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Natural-based solutions to mitigate dietary microplastics side effects in fish

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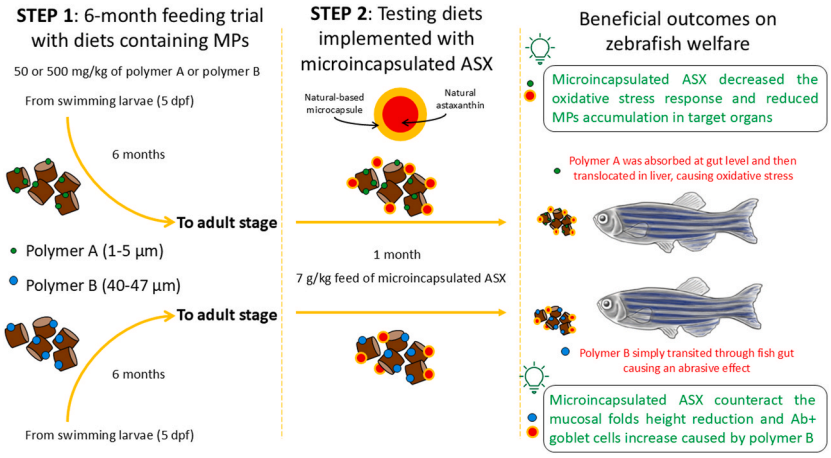
HIGHLIGHTS

- Microencapsulated ASX mitigated side effects of dietary MPs different in size in fish.
- Microencapsulated ASX restored gut epithelium affected by the 40–47 μm MP transit.
- Microencapsulated ASX reduced oxidative stress caused by 1–5 μm MP sited in liver.
- Starch in the microcapsules' wall promoted MPs coagulation in fish gut.
- Coagulation in gut reduced MPs absorption and consequent transfer to fish organs.

GRAPHICAL ABSTRACT



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ABSTRACT

Dietary microplastics (MPs) can be consumed by fish, crossing through the gastrointestinal tract. MPs smaller than 20 μm can easily translocate to other organs, such as liver, commonly triggering oxidative stress in fish. Given the current unlikelihood of their short-term elimination, strategies to mitigate MPs-related issues on fish are of considerable interest to the scientific community. In the present study, to reduce both the dietary MPs-induced oxidative stress and the accumulation of MPs, the effectiveness of microencapsulated astaxanthin (ASX) was evaluated in zebrafish (*Danio rerio*). Specifically, zebrafish were reared from larvae to adults (6 months) and fed diets containing MPs different in range-size (polymer A: 1–5 μm; polymer B: 40–47 μm) at different concentrations (50 or 500 mg/kg). After this period, fish from each experimental group were divided in two sub-groups that were fed, for an additional month, with the previous diets or with the same diets containing implemented with microencapsulated ASX (7 g/kg), respectively. Results showed that microencapsulated ASX

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was able to counteract the negative effects caused by MPs different in size. Particularly, in zebrafish fed diets containing polymer B microbeads, microencapsulated astaxanthin was able to restore the intestinal epithelium, affected by the abrasive role of MPs during gut transit. Differently, in zebrafish fed diets containing polymer A microbeads, absorbed at intestinal level and translocated mainly to the liver, the microencapsulated ASX decreased the oxidative stress response and reduced the MPs accumulation in target organs due to the antioxidant and the coagulant properties of the ASX and microcapsules wall, respectively. Taken together, the results highlighted that the aquafeeds' implementation with microencapsulated astaxanthin is a prospective tool to prevent MPs-related issues in fish.

