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Impact of polypropylene microplastics and chemical pollutants on European sea bass (*Dicentrarchus labrax*) gut microbiota and health.

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Abstract

Plastic pollution has become a global problem for marine ecosystems. Microplastics (MPs) are consumed by several marine organisms, including benthic and pelagic fish species that confuse them with food sources, thus contributing to bioaccumulation along the food chain. In addition to structural intestinal damage, ingestion of MPs represents a pathway for fish exposure to potentially hazardous chemicals, too. Most of them are endocrine disrupters, genotoxic or induce immune depression in fish.

Accordingly, we assessed the combined toxicological effects of microplastics (MPs) and adsorbed pollutants by adding them to marine fish diet. European sea bass (*Dicentrarchus labrax*) juveniles were fed for 60 days with feeds containing polypropylene MPs, either virgin or contaminated with chemical pollutants (a blend of dichlorodiphenyldichloroethylene, chlorpyrifos, and benzophenone-3). The data demonstrated a synergic action of MPs and chemical pollutants to induce an inflammatory-like response in distal intestine of sea bass as shown by the up regulation of cytokine *il-6* and *tnf-α* expression. Morphological analysis detected the presence of a focus of lymphocytes in anterior and posterior intestinal segments of fish fed with contaminants in the diet. With regard to microbiota, significant changes in bacterial species richness, beta diversity, and composition of gut microbiota were

observed as a consequence of both pollutants and polluted MPs ingestion. These perturbations in gut microbial communities, including the reduction of beneficial lactic acid bacteria and the increase in potential pathogenic microorganism (Proteobacteria and Vibrionales), were undeniable signs of intestinal dysbiosis, which in turn confirmed the signs of inflammation caused by pollutants, especially when combined with MPs. The results obtained in this study provide, therefore, new insights into the potential risks of ingesting MPs as pollutant carriers in marine fish.

Keywords: Chemical pollutants · European sea bass · Gut microbiota · Histopathology · Ingestion · Microplastics

1. Introduction

Plastics have become a threat to marine ecosystems, not only because of the physical damage to organisms, but also because of the toxicity due to the associated chemical pollutants. It has been estimated that, globally, from 2 to 5% of plastic waste goes into the oceans every year (Jambeck et al., 2015). For instance, in 2018, almost 62 million tons of plastics were produced in Europe and between 7 and 13.5 million tons of them were emitted as waste into the marine environment (Plastics-the Facts, 2019).

It is generally accepted that the term plastic refers to synthetic polymers composed of various elements, such as carbon, nitrogen, oxygen, and sulphur. In terms of polymers, polyethylene (PE) is the most abundant in the marine environment, representing 23% of the total, followed in decreasing order by polyesters (PEST), polyamide (PA), polypropylene (PP), and polystyrene (PS) (Erni-Cassola et al., 2019).

The recent increasing interest in plastic accumulation in marine environments is mainly related to the insidious and deleterious impacts of micro-sized (1 μm–1 mm) and nano-sized (<1 μm) plastic particles (Jambeck et al., 2015). Microplastics (MPs) are derived either from small particles developed for specific applications (primary MPs), i.e., microbeads used in cosmetics or microfibers shed from clothing and other textiles, such as fishing nets, or produced through the breakdown of larger plastics mainly due to abiotic factors, such as the sunlight, wind, and water (Murphy et al., 2016).

MPs are consumed by marine organisms that confuse them with food sources, thus contributing to bioaccumulation along the food chain (Borrelle et al., 2017). The ingestion of MPs was recently documented in several marine species (Carbery et al., 2018; Setälä et al., 2018; Wang et al., 2019; Wright and Kelly, 2017), including benthic and pelagic fish species (Adika et al., 2020; Cardozo et al., 2018; Dantas et al., 2020; Herrera et al., 2019; Kroon et al., 2018; Zeytin et al., 2020). Overall, fibers represent the most abundant MP type found in fish stomach (Adika et al., 2020; Dantas et al., 2020; Herrera et al., 2019; Kroon et al., 2018; Maaghloud et al., 2020; Ory et al., 2017; Rezania et al., 2018), whereas the most prevalent polymers are PE, PP, PS, and PEST (Kuebutornye et al., 2020; Wang et al., 2020). Generally, the

low number of plastic particles found in the gastrointestinal (GI) tract of fish suggests that the potential for accumulating MPs is close to zero and that the presence of MPs in the GI only indicates recent ingestion (Jovanović, 2017).

Despite the high number of publications on the presence of MPs in fish, little is known about uptake, translocation, and accumulation within fish organs (De Sales-Ribeiro et al., 2020; Jovanović, 2017). Translocation of MPs to other tissues, such as liver and muscle, has been described in some important commercial marine species, such as common mullet (*Mugil cephalus*) (Avio et al., 2015), European anchovy (*Engraulis encrasicolus*) (Collard et al., 2017), gilthead sea bream (*Sparus aurata*) (Jovanović et al., 2018), and European sea bass (*Dicentrarchus labrax*) (Zeytin et al., 2020). Therefore, although current knowledge on MPs seems to suggest a low risk for human health, considering the scarce and controversial findings concerning their accumulation in fish organs, we cannot exclude a possible negative effect on seafood safety.

What is certain is that the intake of MPs causes various adverse effects in fish, such as damage to the GI tract, changes in lipid metabolism, changes in behavior, as well as cytotoxicity (Jovanović, 2017; Wright and Kelly, 2017; Yan et al., 2020). The aforementioned effects are similar in response to both dietary and environmental (water) MP exposure.

The most frequent histopathological alterations observed at the intestinal level comprise detachment of mucosa epithelium, hyperplasia, shortening, swelling of villi, vacuolation of enterocytes, and leukocyte infiltration (Ahrendt et al., 2020; Jovanović, 2017; Pedà et al., 2016); however, other authors did not find any impact of MPs on gut integrity (Ašmonaite et al., 2018; Jovanović, 2017).

Ingestion of MPs also represents a pathway by which fish can be exposed to potentially hazardous chemicals (Carbery et al., 2018; Wright and Kelly, 2017). Owing to their hydrophobic surface, MPs can adsorb hydrophobic organic contaminants (HOCs), such as polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane (DDT) and their metabolites, organochlorine pesticides, and polychlorinated biphenyls (PCBs) (Camacho et al., 2019; Henríquez-Hernández et al., 2017; Hirai et al., 2011; Ogata et al., 2009; Van et al., 2012). They also accumulate heavy metals such as cadmium, arsenic, mercury, zinc, nickel, and lead (Holmes et al., 2012; Rochman et al., 2014). The toxicological effects of several of these chemical compounds, for instance, dichlorodiphenyltrichloroethane (DDT) and their metabolites, or polychlorinated biphenyls (PCBs), organophosphate pesticides, herbicides, and heavy metals, are well known (Camacho et al., 2019). Most of them are endocrine disrupters, genotoxic, or induce immune suppression in fish (Kuo et al., 2012). However, the toxicological assessment of the combined effects of MPs and adsorbed pollutants has not been exhaustively investigated in fish (Barboza et al., 2018; Pedà et al., 2016; Qiao et al., 2019; Rochman et al., 2013; Wang et al., 2020).

Different types of environmental chemicals can effectively induce changes in gut microbiota (Tu et al., 2020). Therefore, a dysbiosis (gut microbial imbalance) might develop in the host's intestine after consuming MPs due to the ingestion of foreign and potentially pathogenic bacteria or to chemicals that make up or adhere to MPs (Fackelmann and Sommer, 2019). This is a negative effect as changes in microbiota balance could play an active role in the pathogenesis of several diseases. It is well known that a well-balanced gut microbiota fulfills a variety of functions in the host and that it is important for nutrition and for the immune system of fish by avoiding inflammation responses and minimizing the presence of pathogens (Nayak, 2010).

Up to now, the effects of exposure to different classes of contaminants (chemicals, heavy metals, and MPs) on gut microbial communities have only been investigated in a few fish species (Evaristo et al., 2019; Gu et al., 2020; Huang et al., 2020; Jin et al., 2018; Lu et al., 2019; Qiao et al., 2019; Wan et al., 2019; Yan et al., 2020). Those studies agree that all classes of contaminants contribute to impairing fish digestive performance, stimulating the immune response, and inducing gut microbiota dysbiosis. However, to our knowledge, no study has taken into account the effects of dietary administration of MPs alone or in combination with chemical pollutants on the composition of the gut microbiome and the immune response in a reared Mediterranean fish species.

Accordingly, we exposed European sea bass juveniles to chemical pollutants and to PP-MPs contaminated or not with a blend of chemical pollutants (dichlorodiphenyldichloroethylene, chlorpyrifos, and benzophenone-3), by adding them to the fish diet. The aim was to determine the individual and combined effects of dietary MPs and chemical pollutants on fish gut morphology and microbiota and on the expression levels of genes coding for the cytokines interleukin (il) *il-6*, *il-1β*, and *il-10*, and tumor necrosis factor-alpha (*tnf-α*), all involved in the fish immune response.

2. Materials and methods

All procedures involving fish complied with the guidelines of the European Union Council (86/609/EU) and Spanish legislation (RD 53/2013) and were approved by Bioethical Committee of the University of Las Palmas de Gran Canaria (Ref. 06/2021 CEBA ULPGC).

2.1. Experimental diets

Four different diets were formulated and manufactured at the ECOAQUA Institute of University of Las Palmas de Gran Canaria (ULPGC), (Las Palmas, Canary Islands, Spain), using as basis a commercial pelleted diet (D-2 Optibream AE 1P, Skretting Spain Spa, crude protein 48%, fat 18%, ash 6.3%, and fibre 3.6%). To assess the effect of MPs without pollutants, a diet (diet MP) was prepared by adding MPs to the commercial diet. To assess the effect of pollutants alone or the effects pollutants combined with MPs, either a mix of three chemical pollutants, or MPs contaminated by the

same mix of chemical pollutants were added to the commercial diet to produce diet P, and diet P+MP, respectively. Commercial diet with no additives was considered as the control diet (diet C).

The synthetic MPs were obtained by grinding 5 mm of low-density PP pellets (LDPP, Sigma-Aldrich®) using a cutting mill (Retsch-SM100, Haan, Germany). Then, the MPs were separated by sieving to obtain the 0.7-1 mm fraction. Characterisation of MPs was performed by visual inspection under a binocular stereomicroscope. The microphotograph (Fig. 1) shows the size and shape of the microplastics obtained. As one can see in the figure, the shape of the microplastics is similar to that of the fragments found in environmental samples, therefore, these fragments simulate the real situation better than the microspheres that are frequently used in microplastic studies.

The level of inclusion of MPs in the diets MP, and P+MP was 10% (w/w), mimicking natural occurrence in the wild, as described by (Herrera et al., 2020). Indeed, in areas of maximum accumulation in the Canary Islands, values of MPs/zooplankton ratio of 0.10 in wet weight, and twice as much MPs as zooplankton in dry weight, were found (Herrera et al., 2020). While it is true that this only occurs in areas of maximum accumulation, it is a realistic value in the worst-case scenario.

Diets P and P+MP were supplemented with a blend of three chemical pollutants: dichlorodiphenyldichloroethylene (p,p'-DDE), chlorpyrifos (CPF), and the emerging contaminant UV filter benzophenone-3 (BP-3). Based on the reference values proposed by Camacho et al. (2019), the dietary inclusion levels for p,p'-DDE, CPF, and BP-3 were 1000, 100, and 300 ng/g, respectively. The purity of the used contaminants was 99.9%, 97.5% and 98.5%, respectively. Once the proportional amount of pollutants was calculated and weighed according to the required concentrations, the first step was to dissolve the compounds in organic solvents: 1 l ethanol (100%) and 10 ml pure acetone per 500 g of solute (MPs or feed). The feed and the MPs were distributed in glass bins (20x20 cm) in a homogeneous layer. The solutions were spread on the bin and removed with a metal spoon. Finally, the ethanol was left to evaporate inside the hood for 5 days.

