which may lead to activation of gut-associated lymphoid tissue and infections (Butt and Volkoff 2019; Tay et al. 2025). Collectively, these findings indicate that microplastic-induced gut dysbiosis can compromise intestinal homeostasis, immune competence, metabolic balance, and overall organismal health.

Summary

Our study demonstrates that exposure to PE and PS microplastics induces molecular, proteomic, and microbiome-level alterations in the intestine of *O. mykiss*. Transcriptomic data revealed the downregulation of genes involved in ion exchange (*slc9a1b*) and appetite regulation (*ghrl*), along with the upregulation of immune- and stress-related genes (*il10*, *tfa*) – all of which were particularly evident at higher microplastic concentrations. Proteomic analysis indicated a broader and more severe impact of PS, including the suppression of digestive enzymes, disruption of lipid and energy metabolism, and activation of proteins associated with oxidative stress and immune response. Microbiome profiling confirmed dysbiosis, characterised by reduced microbial diversity, depletion of beneficial phyla (e.g., *Bacteroidota*, *Actinobacteriota*), and shifts in short-chain fatty acid-producing bacteria.

Altogether, the results indicate that microplastic exposure – especially to PS – can impair gut function, digestion, immunity, and overall homeostasis in rainbow trout. *O. mykiss* was selected as a model species due to the current scarcity of studies addressing microplastic effects in freshwater fish (Hodkovicova et al. 2022).

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Given the observed differences between PE and PS effects, the toxic potential of microplastics should be further investigated, as different polymer types and their physicochemical properties can elicit diverse biological responses across fish species.

Conflict of interest

The authors declare no conflict of interest.

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