1. Introduction

Microplastics (MPs; plastics size <5 mm) are globally diffused (Shahul Hamid et al., 2018; Tang et al., 2023) and have been detected in most living organisms, including aquatic ones (Ma et al., 2020; Oliveira et al., 2020). The aquaculture sector is also affected by this emerging pollutant (Chen et al., 2021; Miao et al., 2023) since MPs can easily reach the farmed fishes (Dehm et al., 2022; Wu et al., 2023), mainly through the water or the feed (Alak et al., 2023a,b; Castelvetro et al., 2021; Hanachi et al., 2019; Wu et al., 2020). Particularly, as regards aquafeed, the intrinsic MPs contamination of each single ingredient, as well as the processing methods and packaging used, are potential sources of MPs exposure to the farmed animals (Castelvetro et al., 2021; Lusher and Covernton, 2022; Mahamud et al., 2022; Walkinshaw et al., 2022; Wang et al., 2022).

To date, several studies have pointed out that size, shape, concentration and chemical features are key factors in determining MPs toxicity in fish which, in turn, may differently react in relation to their life-cycle stage and the species considered (Alak et al., 2023; Bobori et al., 2022; Pannetier et al., 2020; Pirsaheb et al., 2020; Wang et al., 2020; Zhang et al., 2022). Moreover, the increase in water temperature resulting from climate change may enhance the toxicity of MPs in fish (Senol et al., 2023). In addition, fish hardly recognise MPs once they are implemented in aquafeed (Rainieri et al., 2018; Xiong et al., 2019), leading to a transit in the gastrointestinal tract and, depending on the MPs features (Bhagat et al., 2020; Cattaneo et al., 2023), to a consequent absorption at the intestinal level (De Sales-Ribeiro et al., 2020; Lu et al., 2016). Particularly, it has been demonstrated that MPs larger than 20 μm are unable to be absorbed at fish intestinal level and, thus, cannot be translocated to other tissues or organs (Lu et al., 2016; Qiao et al., 2019a; Su et al., 2019). Differently, smaller MPs (*i.e.* 44 nm and 1–5 and 15 μm) (Brandts et al., 2022; Cattaneo et al., 2023) are easily absorbed at intestinal level and transferred to other organs, especially liver (Abbasi et al., 2018; Zitouni et al., 2021) or the muscle (Di Giacinto et al., 2023; Makhdoumi et al., 2021; Zeytin et al., 2020), raising concerns also for human health.

Due to the role of liver in trapping absorbed MPs, one of the most detected disorder in fish exposed to MPs is oxidative stress (Capó et al., 2021; Cattaneo et al., 2023; Espinosa et al., 2019; Iheanacho et al., 2023; Köktürk et al.; Xia et al., 2020; Xiao et al., 2023; Yedier et al., 2023). In fish, oxidative stress can result in impairment of cellular processes, including protein denaturation, lipid peroxidation, and apoptosis (Hoseinifar et al., 2020; Lesser, 2006); all these effects have been demonstrated to be related to the size and concentration of dietary MPs as well as to the exposure time. In fact, the presence of dietary MPs in the fish hepatic parenchyma generally results detectable after about 30–45 days of administration (Cattaneo et al., 2023; De Sales-Ribeiro et al., 2020).