For the experimental diet, after adding 5% of water, the ground ingredients were mixed and pelleted using a 3-mm die. Throughout the process, the temperature was maintained below 50°C and the pressure stable at 2-3 atm. After pelleting, the diets were dried at 37-39 ºC for 20 hours. A total of 5 kg of experimental feed were produced for each diet. Finally, the p,p'-DDE, CPF, and BP-3 concentration obtained in each diet were analysed. The final values in ng/g are presented in the Table 1.

2.2. Fish and sampling

The feeding trial was set in a flow-through marine water system at the ECOAQUA Institute facility (ULPGC, Canary Island, Spain). Three hundred European sea bass (initial mean body weight, 80.91 ± 13.28 g) were randomly distributed

in twelve cylindrical conical tanks (500 l). For the duration of the feeding trial, water conditions were monitored daily (salinity 37 mg l^{-1}, dissolved oxygen 6.0 ± 0.5 ppm, temperature 22 ± 1 °C). Fish were fed *ad libitum* with four different diets in triplicate (3 tanks/diet) for 60 days.

Uneaten pellets were recovered, dried, and weighed to estimate feed intake. At the end of the feeding trial all fish were individually weighed to calculate weight gain (WG = final body weight - initial body weight), specific growth rate (SGR = 100 x [ln (final body weight) - ln (initial body weight)]/days]), and feed conversion ratio (FCR = feed intake/WG).

At the end of the trial, two fish per replicate tank (6 fish/diet) were sacrificed by administering an overdose of anesthetic (clove oil) by immersion.

The whole intestine was aseptically dissected out; then, a portion of proximal and distal intestine was excised and stored in RNA*later*® Stabilization Solution (Thermo Scientific, Italy), at 4°C until RNA extraction.

The whole intestine of six other fish per dietary group was aseptically dissected out using alcohol-disinfected instruments for gut microbiome analysis. As previously described (Rimoldi et al. 2019; Terova et al., 2019), the fecal matter (allochthonous or transient microbiota) was collected by squeezing and then transferred to a sterile 2-ml tube containing 800 µl of Xpedition™ Lysis/Stabilization Solution (Zymo Research, Irvine, CA, USA). The autochthonous (adherent) intestinal bacteria were then collected by scraping intestinal mucosa with a sterile cotton swab. The tip of the swab was immediately transferred into the tube containing the fecal material to mix the digesta- and the mucosa-associated microbiota. The intestinal microbiota samples were then stored at room temperature for up to 48 hrs until bacterial DNA extraction.

To characterize feed-associated bacterial communities, three aliquots of 200 mg each from each feed were taken at the end of the trial and used for bacterial DNA extraction and sequencing.

2.3. Gut morphological studies

Fish posterior gut and rectum were dissected out and separated as preileorectal valve and postileorectal valve segments as detailed previously by (Torrecillas et al., 2019), then fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. From each segment, 4-µm gut sections were stained with hematoxylin and eosin (H&E) for optical examination and with May-Grünwald Giemsa (MGG) to study leukocyte distribution and presence. Digital images of the slides were obtained using an Olympus VS120 digital scanner (Optic system BX61VS, Tokyo, Japan) equipped with VC50 and VS-XM10 cameras and were processed with Olympus VS software (VS-NIS-SQL-V2.6, Tokyo, Japan).

2.4. RNA extraction

Total RNA for gene expression analysis was extracted from 125 mg of proximal and distal gut samples using the semi-automatic system Maxwell® 16 Instrument (Promega, Italy), and Maxwell® 16 LEV simplyRNA Tissue kit (Promega, Italy). The RNA concentration was calculated by measuring the absorbance at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Italy). The integrity of RNA was assessed by agarose gel electrophoresis.

2.5. Generation of standard curves for absolute quantification of gene expression

Standard curves, one per each target gene (*il-6*, *il-1β*, *il-10* and *tnf-α*), were constructed using the known copy number of a synthetic gene-specific mRNA as reported by Rimoldi et al. (2016). In brief, a forward and a reverse primer were designed based on the coding sequence of each gene and used in a conventional RT-PCR to create templates for *in vitro* transcription. The nucleotide sequences of forward primers were engineered to contain a T7 promoter sequence at their 5'end (Table 2). PCR products were TA-cloned using the pGEM®-T easy vector system (Promega, Italy) and subsequently sequenced. *In vitro* transcriptions were performed using T7 RNA polymerase and the RiboProbe® *In Vitro* Transcription System kit (Promega, Italy), according to the manufacturer's protocol. The concentration of the *in vitro*-transcribed RNAs was spectrophotometrically determined. The molecular weights (MW) of the *in vitro*-transcribed RNAs were calculated according to the following formula:

MW = [129 (no. of A bases) × 329.2) + 69 (no. of U bases) × 306.2) + 66 (no. of C bases) × 305.2) + 98 (no. of G bases) × 345.2)] + 159.

The transcript copy number was obtained by multiplying the number of moles for Avogadro's number.

2.5. Quantitative PCR analysis

Quantitative PCR (qPCR) reactions were performed on 100 ng of total RNA and run in parallel to 10-fold-diluted defined amounts of standard mRNAs. To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 25 µl) for each sample. Primers and gene-specific fluorogenic probe were manufactured by Metabion International AG (Germany). The nucleotide sequences of all primers and probes used in this study are reported in Table 3. The qPCR reactions were performed using iTaq™ Universal Probes One-Step Kit (Bio-Rad, Italy). qPCRs were run on a CFX96 Real-Time PCR Detection System (Bio-Rad, Italy) under the following amplification conditions: 10 min at 50°C, 3 min at 95°C followed by 40 cycles consisting of 15 s at 95 °C, and 30 s at 60°C. Raw data from qPCR runs were collected and analysed by Bio-Rad CFX Maestro software (Bio-Rad, Italy). The Ct values from standard curves served as a basis for calculating the absolute amounts of target transcript copies in unknown intestinal samples.

2.6. Bacterial DNA extraction

The bacterial DNA was extracted from 250 mg of intestinal material (faeces + mucosa) and 200 mg of each tested feeds in triplicate using DNeasy PowerSoil Kit (Qiagen, Italy), according to the manufacturer's instructions. Bacterial lysis was performed using PowerBead Tubes supplied by the kit and a TissueLyser II instrument (Qiagen, Italy) set at 25 Hz for 2 min. As negative control of the extraction procedure, a sample with only lysis buffer was processed in parallel with samples. The concentration of extracted DNA was measured using NanoDropTM 2000 Spectrophotometer (Thermo Scientific, Italy). DNA was then stored at - 20 °C until metabarcoding analysis.

2.7. 16S amplicon library preparation and sequencing

The 16S amplicon library preparation and sequencing were carried out by the GalSeq srl company (Italy), using an Illumina MiSeq platform and applying the Illumina protocol "16S Metagenomic Sequencing Library Preparation for Illumina MiSeq System" (#15044223 rev. B). Details of methodology applied for 16S rRNA gene library preparation and sequencing have been previously described (Rimoldi et al., 2018, Terova et al., 2019). For the characterization of microbial communities, the hypervariable region V4 of 16S rRNA gene was amplified, using the oligonucleotides 515F: 5′-GTGYCAGCMGCCGCGGTAA-3' and 806R: 5′-GGACTACNVGGTWTCTAAT-3 ′.

Amplicon libraries were then quantified by qRT-PCR and pooled in one tube at equimolar concentrations. DNA sequencing was performed on the Illumina MiSeq device with pair-ended sequencing (2 × 250) strategy. All sequences were submitted to European Nucleotide Archive (EBI ENA).

2.8. Metabarcoding data processing and analysis

Processing and analysis of raw sequencing data were performed using QIIME™ 2 (v. 2018.4) pipeline (Bolyen et al., 2019). After trimming at 3' and 5' ends, the reads were filtered for base quality (Q>30) and merged. QIIME DADA2 denoise-paired command was applied to denoise and dereplicate single-end sequences. The quality-filtered fastq reads were then clustered into operational taxonomic units (OTUs) at 97% sequence identity. Each OTU was aligned to Greengenes database v. 13.8 (http://greengenes.lbl.gov/) down to genus taxonomical level. OTUs comprising less than 0.005% of total reads as well as OTUs corresponding to eukaryotic sequences were removed from the dataset. To evaluate the adequacy of sequencing depth, alpha rarefaction curves were plotted and good's coverage estimator was calculated to assess the percentage of the total species that are represented in a sample. Alpha diversity indexes (Chao1, Observed OTUs, Shannon, Faith-PD, and Evenness) were calculated to explain species richness and diversity within microbial communities.

A principal coordinates analysis (PCoA) was performed to show the differences between microbial communities (beta diversity) based on both unweighted UniFrac and weighted UniFrac distance metric (Lozupone and Knight, 2005; Lozupone et al., 2007).

2.9. Inference of metagenomics by PICRUSt

Predictive functional profiling of microbial communities was performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software tool (Langille et al., 2013). This analysis was performed as previously described by (Rimoldi et al., 2021). The inferred metagenomics functions were assigned using the KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologues (KO). The differences between the control and experimental groups were tested by a two-sided Welch t test using the Statistical Analysis of Metagenomics Profiles (STAMP) software package (Parks et al., 2014). The extended error bar plots were generated to show statistically significant differences.

2.10. Statistics

All data were checked for normality and homoscedasticity by Shapiro–Wilk's and Levene's test, respectively. Statistical analysis was performed using R (version 6.1.1) and PAST3 software. One-way ANOVA test was applied to determine if there were significant differences (p value < 0.05), and Tukey's post hoc for multiple comparisons. When normality and homoscedasticity assumptions were not met, the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons was applied. To perform statistics on microbial relative abundance data, the percentage values were firstly angular transformed. Only those taxa with an overall abundance of more than 1% (up to order level) and 0.5% at family and genus level were considered for the analysis.

Non-parametric PERMANOVA and analysis of similarities (ANOSIM) test with 999 permutations were performed using QIIME script 'compare_categories.py' to assess beta-diversity dissimilarities.

3. Results

3.1. Growth performance

All diets were well accepted by fish for the entire duration of the feeding trial. Regardless of the diet, fish grew properly and by the end of the feeding trial (60 days) had approximately doubled their weight. No significant differences were found among dietary groups in terms of growth performance (K, WG, and SGR) and feeding efficiency (FCR). In Table 4 the mean values of growth performance are reported and the FCR calculated for all experimental groups of fish.

3.2. Gut morphological studies

Morphological evaluation of H&E/MGG-stained sections of fish gut showed a well-organized folding pattern, lack of debris, no accumulation of MPs, and an intact intestinal epithelial barrier for all the fish experimental groups. For anterior gut, fish fed diets with contaminants presented a wider *lamina propria* than fish fed control and MPs diets, especially when MPs were combined with the contaminants (Fig. 2a-d). A higher presence of rodlet cells on both the basal area and along the fold was observed in the anterior gut of fish fed diets with contaminants (Fig. 2e). Furthermore, we commonly observed higher lymphocytic focus scores in the submucosa of fish fed with contaminants alone or in combination with MPs (Fig. 2f, g) than in fish fed the rest of the dietary treatments. In the intestinal segment of the postileorectal valve, the *lamina propria* was not clearly engrossed and depended on the contaminant being present in the anterior gut; however, a tendency was observed (Fig. 3). As observed in anterior gut, the presence of a focus of lymphocytes was detected in fish fed contaminated feed in both segments associated with the presence of contaminants in the diet. (Fig. 3b). In the rectum of fish fed control diet supranuclear vacuolization tended to be higher than in fish fed the rest of the dietary treatments (Fig. 3c).

3.3. Expression of immune-relate genes

Expression of *il-1β* gene was significantly affected by diet in proximal, but not in distal intestine (Fig.3a). In particular, *il-1β* mRNA copies were decreased in the proximal intestine of fish fed diet MP (containing synthetic MPs with no contaminants), in comparison to fish of the control group.