Since a short-term complete disappearance of MPs pollution is currently utopic, mitigation strategies able to preserve fish welfare and to potentially reduce MPs accumulation in fish, are looked with great interest by the scientific community. On this regard, to both mitigate the dietary MPs-induced oxidative stress and the MPs accumulation in fish, the concomitant inclusion of antioxidant molecules and natural organic coagulants in aquafeeds can represent a valid and innovative approach. Several molecules with antioxidant properties have been tested within the aquaculture sector (even if not in response to dietary MPs) like antimicrobial peptides (García-Beltrán et al., 2023; Valero et al., 2020), ferulic acid (Abasubong et al., 2023; Fu et al., 2022; Yu et al., 2020), coenzyme Q10 (Aramli et al., 2023; Y. Huang et al., 2023), and the carotenoid astaxanthin (ASX) (Campos-Sánchez and Esteban, 2024; Harith et al., 2024). Particularly, ASX is widely used in aquaculture and its beneficial effects on farmed species are well reported, including enhanced pigmentation, growth and reproductive performances, immune response, and tolerance to abiotic and biotic stressors (Elbahnaswy and Elshopakey, 2023; Kalinowski et al., 2019; Lu et al., 2021; Mularczyk et al., 2020; Verlhac Trichet and Amaya, 2022). ASX included in aquafeeds can be of natural or synthetic origin (Snell and Carberry, 2022). The synthetic form has currently a wider application compared to the natural one due to its lower price (Patel et al., 2022) but it should be pointed out that synthetic ASX is less effective (Snell and Carberry, 2022) and is characterized by a lower bioavailability and stability compared to the natural one (Capelli et al., 2019; Long et al., 2023; Stachowiak and Szulc, 2021). The natural ASX molecule is a red carotenoid (chemical formula: 3,3′-dihydroxy-β, β′-carotene-4,4′-dione; molecular formula: $C_{40}H_{52}O_4$) from the xanthophyll family and is naturally synthetised by a number of microalgae such as *Haematococcus pluvialis*, *Haematococcus lacustris*, *Chlorococcum* sp., *Chlorella zofingiensis*, the yeast *Phaffia rhodozyma*, and the bacterium *Agrobacterium aurantiacum* (Nair et al., 2023). Prompted by the ecological benefits and the market requirements, ASX production from microalgae (low carbon footprint) and its practical application in the aquaculture sector have become pivotal points of current studies in this sector (Lu et al., 2021).

However, the natural ASX is subjected to a rapid degradation because of its unsaturated disposition and highly conjugated structure, especially when separated from its matrix and exposed to industrial conditions (Yao et al., 2023). This property represents the main drawback of natural ASX which can still limit its application as aquafeed supplement (Martínez-Delgado et al., 2017). Therefore, it is essential to preserve ASX integrity in different settings through stabilization processes (Xu et al., 2023). Particularly, the microencapsulation of ASX can represents a valid solution to save, over the time, its natural features (Abdol Wahab et al., 2022; Martínez-Álvarez et al., 2020). Beside avoiding ASX leaching, the encapsulation process provides a prolonged release of the molecule in fish's digestive tract (Mahotra et al., 2022). Microencapsulation can be applied using different techniques (Khalid and Barrow, 2018) among which the conversion of natural ASX into a dry powder enclosed in microcapsules composed of natural substances, including starch, Arabic gum, chitosan, or maltodextrin, has emerged as a promising solution for the aquaculture sector. In fact, starch is gathering interest as an organic and sustainable substance able to coagulate water-suspended microplastics (Tang et al., 2024). In addition, it has been recently demonstrated that starch-based microcapsules (containing ASX) were able, due to their wall matrix composition, to promote MPs coagulation in the gut of European seabass (*Dicentrarchus labrax*) fed diets including fluorescent MPs microbeads (1–5 μm; 50 mg/kg feed), limiting their absorption and the consequent accumulation in different tissue, including liver (Zarantoniello et al., 2024).

Within this context, the primary objective of the present study was to explore the combined impact of incorporating ASX as a natural antioxidant and natural-based microcapsules into aquafeed formulation to

potentially influence both fish overall health and MPs accumulation in target tissues. The positive attributes of natural ASX in mitigating the adverse effects of MPs exposure in aquatic organisms are supported by only a limited number of studies. On this regard, it has been demonstrated that natural ASX was able to neutralize inflammatory responses caused by MPs exposure in head kidney-cultured cells of Nile tilapia (*Oreochromis niloticus*) (Deng et al., 2023) and to reduce oxidative stress in the liver and skin of discus fish (*Symphysodon aequifasciatus*) exposed to environmental MPs (Huang et al., 2023a,b). More recently, Zarantoniello et al. (2024) demonstrated that the dietary implementation of microencapsulated ASX led to a mitigation of MPs-induced oxidative stress in European seabass juveniles fed diets including fluorescent MPs microbeads (1–5 μm; 50 mg/kg feed) over a 60-day feeding trial. In this study, a comprehensive laboratory-based approach was employed to investigate the potential synergistic benefits of dietary natural microencapsulated ASX administration in zebrafish fed diets containing MPs of varying sizes and concentrations over the whole life cycle, from hatching to adulthood. This investigation placed a particular emphasis on fish overall health status, MPs accumulation, and translocation to different organs and tissues, utilizing zebrafish (*Danio rerio*) as the experimental model. Zebrafish was chosen due to its well-established status as a model organism for nutritional (Chemello et al., 2022; Randazzo et al., 2020) and toxicological studies (Bhagat et al., 2020; Qiao et al., 2019b), boasting a short life cycle, high reproductive rate, and a fully sequenced genome (Ribas and Piferrer, 2014; Ulloa et al., 2014).

2. Materials and methods

2.1. Ethics

All procedures conducted on fish were approved by Ethics Committee of the Marche Polytechnic University (Ancona, Italy) and Italian Ministry of Health (Aut. n. 391/2023-PR) and were in accordance with the Italian legislation on experimental animals. To minimize the suffering of the animals an anaesthetic was used (MS222; Merck KGaA, Darmstadt, Germany).

2.2. MPs features

Two distinct fluorescent MPs microbeads were acquired from Cospheric LCC (Goleta, CA, USA): (i) polymer A: amino formaldehyde polymer (FMv-1.3; size range: 1–5 μm; emission peak: 636 nm; excitation peak: 584 nm; 1.22×10^{11} microspheres/g); (ii) polymer B: polyethylene (UVPMS-BR-0.995; size:40–47 μm; emission peak: 607 nm; excitation peak: 575 nm; 1.62×10^{7} microspheres/g).

2.3. Microencapsulated astaxanthin

The natural astaxanthin (ASX; AstaReal® L10, Nacka, Sweden) underwent microencapsulation by STM Aquatrade S.r.l. in Ancona, Italy, using an innovative technology named Co.M.E. (Coating Made Easy), which was developed as part of a private project called + POP (Powder on Pellets). The microcapsules' wall featured a unique matrix composed mainly of Arabic gum (55%) and starch (22%), with smaller proportions of cellulose, sodium ascorbate, and vitamin E, listed in declining order of abundance. The detailed process of preparing microcapsules is safeguarded due to intellectual property concerns. Importantly, all components utilized in the microcapsule preparation are deemed safe for both humans and animals. The encapsulation method was designed to protect the carried molecule, in this case, ASX, while ensuring optimal delivery to the feed. This technology facilitated excellent adhesion between the dry microcapsules and any aquafeed through tailored physical and chemical interactions. Due to their unique chemical properties, the microcapsules released their contents within approximately 90 s upon contact with water. Providing 1 g of microcapsules containing ASX resulted in a direct transfer of 25 parts per million (ppm) of ASX to the feed.

2.4. In vitro MPs coagulation

To assess the potential role of the microcapsules (empty or including ASX) and of the two main microcapsule components (Arabic gum and starch) an *in vitro* experiment was conducted considering only the smaller MPs (polymer A; size range: 1–5 μm) that are known to be effectively absorbed at intestinal level (Cattaneo et al., 2023). Two different stock solutions were prepared by diluting polymer A microbeads in distilled water to obtain final concentrations of 50 mg/L (solution 50) or 500 mg/L (solution 500) (same concentration used for the preparation of the tested diets; see details in further sections). The pH was adjusted at 7.8 to mimic the pH conditions of zebrafish gut lumen (Brugman, 2016; Kristina Hamilton et al., 2022).

To 1 mL of both 50 and 500 solutions (in triplicate), ASX, microcapsulated ASX, empty microcaspules, starch and Arabic gum were added at three different concentrations: (i) concentration 1 × that was exactly the concentration of the microcapsules or of their different components used for feed preparation in the present study; (ii) 5 × and 10 × concentrations that were 5 and 10 times higher, respectively (Table 1). Each solution was immediately placed in a 35 mm glass bottom dish (ibidi GmbH, Gräfelfing, Germany), on a shaker plate (SO3 Orbital Shaker; Stuart Scientific Co. Ltd., Redhill, England) to mimic fish gut movements.