The transcript levels of *il-6* resulted higher than the control in proximal and distal intestine of European sea bass fed with diet P+MP, containing MPs and contaminants (Fig.3b). Similarly, *tnf-α* was affected by diet both in proximal and distal gut portions. In particular, *tnf-α* expression decreased in the proximal intestine of fish receiving diet MP, whereas a two-fold increase of mRNA copies was found in fish fed diet P+MP as compared to the control fish (Fig. 4d). On the contrary, the experimental diets had no significant effect on *il-10* expression as compared to the control group fed with diet C (Fig. 4c).

Overall, these results indicated that the major changes in interleukin genes were due to the chemical contaminants rather than to the MPs in the diet and this effect was much more severe in the proximal intestine.

3.4. Metabarcoding analysis of microbial communities

The raw data, generated as fastq files, from Illumina sequencing of 36 samples (24 gut + 12 feeds) were elaborated and analysed with QIIME 2™ software pipeline. Only two intestinal samples, one from group P and one from P+MP, failed. The total number of high-quality reads taxonomically classified according to the Greengenes database, was 543,665

(15,102 ± 4,897 reads per sample), corresponding to an overall of 158 OTUs (Supplementary Table S1). Rarefaction analysis of chao1 index showed that all curves approximated saturation, indicating that the number of sequences recovered from MiSeq sequencing was adequate to achieve a good coverage (Supplementary Fig. S1). All fastq sequencing files were deposited in the EBI ENA public database under the accession project code: **PRJEB41533**.

3.4.1. Feed-associate bacteria

The microbial community profiles of each feed were outlined at the phylum, class, order, family, and genus. By taking into account only the most representative taxa, the overall feed-associate microbial community comprised 3 phyla, 6 classes, 9 orders, 21 families, and 8 genera. In Supplementary Table S2, is reported the list of the most abundant bacterial taxa, their relative abundances, and statistical analysis.

Firmicutes and Proteobacteria were the dominant phyla of feed-associate bacterial communities, with a relative abundance ranged between 45-59% and 37-54% respectively. The phylum Bacteroidetes was detectable only in feed samples C and MP (Fig. 5a, Fig. 6). At class level, the most representative taxa harbored in feed were Bacilli (43-56%) followed by Alphaproteobacteria (21-28%) and Gammaproteobacteria (11-32%). Lactobacillales (42-50%) and Enterobacteriales (5-32%) were the most abundant bacterial orders (Table S2). The Flavobacteriales, Bacillales, Pseudomonadales, Burkholderiales, and Aeromonadales were significantly higher in C and MP feeds (Table S2). Accordingly, the Lactobacillaceae and Enterobacteriaceae were the most representative bacterial families in feed samples. Flavobacteriaceae, Bacillaceae, Moraxellaceae, Pseudomonadaceae, Weeksellaceae, and Xanthomonadaceae families differed significantly between feeds, being higher represented in C and MP (non-contaminant) than in P and P+MP feeds (contaminant-containing diets) (Fig. 5b). At genus level, feed-associate bacterial communities were dominated by *Lactobacillus* (38-44%), regardless of the diet. Feeds C and MP were characterized by higher relative abundance of *Flavobacterium, Photobacterium*, and *Bacillus* genera (Fig. 5c, Fig. 6).

Despite the aforementioned differences in terms of taxa relative abundance, in general the feed-associated microbiome profiles did not show great differences in taxonomic composition among the feeds.

3.4.2. Microbial profile and dietary modulation of gut bacterial communities

The overall intestinal microbial community, considering only the most representative taxa, was mainly composed of 3 phyla, 6 classes, 12 orders, 17 families and 16 genera. The profiles of the intestinal microbial communities for each dietary group are shown at phylum, family, and genus level in Fig. 7. In Supplementary Table S3, is reported the list of the most abundant bacterial taxa, their relative abundances and statistical analysis. To calculate alpha rarefaction indices (alpha diversity), samples were normalized at a sequencing depth of 9,300 reads. The administration of contaminant-

containing diets (P and P+MP) significantly decreased the species richness (Chao 1 index), observed OTU number, and phylogenic diversity index of gut microbial communities. On the contrary, the entropy (Shannon diversity index and Eveness) was not affected by dietary treatment (Table 5).

Analysis of beta-diversity revealed an overall effect of diet on microbial communities both in relative abundance (weighted UniFrac) and in presence/absence (unweighted UniFrac) of specific OTUs (Fig. 8). However, the major effect of the diet was observed in terms of relative abundance of taxa. The first principal coordinate PC1 of weighted UniFrac PCoA plot explained up to 77.2% of the variation between individuals (Fig. 8a). Interestingly, control diet C and diet MP (with no contaminants) clustered together and distinctly from other samples (Fig. 8a). Additionally, both in weighted (Fig. 8a) and unweighted (Fig. 8b) UniFrac PCoA, intestinal samples appeared clearly separated from feed ones, thus indicating that observed differences between intestinal bacterial communities were not simply a consequence of undigested feed-associate bacteria that might have been present. The permutational multivariate analysis applying ANOSIM and PERMANOVA tests on UniFrac distance data, wholly confirmed the PCoA results. Multivariate analysis on weighted data revealed significant differences between diet C and MP versus other dietary groups . Results of pairwise comparisons on phylogenetic distances are summarized in Table 6.

Gut microbial community of sea bass was mainly dominated, regardless of the diet, by three phyla: Proteobacteria, Firmicutes, and Actinobacteria (Fig. 7a, Table S3). Among them, Proteobacteria were the most abundant bacteria in all samples with a relative abundance range between 82 and 28%, followed by Firmicutes (68–17%), and Actinobacteria (2.5–0.2%). At the phylum level, the amounts of Proteobacteria and Firmicutes were significantly influenced by the diet. Specifically, fish fed diets P and P+MP (with contaminants) showed higher relative abundance of Proteobacteria (81%), but lower amount of Firmicutes (15%) than the other experimental groups (Fig. 7a, Table S3). On the contrary, gut microbiome of control group (diet C) showed an inverted ratio of Proteobacteria and Firmicutes, with predominance of the latter. Fish receiving diets MP showed intermediate values. As revealed by statistical analysis, three groups were clearly distinguished: one group with an intestinal microbiome dominated by Firmicutes (control), a second group enriched in Proteobacteria (P and P+MP), and an intermediate group (MP). Same pattern of differences in taxa abundance was maintained even at lower taxonomical levels.

Specifically, the most representative classes harbored in intestine of sea bass were Gammaproteobacteria and Bacilli (Fig. 9, Table S3). The lowest percentage of Bacilli was found in fish fed diets P (15%) and P+MP (17%), whereas the same feeding groups showed the highest amount of Gammaproteobacteria (73-76%) (Fig. 9, Table S3). Accordingly, the percentage of bacteria belonging to the orders Bacillales and Lactobacillales were negatively affected, whereas the order of Vibrionales was significantly enriched in intestine of fish fed diets containing contaminants. Similarly, at family level, Bacillaceae, Staphylococcaceae, and lactic acid bacteria (LAB), together with Clostridiaceae,

Peptostreptococcaceae, and Pseudomonadaceae were less abundant in gut of fish fed diet P and P+MP (Fig. 7b). At genus level, in comparison to control, fish fed diet with pollutants had a lower amount of genera *Bacillus*, *Staphylococcus*, *Lactobacillus*, and *Clostridium* (Fig. 7c, Fig. 9). Genus *Streptococcus* was negatively affected by diet P+MP, whereas a reduction in bacteria assigned to genus *Photobacterium* was found in the intestine of fish fed with diet P (Fig. 7c, Fig.8).

3.5. Microbial functional analysis

PICRUSt tool was applied to predict the functional potential of the intestinal microbiome of rainbow trout. Level 3 KEGG orthologue function prediction was used. In accordance with the bacterial gut taxonomic composition data, the microbial metabolic pathway profile of control fish gut was significantly influenced by contaminants in the diet (P, P+MP), not by virgin MPs. Applying a cutoff of 0.2 to the difference in mean proportion, a total of 22 and 28 pathways were significantly different between control and P and P+MP groups, respectively (Fig. 10).

The ingestion of chemical contaminants or contaminated MPS upregulated the abundance of genes responsible for pathways involved in transporters, transcription, secretion, two-component system, energy metabolism, lipopolysaccharide biosynthesis, and flagellar assembly. In contrast, photosynthesis, chlorophyll, cysteine, methionine, and purine metabolism, prokaryotic defense system, and peptidoglycan biosynthesis decreased in the P and P+MP groups. Contaminants alone caused an over-representation (difference in mean proportion > 0.2) of bacterial chemotaxis and a reduction in starch and sucrose metabolism (Fig. 10a), whereas in combination with MPs, they were responsible for enhancing a *Vibrio cholera* biofilm formation pathway (Fig. 10b).

4. Discussion

It is undeniable that plastic pollution has become one of the most pressing environmental issues. Polypropylene (PP), together with polyvinylchloride (PVC), polystyrene (PS), and polyethylene (PE) are the most commonly and frequently found plastics in marine environments.

Particularly in the last decade, the presence of MPs in the marine environment has triggered scientific interest because they are frequently detected in fish **s**pecies destined for direct human consumption (Carbery et al., 2018). Additionally, MPs seem to be capable of absorbing and concentrating organic pollutants, such as pesticides or pharmaceuticals (Hirai et al., 2011; Ogata et al., 2009; Van et al., 2012). This raises concerns about the role of MPs in bioaccumulation and biomagnification of toxic chemicals, which could have a negative effect on fish intestinal microbiota (Hirt & Body-Malapel, 2020). Indeed, the transfer of contaminants from plastic to biota has been demonstrated (Betts, 2008; Teuten et al., 2009). However, we still lack an understanding of the interactive effects of MPs and contaminants, and discordant

results have often been reported about their combined effects, indicating either increased or decreased toxicity (Bellas and Gil, 2020; Guven et al., 2018; Karbalaei et al., 2021).

Therefore, the present study investigated the individual and combined effects of dietary administration of chemicals, pollutants, and PP-MPs on growth performance, immune response, and gut microbial communities of European sea bass, one of the most important species for Mediterranean aquaculture.

During the present feeding trial, all experimental diets were well accepted and no significant differences in survival, growth, and feed efficiency were recorded among groups. Indeed, the effects of plastic exposure on fish growth are likely to be concentration-dependent, with chronic exposure having no effects on fish growth (Critchell and Hoogenboom, 2018).

Our results are consistent with data previously reported in the same species fed with diets containing virgin PVC- or PE-MPs (Espinosa et al., 2019). In agreement with Guven et al. (2018), the combination of MPs and pollutants did not magnify the adverse effects of singularly used chemical contaminants or plastics on fish feeding and growth performance. However, in barramundi (*Lates calcarifer*), MP and pyrene (a polycyclic aromatic hydrocarbon), used alone or in combination, reduced feed intake (Guven et al., 2018). In juvenile large yellow croaker (*Larimichthys crocea*), in contrast, exposure to PS-MPs reduced both lysozyme activity and specific growth rate and significantly increased mortality (Gu et al., 2020). Digestive performance was also reduced in juvenile guppy (*Poecilia reticulata*) after exposure to MPs (Huang et al., 2020).

Although chronic exposure to MPs and associated pollutants does not affect fish growth and digestion, there is evidence showing that accumulation of MPs in the digestive tract can lead to a nonspecific immune response in fish (Ahrendt et al., 2020; Espinosa et al., 2019, 2017; Huang et al., 2020; Jin et al., 2018). In the present study, ingestion of contaminated MPs increased the mRNA levels of *il-6* in the proximal and distal intestine, whereas *tnf-α* transcripts increased solely in the distal intestine. This result agrees with the tendency of fish intestine to present a wider *lamina propria*. The effects of certain contaminants on gut cytokines have been described in mammals, with an increase in proinflammatory cytokines, including *il-6* or *tnf-α* associated with CPF exposure (Li et al., 2019) or organochlorine pesticides (Téllez-Bañuelos et al., 2016). Conversely, virgin MPs and chemical pollutants used singularly did not induce the expression of tested immunity-related cytokines. These data seem to indicate that MPs and contaminants act synergistically in the gut of sea bass to promote inflammation and that such action was evident in the posterior part. This suggests that MPs transfer the contaminants to the posterior intestine instead of allowing them to be absorbed in the anterior intestine. The evidence of a potential "cleaning" effect of plastics was also reported in a study by Gouin et al. (Gouin et al., 2011), who predicted that the bioaccumulation of hydrophobic pollutants associated with MPs could help reduce concentrations in the body.

Different studies have described the role of MPs as vectors of pollutants to marine organisms (Ašmonaitė et al., 2020; Bellas and Gil, 2020) that are able to alter the availability of certain toxicants. The chemical sorption, desorption, and subsequent transfer of chemicals *in vivo* depends on multiple, interconnected factors, including physicochemical properties of particles and contaminants (Ašmonaitė et al., 2020); here, the GI tract is an important organ where chemical desorption and metabolism of particle-bound chemicals take place. Hydrophobic pollutants, such as DDE, CPF, and BP-3, are taken up from the intestinal lumen into the enterocytes via coabsorption with dietary lipids as they dissolve in micelles made of hydrolyzed dietary lipids and bile acids (Andreas Moser and McLachlan, 2001). This absorption process depends on the physiological conditions in the GI tract, the digesta retention time, and desorption of HOCs from ingested plastics. The latter could be rate-limited, limiting the uptake of chemicals into the fish (Mohamed Nor and Koelmans, 2019). Indeed, there is evidence that CPF-contaminated MPs produce an increased immune response in the crustacean *Porcellio scaber*, despite the reduced bioavailability of pollutant (Dolar et al., 2021).

Recently, hydrophobic contaminants were reported to increase proinflammatory cytokines (Li et al., 2019) and to enhance *cyp1a* in intestinal mucosal epithelia (Ašmonaitė et al., 2020). *Cyp1a* is involved in the metabolism of polycyclic aromatic hydrocarbons and other xenobiotics, as well as in the immune response via AhR (Manzella et al., 2018). Ašmonaitė and coauthors described an enhanced effect when pollutants (including chlorpyrifos) were associated to MPs (either glassy polystyrene, rubbery polyethylene, or silica glass particles) rather than when plastics or pollutants were used separately. Their results agree with the inflammatory signs related to hydrophobic pollutants observed in our study, mainly when pollutants were combined with MPs.

Our results are in line with Pedà et al. (Pedà et al., 2016), who described pathological alterations in the intestine of European sea bass fed with virgin or polluted PVC pellets. They found numerous leukocyte infiltrations and an increase in the population of rodlet cells after sixty days of exposure to polluted MP pellets. Although the alterations were observed both in MPs and polluted MPs, the worst condition was in the distal GI of sea bass fed with polluted pellets, which is in line with the histological alterations found in our study. Accordingly, the ingestion of synthetic MPs for 45 days did not induce any noticeable histopathological damage or effect on immune-related genes in gilthead sea bream (Jovanović et al., 2018). High numbers of histopathological lesions, including massive necrosis, infiltration of inflammatory cells, and shedding of villi tips, were also observed in the gut of rainbow trout exposed to high CPF concentrations combined with high polystyrene-MP concentrations (6 µg/L CPF + 300 µg/L PS-MPs) (Karbalaei et al., 2021). However, in the same study, fish treated with MPs or pollutants alone showed a normal histological structure of the gut, except for a decrease in villi diameter as compared to their control. In contrast, some studies reported histopathological lesions in fish, suggesting that ingestion or exposure to virgin MPs may stimulate the immune response (Pirsaheb et al., 2020). For instance, histological lesions, such as leukocyte infiltration or villi cell loss, were

described in the intestine of intertidal fish *Girella laevifrons*, with a dose-response relationship with PS-MPs in the diet (Ahrendt et al., 2020).

These apparently contradictory results might be related to the shape and type of the MPs studied. MPs are usually of irregular shape and may likely cause mechanical abrasions and ulcerations in fish gut mucosa. Espinosa and coauthors (Espinosa et al., 2019) showed an effect for dose and type of MPs on sea bass gut integrity. Exposure to PVC-MPs triggered different signs of intestinal injury only at high levels of dietary inclusion (500 mg PVC-MPs kg^{-1} diet), whereas, regardless of the dose, PE-MPs generated high levels of enterocyte vacuolization in the apical part of the villus.

Exposure to PS-MPs for 28 days significantly increased the protein levels of TNF-α, IFN-γ, TLR4, and IL-6 in the gut of guppy (*Poecilia reticulate*) (Huang et al., 2020). In zebrafish (*Danio rerio*), PS-MPs at a concentration of 1000 μg/L for 14 days increased both protein and transcript levels of IL-1α, IL-1β, and IFN-γ in the intestine, without any histopathological signs in the intestine except for an increase in the volume of gut mucus when exposed to 50 μm PS-MPs (Jin et al., 2018). In our case, *tnf-α* and *il-1β* gene expression were downregulated in the proximal intestine of fish fed with the diet containing virgin MPs.

In addition to MPs, the fish immune system is also susceptible to several contaminants. In the present study, European sea bass was exposed to three chemical pollutants, all of which are known to produce immune responses. For instance, BP-3 has been associated with lower cell viability and increased activity of caspase-3, which is a key mediator of cell apoptosis (Broniowska et al., 2016). The organochlorine pesticides, such as DDE, have been shown to be capable of either decreasing the secretion of NK-stimulatory ILs (IL-2, IL-12 and/or IL-10) and/or increasing secretion of the NK-inhibitory cytokine IL-4 (Beach and Whalen, 2006).

Lastly, exposure to the insecticide CPF has always activated inflammatory and stress responses associated with the induction of inflammatory cytokines in fish. In an *ex vivo* study in rainbow trout (*Oncorhynchus mykiss*), induction of *tnf-α* and *il-6* mRNAs in the liver in response to CPF (20 μg L^{-1}) exposure was described (De Anna et al., 2021). Previously, Wang et al. (2011) found an up regulation of *il-1β* and *il-1r* and *ifn-γ* in spleen and kidney of common carp (*Cyprinus carpio*) after exposure to a high concentration of atrazine, CPF, and a mixture thereof. Actually, many endocrine-disrupting compounds, such as DDE, have been shown to possess both immunosuppressive and inflammatory properties. Endocrine disrupters may modulate the immune system by acting at different levels, including cytokine synthesis by the immune cells (Kuo et al., 2012). Our results indicate that the expression of proinflammatory cytokines *il-6* and *tnf-α* was significantly increased after exposure to chemical pollutants paired with MPs. Furthermore, inflammation processes in the gut are often related to shifts in the intestinal bacterial communities (dysbiosis) (Ni et al., 2017), a condition that can seriously affect the host's health.

Like in mammals, fish intestinal microbiota plays a pivotal role in the digestion/absorption of nutrients, synthesis of functional metabolites, such as short-chain fatty acids, and in the immune response (Ghanbari et al., 2015; Gómez and Balcázar, 2008; Nicholson et al., 2012). Our metagenomic analysis showed alterations in sea bass gut microbiome composition up to the genus taxonomical level and changes in bacterial species richness following ingestion of pollutants or polluted MPs. The Chao 1 index decreased in gut microbiota of fish exposed to dietary pollutants, but no difference in bacterial community diversity was found among the four experimental groups. In line with our findings, the intake of nanoparticles or MPs of polystyrene in yellow croaker and zebrafish reduced gut bacterial richness without changing the biodiversity (Shannon index) (Gu et al., 2020; Wan et al., 2019). Furthermore, the PCoA analysis in the present study revealed that both pollutant treatments (diets C and D) significantly altered gut microbiome community structures that clearly separated them from the control group.

Although the research linking MPs with the fish gut microbiome is still limited, all studies focused on this topic have common denominators, i.e., dysbiosis, altered beta diversity, and reduced alpha diversity of gut microbial communities (Fackelmann and Sommer, 2019; Gu et al., 2020; Huang et al., 2020; Jin et al., 2018; Qiao et al., 2019; Wan et al., 2019). The only exception is a study conducted solely in cultured European sea bass in which neither dysbiosis nor altered bacterial diversity was observed after exposure to MPs (Caruso et al., 2018). This agrees with our study, although we observed a slight decrease in Bacillus and LAB.

In the present study, irrespective of the diet, the gut microbiota of European sea bass was dominated by Firmicutes, Proteobacteria, and Actinobacteria phyla, thus confirming what was reported in previous microbiome studies in this species (Carda-Diéguez et al., 2014; Gatesoupe et al., 2016; Parma et al., 2019; Rimoldi et al., 2020). However, their relative abundances were significantly changed by ingesting pollutants. In particular, in comparison to the controls, we found a reduction in Firmicutes and an increase in Proteobacteria in the intestine of fish fed both polluted diets. This finding is in line with the described increase in Proteobacteria phylum in the gut microbiome of mice induced by CPF (Liang et al., 2019). Furthermore, a decrease in Bacillaceae, Staphylococcaceae, Lactobacillaceae, Streptococcaceae, Clostridiaceae, Peptostreptococcaceae, and Pseudomonadaceae families was observed in the same fish groups. In contrast, the dietary inclusion of virgin MPs did not cause relevant changes in microbiome composition as compared to controls. Only a slight decrease in Firmicutes and a tendency for Proteobacteria to increase can be perceived. These results are in line with those reported for yellow croaker (*Larimichthys crocea*) and adult zebrafish (*Danio rerio*) exposed to PS-MPs (Gu et al., 2020; Jin et al., 2018; Qiao et al., 2019). In agreement with our findings, the relative abundance of Firmicutes in the gut was reduced and Proteobacteria increased in zebrafish exposed to the organic contaminant 9-nitroanthracene, alone or in combination with PE-MPs, showing an exacerbated effect in the latter case (Zhang et al., 2021).

Actually, our metabarcoding data are in agreement with the upregulation of cytokines in intestine and also with observed gut morphology as Proteobacteria phylum is considered a microbial signature of gut inflammation (Shin et al., 2015). As proposed by Ni et al. (2017), the inflammation processes and derived oxidative stress could promote the growth of Proteobacteria and other taxa that can cope with such a hostile host environment. In addition, members of Firmicutes phylum are known for making short-chain fatty acids such as butyrate, acetate, and propionate, which are the end products of fiber fermentation (Refstie et al., 2005; Wong et al., 2006). The short-chain fatty acids, especially butyrate, have anti-inflammatory potential and play a key role in regulating the host immune system (Canani et al., 2011; Gonçalves et al., 2018; Lazar et al., 2018; Rimoldi et al., 2016; Terova et al., 2016). Therefore, Firmicutes are generally considered beneficial and are associated with a healthy intestinal epithelium and therefore a decrease in levels of this microorganism is not desirable. Indeed, Firmicutes phylum includes different genera of LAB, such as *Streptococcus*, *Lactobacillus*, and *Carnobacterium*, which are often used as probiotics in cultured fish feeding, like in other vertebrates (Kim et al., 2012; Ringø and Gatesoupe, 1998). In our study, the ingestion of pollutants associated or not to MPs markedly affected the beneficial LAB. The amount of *Lactobacillus* and *Streptococcus* was significantly reduced in the intestine of fish that ingested pollutants, but it was only slightly affected by MPs alone.

It is well known that LAB, in addition to producing short-chain fatty acids, have an active role in mounting a host defense against pathogen invasion. They are able to produce antimicrobial compounds such as lactic acid, hydrogen peroxide, and bacteriocins and release biosurfactants that prevent invading microorganisms from adhering to the intestinal surface (Bermudez-Brito et al., 2012). In contrast, Gu and colleagues (2020) found an enrichment of LAB in the intestine of yellow croaker exposed to PS nanoplastics. However, their finding collided with the increased number of potential pathogens observed. In our study, the addition of virgin MPs did not affect the gut as severely as pollutants. An enrichment in Vibrionales, an order including several potentially pathogenic bacteria, was found in fish fed pollutants (either with or without MPs) when compared to the control fish. Nevertheless, this did not correspond to an increase in Vibrionaceae or *Vibrio*. The lack of correspondence might be due to a taxonomical assignment failure.