All the solutions were observed under a Nikon A1R (Nikon) confocal microscope (Nikon Corporation, Tokyo, Japan) at T_0 and after 6 h shaking (T_1), exciting them with 561 nm wavelength and collecting emissions at 615 nm to visualize in red the fluorescent polymer A microbeads. The shaking time was chosen in relation to the gut transit time of feed reported in literature in stomach-less fish (Logothetis et al., 2001). At T_1, coagulation events were verified, and possible coagula were eventually measured. The images were processed with NIS-Element software (version 5.21.00, Nikon)

2.5. Experimental diets

Starting from the *in vitro* results, five test diets were prepared from the same single ingredients. A control diet free of fluorescent MPs (named CTRL) was formulated to resemble the proximate composition of a commercial standard diet available for zebrafish (Zebrafeed; Sparos LDA, Olhão, Portugal), according to a previous study (Zarantoniello et al., 2021). Additionally, four experimental diets (set 1 diets) containing fluorescent MPs were then prepared according to Cattaneo et al. (2023), by adding the A or B fluorescent polymer at two different concentrations to the CTRL diet formulation. Particularly: (i) diet A50: 50 mg/kg feed of polymer A; (ii) diet A500: 500 mg/kg feed of polymer A; (iii) diet B50: 50 mg/kg feed of polymer B; (iv) diet B500: 500 mg/kg feed of polymer B.

All the powdered ingredients used to produce the experimental diets were well mixed (GastroNorm 30C1PN, ItaliaGroup Corporate S.r.l.) for 20 min and then oil and water were added to attain appropriate consistency for pelleting. Water was used to include A or B polymers in the mixture. Pellets were obtained by using a 3-mm-die meat grinder, dried

Table 1
Quantity (g) of microcapsules or microcapsules components used to prepare 1 mL of each solution staring from the two stock solutions of polymer A (50 and 500 mg/L of polymer A, respectively).

	1 ×	5 ×	10 ×
ASX	0.175	0.875	1.750
ASX microcapsules	7.000	35.000	70.000
Empty microcaspules	6.825	34.125	68.250
Starch	1.540	7.700	15.400
Arabic gum	3.850	19.250	38.500

at 37 °C for 48 h in a ventilated heater, and then ground and sieved through a battery of sieves to obtain particles of the right size for fish rearing (as specified in the *2.6. Experimental design* section). More specific details about this set of diets have recently been published in Cattaneo et al. (2023).

A second set of diets was prepared, starting from the previously described ones (set 1 diets), by adding 7 g of microencapsulated ASX per kg of feed (corresponding to a transfer of 175 mg of ASX per kg of feed), and were accordingly named as follows: CTRL-ASX, A50-ASX, A500-ASX, B50-ASX, and B500-ASX, respectively. The first set of diets were firstly weighted and then transferred to glass airtight jars for the addition of weighted ASX microcapsules. Each diet was then vigorously mixed to allow ASX microcapsules to uniformly cover each single feed pellet. Analysis for the ASX content determination were performed on 3 subsamples of each diet according to Du et al. (2016) and confirmed the presence of the molecule at an average concentration of 170 ± 8 mg/kg per diet. In addition, to confirm the absence of eventual contamination of fluorescent MP microbeads, three subsamples of both CTRL and CTRL-ASX diets were checked through a confocal microscope (Nikon A1R; Nikon Corporation, Tokyo, Japan) and to chemical digestion followed by filtration on fiberglass filters with 0.7 μm-pores (Whatman GF/A, Merck KGaA). Filters, after being dried at room temperature, were analysed through a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) to confirm the absence of fluorescent polymers in the analysed diets (for details on the procedure, please refer to the dedicated section).