The genus *Bacillus*, another member of Firmicutes, was scarcely represented in the intestinal microbiome of European sea bass that ingested MPs and/or contaminants. Like LAB, several *Bacillus* species represent the most commonly used probiotics in aquaculture. The use of Bacillus as probiotics in aquaculture is a relatively recent development; nevertheless, their role in mitigating pathogenic bacteria and in enhancing the immunity of aquaculture fish species is overwhelming (Kuebutornye et al., 2020).

The encoded metabolic pathway profile of microbial communities reflected the changes in gut microbiota composition found in fish fed pollutants containing diets (P, P+MP). In agreement with the reduction in beneficial bacterial taxa belonging to Fimicutes phylum, PICRUSt analysis predicted a decrease in starch and carbohydrate metabolism as well

as in cysteine, methionine, and purine metabolism. These metabolic pathways usually correlated positively with the abundance of Firmicutes. The two-component and secretion system pathways were instead greatly upregulated. The two-component signal transduction system constitutes a major strategy of microbes for controlling their expression profiles in response to changes in the environment; it enables bacteria to detect physical and/or chemical changes (Liu et al., 2019). Bacterial secretion systems are membrane protein complexes used by pathogenic bacteria to secrete their virulence factors (mainly of proteins) to invade the host cells. This would agree with the gut inflammatory signs and microbiome profiles observed in fish fed diets containing chemical pollutants. Likewise, an increase in lipopolysaccharide biosynthesis could be related to the inflammatory status of intestine and to dysbiosis. In fact, the increased Proteobacteria levels are associated with the production of lipopolysaccharides that triggered inflammation, disrupted the intestinal mucosal barrier, and increased intestinal permeability (Shin et al., 2015). The situation worsened in the P+MP group, in which an improvement of biofilm formation related to *Vibrio cholera* species was predicted. Interestingly, genus *Vibrio* have been identified as members of the plastisphere (a term coined to indicate the microbial community that adheres to MPs) (Fackelmann and Sommer, 2019; Zettler et al., 2013), and this was in line with the enrichment in Vibrionales found in the intestines of these fish.

5. Conclusions

In summary, our results clearly demonstrate that PP-MPs and chemical pollutants act synergistically to generate inflammation in the intestine of European sea bass. The upregulation of cytokine *il-6* and *tnf-α* gene expression in this tissue, the increase in rodlet cells and lymphocytes in submucosa, and the increase in *lamina propria* width, mainly in the proximal, but also in the distal intestine, are commonly associated with the inflammatory response. Significant changes in richness, composition and beta diversity of the gut microbiome were also observed as a consequence of ingesting contaminants (P and P+MP diets). These perturbations in gut microbial communities, including the reduction in beneficial bacteria genera (LAB) and the increase in potentially pathogenic microorganisms (Proteobacteria and Vibrionales), were undeniable indicators of intestinal dysbiosis, which in turn confirmed the signs of inflammation caused by pollutants, especially when combined with MPs. The results obtained in this study provide, therefore, new insights into the potential risks of ingesting MPs as pollutant carriers in marine fish.

Data availability

The raw sequencing data are available in the European Nucleotide Archive (EBI ENA) public database under the accession project code: PRJEB41533

CRediT authorship contribution statement

Daniel Montero: conceptualization, data curation, and writing. **Simona Rimoldi, Silvia Torrecillas:** experimental investigation, methodology, data curation, and writing. **Jorge Rapp, Federico Moroni:** experimental investigation, methodology, formal analysis. **Alicia Herrera, May Gómez:** conceptualization and experimental investigation, review and editing. **Álvaro Fernández-Montero:** review. **Genciana Terova:** data curation, and writing—review and editing. All authors read and approved the final manuscript.

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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Tables

Table 1
Concentration of pollutants in the experimental feeds

		Concentration (ng/g of feed)		
Pollutant	**Diet**	**Mean**	**Standard deviation**	**Median**
3- Benzophenone (BP-3)	C	2.8	9.9	26.9
3- Benzophenone (BP-3)	MP	16.8	10.2	11.9
3- Benzophenone (BP-3)	P	156.4	4.8	154.9
3- Benzophenone (BP-3	MP+P	1064.9	15.2	1062.0
Chlorpyrifos (CFP)	C	0.0	0.0	0.0
Chlorpyrifos (CFP)	MP	0.0	0.0	0.0
Chlorpyrifos (CFP)	P	42.0	5.5	39.2
Chlorpyrifos (CFP)	MP+P	204.3	8.3	203.6
DDE-p,p'	C	3.0	0.2	3.0
DDE-p,p'	MP	20.8	0.9	20.8
DDE-p,p'	P	297.6	10.6	296.4
DDE-p,p'	MP+P	2630.7	77.6	2590.2

Table 2
Nucleotide sequences of the primers used for in vitro synthesis of standard mRNAs.

Gene	**Symbol**	**GenBank Acc. n.**	**Primer nucleotide sequence (5'-3')**
Interleukin-6	*il-6*	AM490052	F: gtaatacgactcactatagggACTT CCAAAACATGCCCTGA R: CCGCTGGTCAGTCTAAGGAG
Interleukin-1β	*il-1β*	AJ269472	F: gtaatacgactcactatagggTGC CATGGAGAGACTGAAGG R: ACTGGGTGTACGGTCCAAGT
Interleukin-10	*il-10*	AM268529	F: gtaatacgactcactatagggCAGT GCTGTCGTTTTGTGGA R: TCACTCTTGAGCTGGTCGAAG
Tumor necrosis factor alpha	*tnf-α*	DQ070246	F: gtaatacgactcactatagggCACTA CACACTGAAGCGCAT R: CTGTAGCTGTCCTCCTGAGC

Table 3
Nucleotide sequences of primers and probes used for qPCR.

Gene	Symbol	Primer nucleotide sequence (5'-3')
Interleukin-6	*il-6*	F: gcctgctcacttacacagctcttc R: tcttgaaactgtggccctctga Probe: 6-Fam-agaaggagtcccccagctcgatccg-BHQ-1
Interleukin-1β	*il-1β*	F: ttgtgtttgagcgcggaaca R: tgtcggtcacgctgcattg Probe: 6-Fam-ctccaacagcgcagtacagcaagcga-BHQ-1
Interleukin-10	*il-10*	F: agcgctgctagaccagactgt R: cggcagaaccgtgcttagat Probe: 6-Fam-agacactttaagg[illegible]cccgttcgcttgc-BHQ-1
Tumor necrosis factor alpha	*tnf-α*	F: aaaccggcctctacttcgtcta R: tcccgcactttcctcttcac Probe: 6-Fam-agccagcgtcgttcagagtctcc-BHQ-1

Table 4
Growth performances and feed utilization of European sea bass fed the experimental diets. Values are expressed as mean ± SD (n = 3 tanks). Initial body weight (W_i), final body weight (W_f), weight gain (WG), specific growth rate (SGR), condition factor (K), and feed conversion ratio (FCR).

Diet	W_i (g)	W_f (g)	WG (g)	K	SGR	FCR
C	77.9 ± 11.0	133.2 ± 19.7	71.0 ± 8.4	1.33 ± 0.03	0.89 ± 0.08	1.26 ± 0.02
MP	79.5 ± 13.5	138.1 ± 22.[illegible]	73.7 ± 12.7	1.32 ± 0.03	0.92 ± 0.12	1.28 ± 0.13
P	81.3 ± 13.1	127.8 ± 19.[illegible]	57.2 ± 5.5	1.28 ± 0.02	0.75 ± 0.06	1.66 ± 0.20
P+MP	80.0 ± 13.9	136.[illegible] ± 18.4	70.5 ± 6.2	1.29 ± 0.04	0.89 ± 0.06	1.39 ± 0.03

Table 5
Alpha diversity metrics values (mean ± SD, rarefied at 9300 reads) of gut microbial community of European sea bass fed diet C (n = 6), diet MP (n = 6), diet P (n = 5), and diet P+MP (n = 5). The mean values with different superscript letters in the same row are significantly different ($p<0.05$).

Item	C	MP	P	P+MP
Observed_OTUs	458 ± 28^{a}	422 ± 67^{a}	267 ± 209^{ab}	251 ± 136^{b}
Chao1	564 ± 27^{a}	504 ± 63^{a}	326 ± 234^{b}	305 ± 161^{b}
Shannon	5.9 ± 0.3	5.8 ± 0.4	4.8 ± 1.4	5.0 ± 1.1
Eveness	0.67 ± 0.03	0.67 ± 0.04	0.61 ± 0.08	0.64 ± 0.07
Faith's PD	7.68 ± 0.52^{a}	6.80 ± 0.75^{ab}	4.85 ± 3.12^{b}	4.78 ± 2.06^{b}

Table 6

Results of non-parametric multivariate analysis ANOSIM and PERMANOVA on weighted and unweighted UniFrac data of intestinal microbiomes of fish fed with different experimental diet. Diet C (n = 6), diet MP (n = 6), diet P (n = 5), and diet P+MP (n = 5). Significant *p*-values ($p \leq 0.05$) are in bold.

ANOSIM		
Unweighted		
No differences	*p*-value > 0.05	
Weighted	R	*p*-value
C vs MP	-0.05	0.682
C vs P	0.59	**0.004**
C vs P+MP	0.49	**0.010**
MP vs P	0.37	**0.038**
MP vs P+MP	0.19	0.124
P vs P+MP	-0.13	0.926
PERMANOVA		
Unweighted	pseudo-F	*p*-value
C vs MP	1.04	0.397
C vs P	2.76	0.061
C vs P+MP	2.31	**0.012**
MP vs P	2.32	0.094
MP vs P+MP	1.91	**0.045**
P vs P+MP	0.38	1.000
Weighted		
C vs MP	1.14	0.306
C vs P	10.61	**0.009**
C vs P+MP	9.37	**0.005**
MP vs P	5.15	0.058
MP vs P+MP	3.99	0.077
P vs P+MP	0.17	0.748

Figure captions

Fig. 1 Detailed micrograph showing the size and shape of the microplastics obtained after separation. The microplastics were separated by sieving to obtain the 0.7-1mm fraction.

Fig. 2 Detailed micrograph of anterior gut (a-d) and main morphological findings (e-g) stained with May-Grünwald Giemsa. Observe the wider *lamina propria* (→) in fish fed contaminated fish feed (b, d) especially when microplastics (MPs) are combined with the contaminants (d). Scale bar 100 μm. (e) Detailed micrograph of anterior gut of fish fed diets with contaminants, stained with May-Günwald Giemsa. Observe the high presence of rodlet cells (→) on the fold basal area and along the fold. Scale bar 50 μm. (f, g) Detailed micrographs of anterior gut of fish fed fed diets with contaminants, observe the concentrated areas of lymphocytes in the submucosa (→). Scale bar 50 μm.

Fig. 3 Detailed micrograph of the main morphological patterns observed for the fish preileorectal valve gut segment (a, b) and post ileorectal valve gut segment (c, d) stained with May-Grünwald Giemsa. (a) Corresponds to fish fed control and microplastics (MPs) diets and (b) corresponds to fish diets with contaminants or with MPs plus contaminants. There was a trend to wider lamina propria on fish fed contaminants, however not as much evident as on anterior gut. Observe the focus of lymphocytes (→) found only in fish fed contaminated feed (b). Scale bar 50 μm. (c) Corresponds to postileorectal valve gut segment fish fed control and microplastics (MPs) diets. Observe a higher level of supranuclear vacuolization (→) which was observed mainly in fish fed control diet. (d) Corresponds to fish diets with contaminants or with MPs plus contaminants. There was a trend to wider lamina propria on fish fed contaminants, however as observed in posterior gut, it was not as much evident as on anterior gut. Observe the higher density of lymphocytes (→) in the lamina propria observed only in fish fed contaminated fish feed (b). Scale bar 50 μm.

Fig. 4 Expression levels (mean ± SD, n = 6) of *il-1β*, *il-6*, *il-10*, and *tnfα* genes in proximal and distal intestine of European sea bass (*D. labrax*) fed for 60 days with different experimental diets. Different letters indicate significantly differences within the same tissue ($p < 0.05$).

Fig. 5 Mean relative abundance (%) (n=3) of the most prevalent bacterial taxa in experimental feeds at phylum (a), family (b), and genus (c) level. Only bacteria with an overall abundance ≥ 0.5% were reported. Bacteria with lower abundance were pooled together and indicated as "Others".

Fig. 6 Bubble plot of relative abundance (%) of the most prevalent bacterial genera associate to experimental feeds. Different superscript letters indicate significant difference among mean values on the same row ($p < 0.05$).

Fig. 7 Mean relative abundance (%) of the most prevalent bacterial taxa in gut mucosa of European sea bass fed diet C (n = 6), diet MP (n = 6), diet P (n = 5), and diet P+MP (n = 5) at phylum (a), family (b), and genus (c) level. Only

bacteria with an overall abundance ≥ 0.5% were reported. Bacteria with lower abundance were pooled together and indicated as "Others".

Fig. 8 Beta diversity metrics. Principal coordinate analysis (PCoA) of weighted (a) and unweighted (b) Unifrac distances of gut microbial communities associated to different diet. The figures show the bi-dimensional plot of individual fish and feed samples according to their microbial profile.

Fig. 9 Bubble plot of relative abundance (%) of the most prevalent bacterial genera in gut mucosa. Different superscript letters indicate significant difference among mean values on the same row ($p < 0.05$).

Fig. 10 Extended error bar graphs, KEGG level 3 significant functional pathways of sea bass gut microbiome.

Online resources

Table S1: List of OTUs found and their frequencies in all analysed samples (gut mucosa + experimental feeds)

Fig. S1. Alpha diversity metrics. Rarefaction curves of chao1 index in feed and gut mucosa samples.

Table S2 Relative abundance (mean ± SE) of the most prevalent taxa associate to experimental feeds. Different superscript letters indicate significant difference among mean values on the same row ($p < 0.05$).

Table S3 Relative abundance (mean ± SE) of the most prevalent taxa found in gut mucosa of sea bass fed four experimental diets. Different superscript letters indicate significant difference among mean values on the same row ($p < 0.05$).

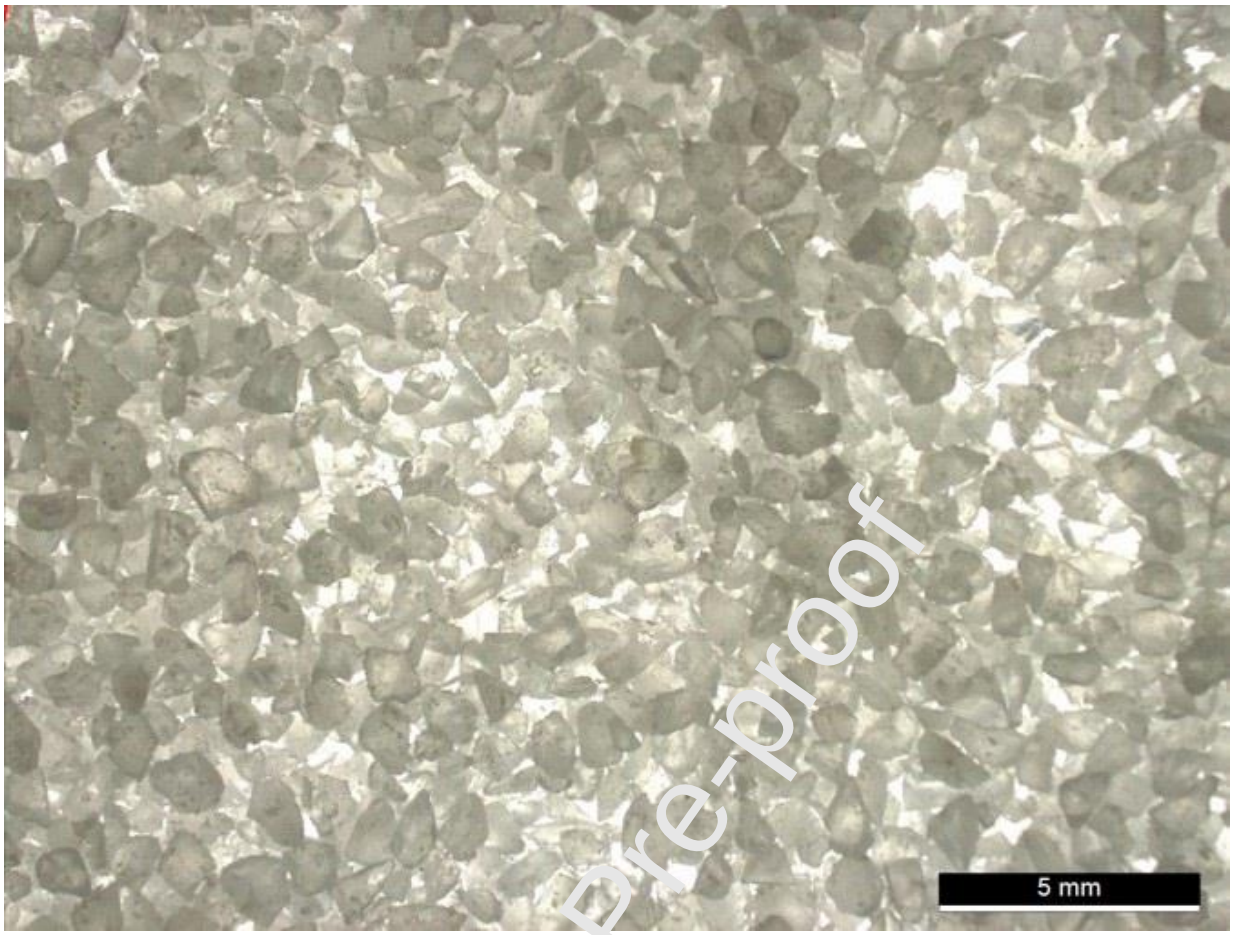


Fig. 1

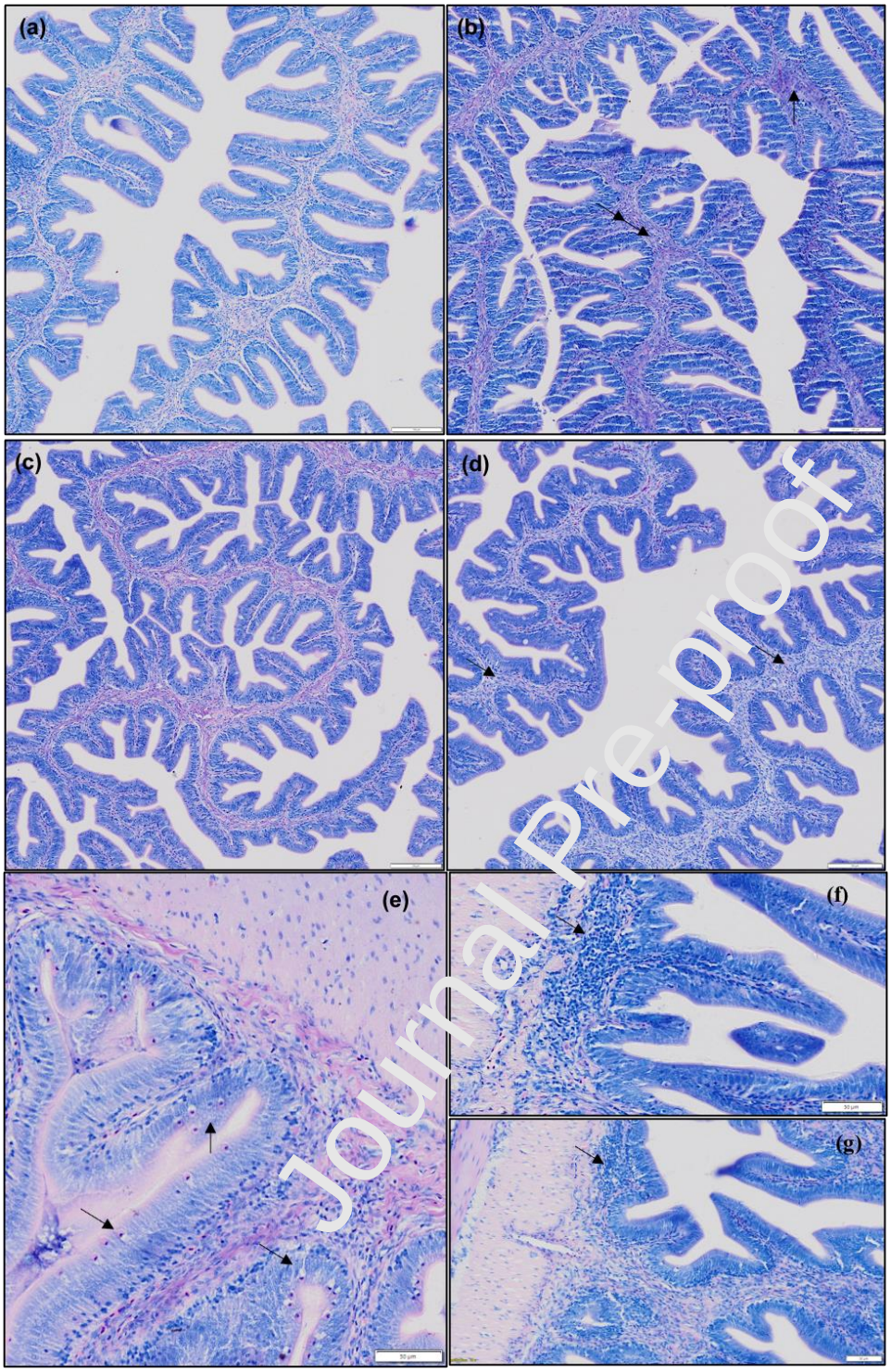
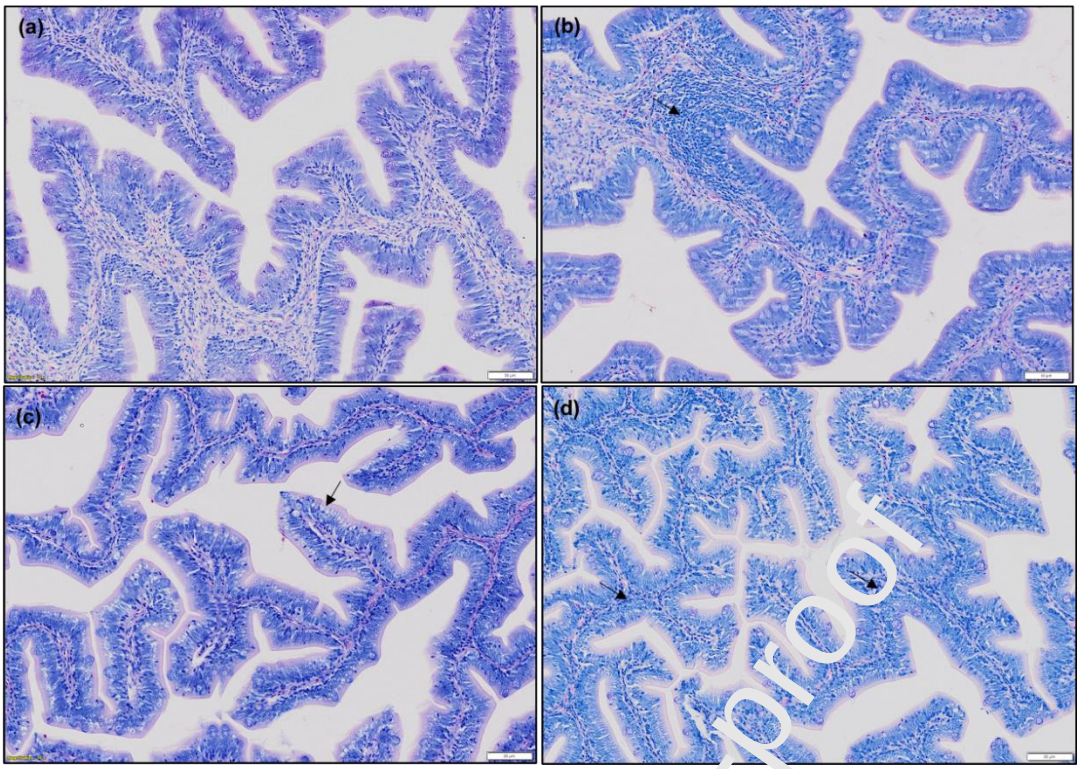