2.6. Experimental design

Zebrafish embryos (wild type strain AB) were obtained from the University broodstock colony and maintained for 48 h in a Tecniplast system (Varese, Italy) at the following parameters: 28 ± 0.5 °C, pH 7 ± 0.1, ammonia and nitrite concentrations <0.01 mg/L, nitrate concentration <10 mg/L, and light:dark period of 12:12 h. Subsequently, developing embryos were collected with the support of a stereomicroscope (Leica Wild M3B; Leica Camera AG, Wetzlar, Germany) and randomly assigned to five experimental groups (named CTRL, A50, A500, B50, and B500 according to the set 1 dietary treatment; 1500 embryos per group, 500 embryos per tank). Fish were maintained in 20L independent tanks (3 tanks per experimental group) at the same chemical-physical parameters applied to the Tecniplast system from hatching to 30 days post fertilization (dpf). Each 20 L were set up to avoid light reflection and to water change according to Olivotto et al. (2004).

After 30 dpf, fish from each group (CTRL, A50, A500, B50, and B500) were transferred to 200 L tanks (three tanks per experimental group) each equipped with biological and mechanical filters (Panaque, Rome, Italy), and reared for 6 months. During the feeding trial, the feed size of CTRL, A50, A500, B50, and B500 diets was adjusted in response to the fish development as follows: pellets <100 μm from 5 to 15 dpf, 101–200 μm from 16 to 30 dpf, 201–400 μm from 31 dpf to 60 dpf, and 401–600 μm from 61 dpf to 6 months according to Chemello et al. (2022). The daily feeding rate corresponded to 3% of the total fish body weight (divided in two equal amounts, one given in the morning and one in the afternoon). In addition, from 5 to 10 dpf, zebrafish at larval stage were fed also rotifers (*Brachionus plicatilis*; 5 individuals/L), according to Zarantoniello et al. (2021).

At 6 months 1200 fish from groups CTRL, A50, A500, B50, and B500 were randomly collected and divided in two sub-groups (each of 600 fish, 200 fish per tank in triplicate) that were fed (3 % daily) for one more month as follows: (i) the same diets (CTRL, A50, A500, B50, or

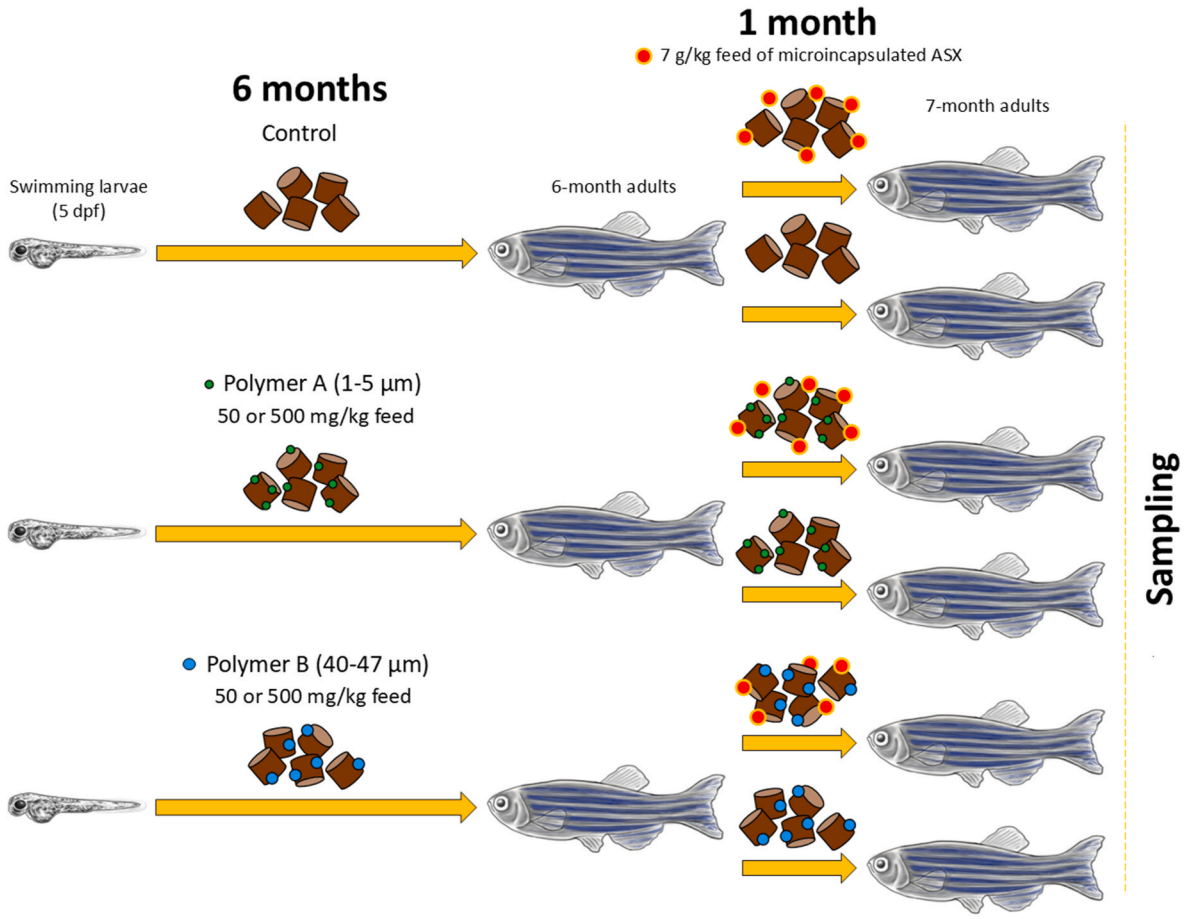

Fig. 1. Scheme of the experimental design.

B500); (ii) diets CTRL, A50, A500, B50, and B500 implemented with microencapsulated ASX (7 g of microencapsulated ASX per kg of feed). The experimental design is represented in Fig. 1.

During the whole trial, zebrafish were daily inspected and, if present, dead specimens were removed from each tank and recorded for survival rate calculation. At the end of the whole trial (7 months), after 24h fasting, all fish were collected, euthanised (lethal dose of MS222; 0.3 g/L) and liver, intestine, and muscle were sampled and properly stored for the laboratory analysis.

2.7. Survival rate and growth

At the end of the experiment (7 months), 20 fish from each tank (60 per experimental group) were weighted with a OHAUS Explorer analytical balance (Greifensee, Switzerland), accuracy: 0.1 mg. Survival rate was calculated by removing the dead fish over the feeding trial to the initial number of fish per tank (at 6 months).

2.8. Confocal microscopy

Intestine, liver, and muscle were sampled from 5 fish per tank (15 fish per experimental group). Samples were fixed in 4% PFA at 4 °C for 24 h and then stocked at 4 °C in 1 × phosphate-buffered saline (PBS) solution. For confocal microscopy analysis, each sample was positioned in a concave glass slides with two drops of glycerol:PBS solution (90:10) and mounted with a glass coverslip. The detection of fluorescent MPs microbeads was performed with a Nikon A1R confocal microscope (Nikon Corporation, Tokyo, Japan). Samples were excited with 561 and 647 nm wavelengths simultaneously, and emissions were collected at 615 and 670 nm to visualize in red the fluorescent MPs microbeads and in blue (chosen pseudocolor for the far-red) the tissue texture. Finally, the images were processed with NIS-Element software (version 5.21.00; Nikon).