Fig. 2



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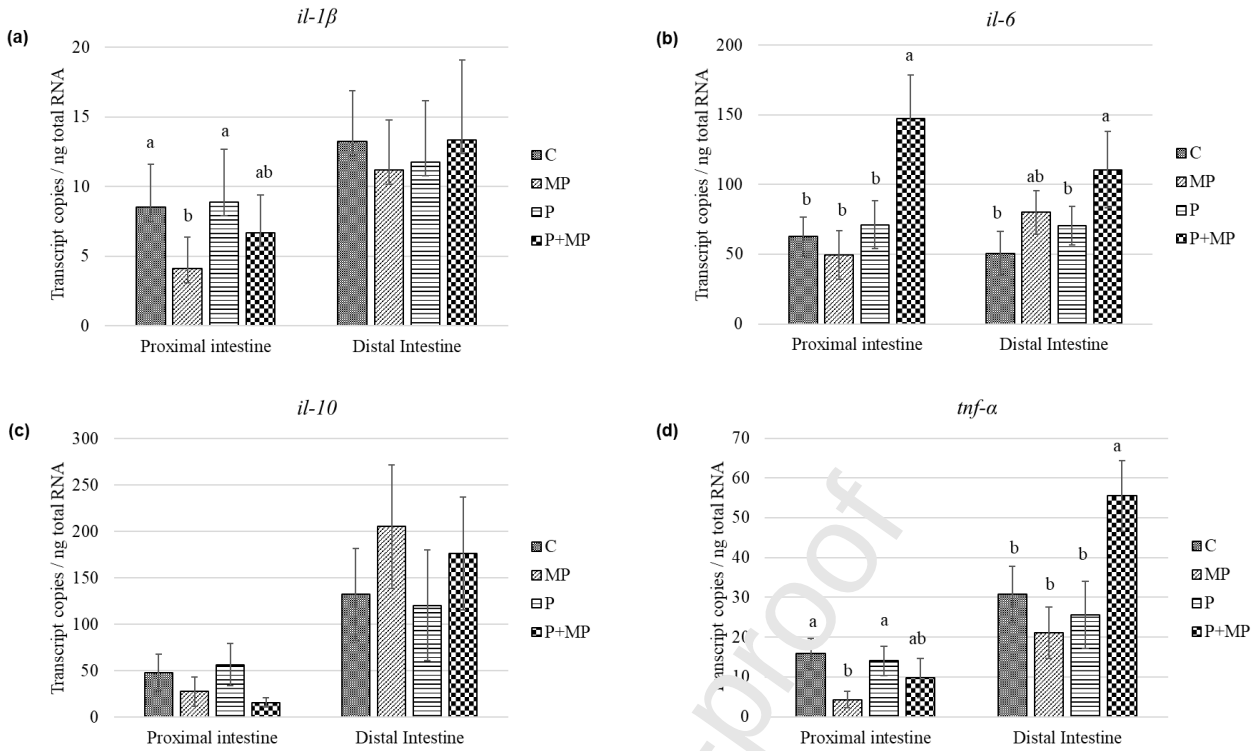


Fig. 4

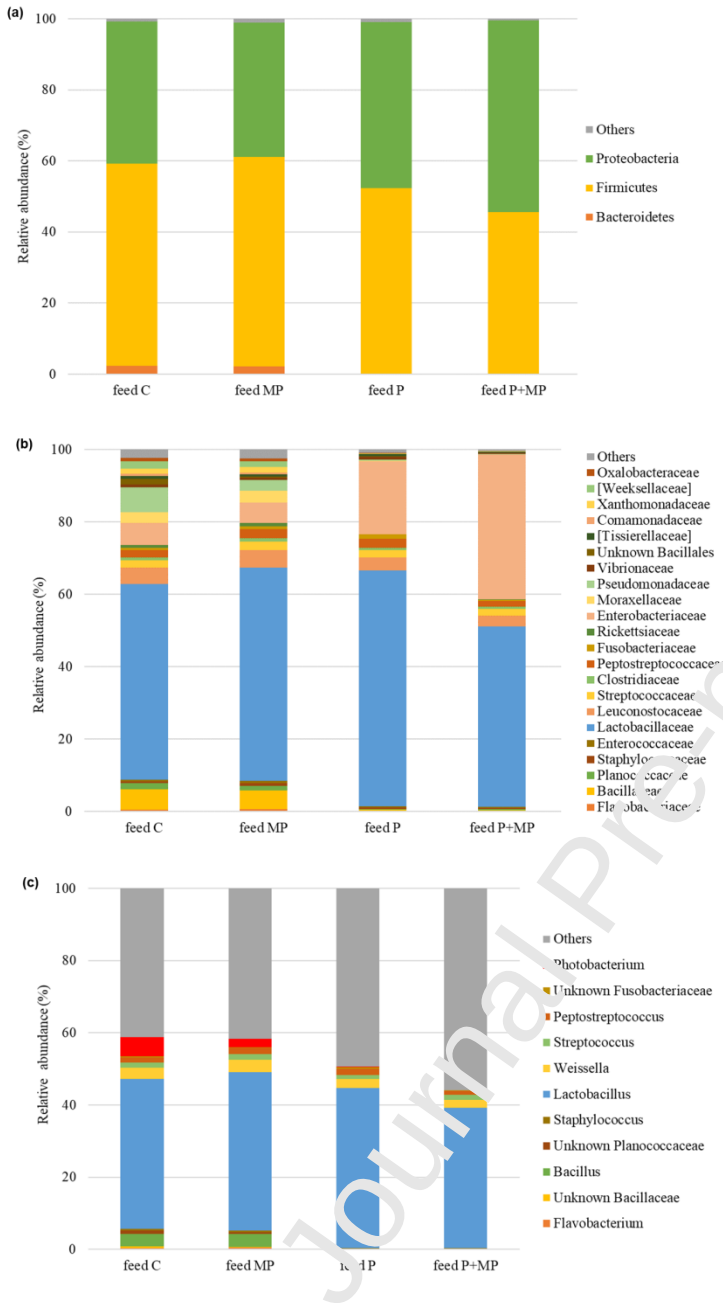

(a)
100
80
60
40
20
0
Relative abundance (%)
feed C
feed MP
feed P
feed P+MP
Others
Proteobacteria
Firmicutes
Bacteroidetes
(b)
Relative abundance (%)
Others
Oxalobacteraceae
[Weeksellaceae]
Xanthomonadaceae
Comamonadaceae
[Tissierellaceae]
Unknown Bacillales
Vibrionaceae
Pseudomonadaceae
Moraxellaceae
Enterobacteriaceae
Rickettsiaceae
Fusobacteriaceae
Clostridiaceae
Leuconostocaceae
Lactobacillaceae
Enterococcaceae
(c)
Relative abundance (%)
Others
Photobacterium
Unknown Fusobacteriaceae
Peptostreptococcus
Streptococcus
Weissella
Lactobacillus
Staphylococcus
Unknown Planococcaceae
Bacillus
Unknown Bacillaceae
Flavobacterium

Fig. 5

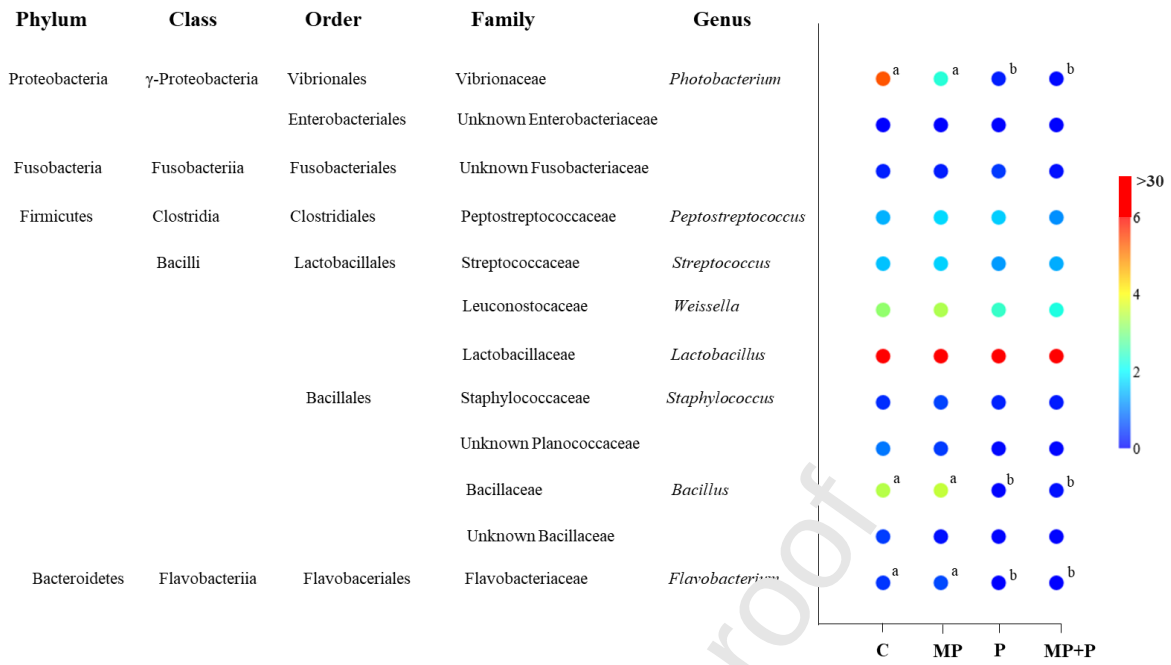
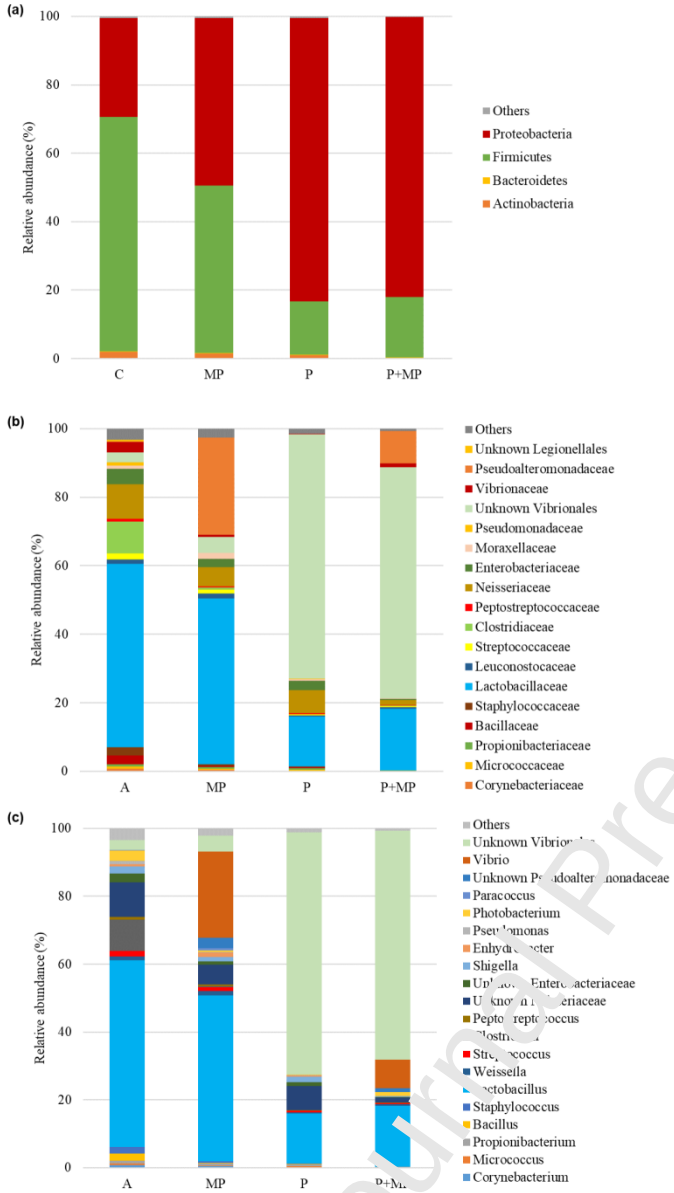


Fig. 6



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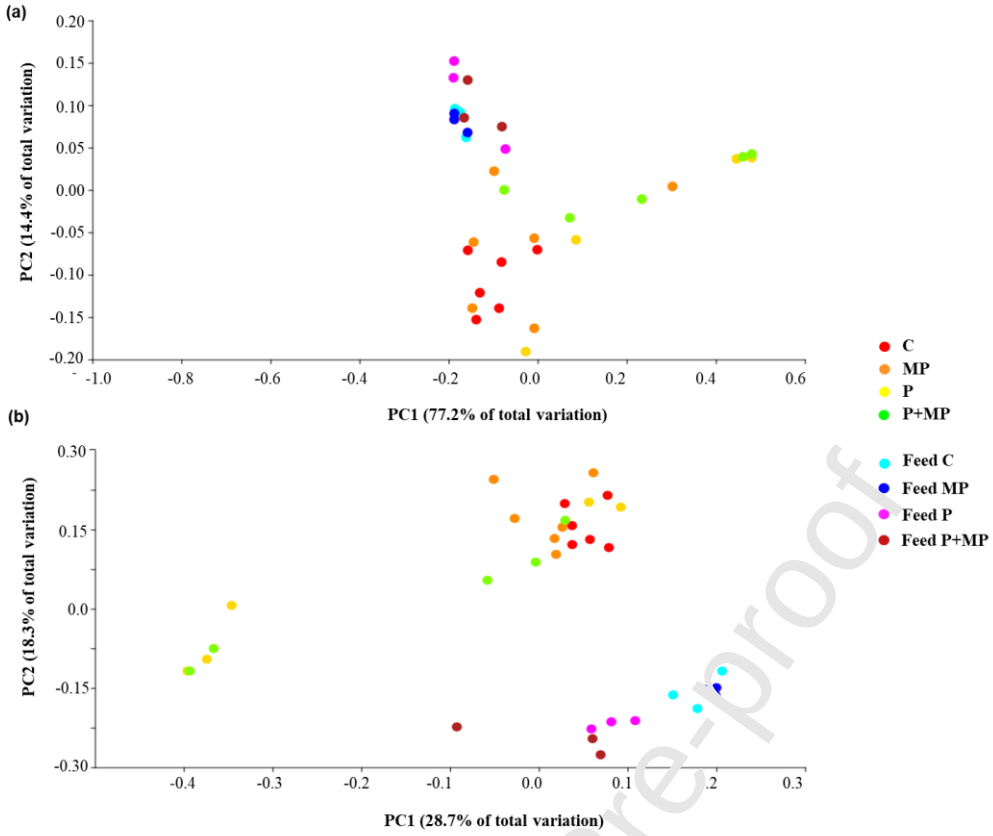

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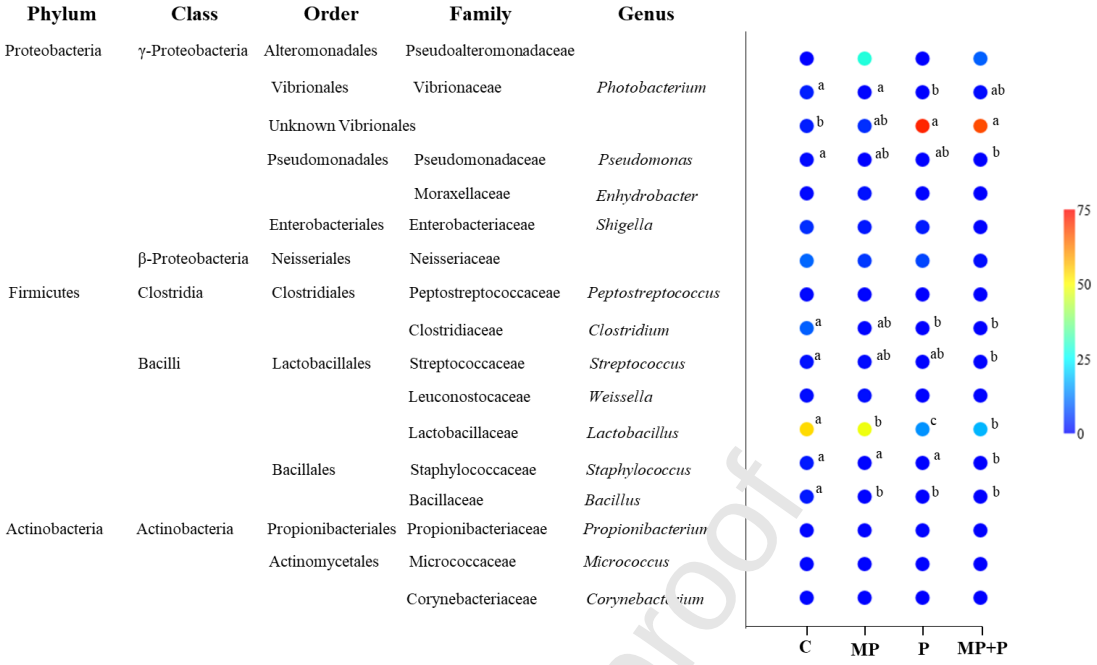


Fig. 9

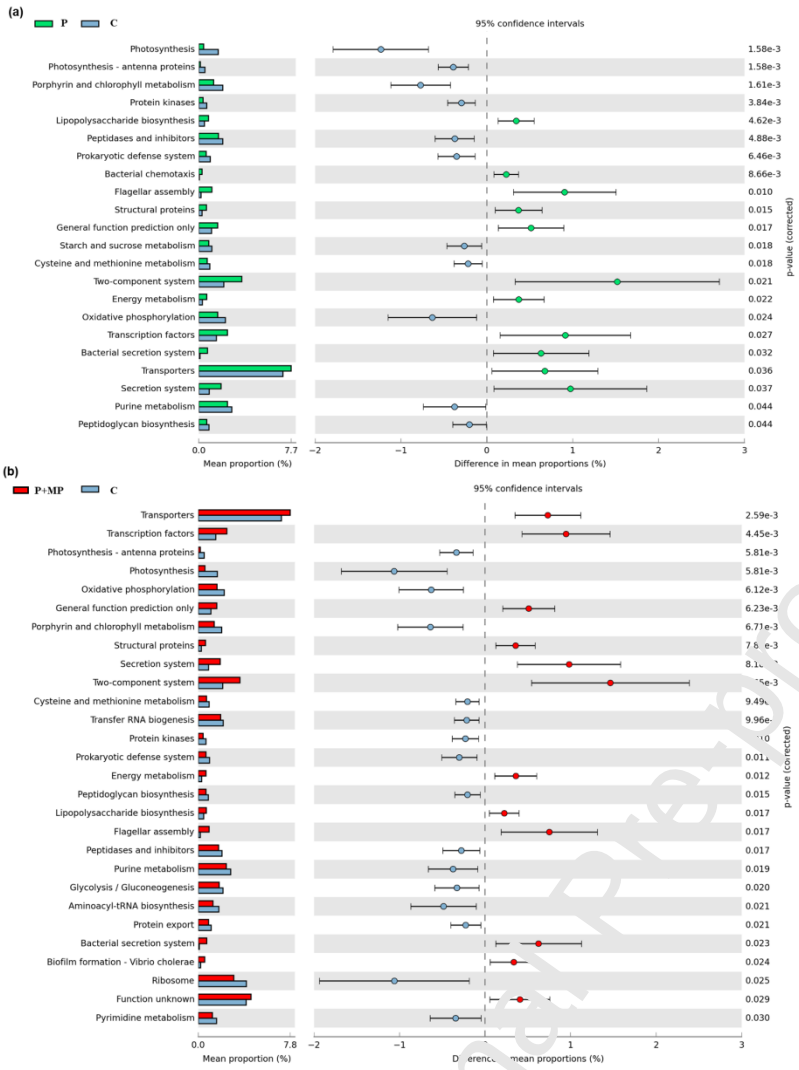


Fig. 10

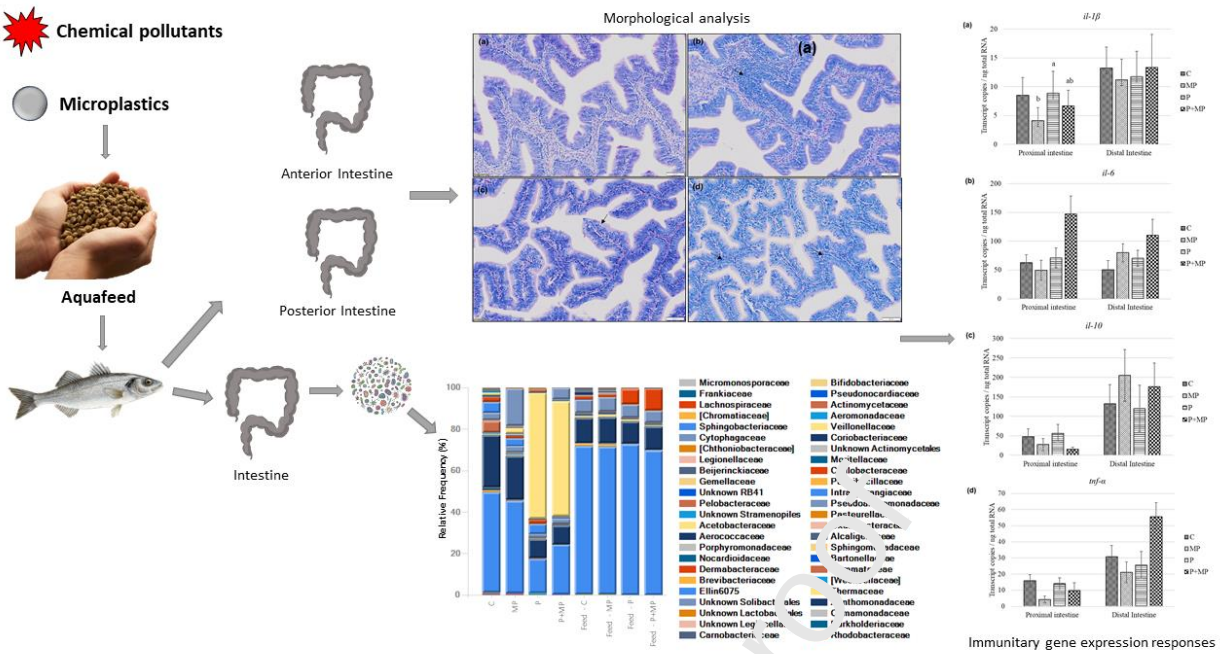
CRediT authorship contribution statement

Daniel Montero: conceptualization, data curation, and writing. **Simona Rimoldi, Silvia Torrecillas:** experimental investigation, methodology, data curation, and writing. **Jorge Rapp, Federico Moroni:** experimental investigation, methodology, formal analysis. **Alicia Herrera, May Gómez:** conceptualization and experimental investigation, review and editing. **Álvaro Fernández-Montero:** review. **Genciana Terova:** data curation, and writing—review and editing. All authors read and approved the final manuscript.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Immunitary gene expression responses

Graphical abstract

Highlights

- There is a synergic action of polypropylene microplastics (MPs) and chemical pollutants.
- MPs and chemical pollutants induced an inflammatory-like response in fish intestine.
- Ingestion of polluted MPs caused significant changes in fish gut microbiome.
- MPs act as pollutant carriers in marine fish.