2.9. Chemical digestion of samples and MPs quantification

Intestine, liver, and muscle samples from 5 zebrafish per tank (15 fish per experimental group) were collected and frozen at −80 °C. Each sample was then weighed and digested following the procedure reported in Cattaneo et al. (2023). Briefly, the samples were placed in glass tubes, adding a 10% KOH solution in a ratio of 1:10 wt/volume with the sample. Samples were incubated at 40 °C for 48 h and then the resulting digestate was filtered through fibreglass filter with pores of 0.7 μm (Whatman GF/A, Merck KGaA, Darmstadt, Germany), using a vacuum pump connected to a filter funnel. The filters were stocked in glass Petri dishes after being dried at room temperature. MPs quantification on filters was performed through a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) using FITC (491 nm) and Texas Red (561 nm) channels. The MPs were counted using the ZEN Blue 2.3 software (Zeiss) and the acquisition of images was made by the Axiocam 503 digital camera (Zeiss).

2.10. Histology

Intestine and liver samples from 5 zebrafish per tank (15 fish per experimental group) were fixed and stocked according to Piccinetti et al. (2014). Then, samples were dehydrated, washed with xylene (Bio--Optica, Milano, Italy), and, finally, embedded in paraffin (Bio-Optica). Sections (thickness 5 μm, obtained using a Leica RM RTS microtome; Leica, Nussloch, Germany) were stained with (i) Mayer haematoxylin and eosin Y (Merck KGaA; H&E) to assess potential alterations in the tissues' architecture and the eventual occurrence of inflammatory infiltration in both the intestinal tract and the hepatic parenchyma; (ii) Alcian Blue (Bio-Optica) to count the relative abundance of Alcian blue positive (Ab+) goblet cells in intestine sections (Zarantoniello et al., 2023). The evaluation of all the histological parameters considered was performed on three sections per fish (15 fish per group) collected at a distance from each other of 50 μm. The ZEN 2.3 software (Zeiss) was used for the morphometric evaluation of the height of undamaged and non-oblique mucosal folds. Regarding the semi-quantitative analysis of the relative abundance of Ab + goblet cell, scores were assigned as follows: Ab + goblet cells: + = scarce; ++ = diffused; + + + = highly abundant.

2.11. Molecular analysis

Intestine and liver samples were collected from 3 zebrafish per tank (9 fish per experimental group), stored at −80 °C and processed according to Cionna et al. (2006). Briefly, total RNA extraction from the samples was performed using RNAzol™ reagent (Merck KGaA), the possible contamination with genomic DNA was avoided by the DNase treatment (10 IU at 37 °C for 10 min; MBI Fermentas, Milano, Italy), and the final RNA concentration (NanoPhotometer P-Class; Implen, München, Germany) and integrity (running 1 μg of total RNA stained with GelRed™ on a 1% agarose gel) were finally checked. RNA samples were stored at −80 °C. The cDNA synthesis was performed using 1 μg of RNA sample using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

Real-time quantitative PCR reactions were performed in an iQ5 iCycler thermal cycler (Bio-Rad), using reaction set up and thermal profile according to Zarantoniello et al. (2022). Two no template controls (NTCs) were added in each run to guarantee absence of contamination (no peaks detected for the NTC in each reaction). One single peak was detected for each qPCR product in the melting curve analyses. Amplification products were sequenced, and homology was verified. The elative quantification of genes involved in immune response (interleukin 1β, *il1b*; interleukin 10, *il10*; lipopolysaccharide-induced TNF factor, *litaf*) was performed on intestine samples, whilst the relative quantification of genes involves in stress (glucocorticoid receptor, *nr3c1*; heat shock protein 70, *hsp70.1*) and oxidative stress (superoxide dismutase 1, *sod1*; superoxide dismutase 2, *sod2*; catalase, *cat*) was performed on liver samples. The sequences of all the primers used in the present study are reported in Table 2. For each pair of primers: (i) the annealing temperature (reported in Table 2) was priorly optimized with a temperature gradient assay; (ii) the specificity was evaluated by checking the absence of primer–dimer formation and dissociation curves; (iii) the efficiency was verified with a mix of cDNA (Control group) (efficiency around 90% for all the primers, with an R^2 that ranged from 0.995 to 0.998) at different concentrations (1:1, 1:10, 1:100, 1:1000). The mRNA levels of target genes analysed were calculated using the geometric mean of the two reference genes (actin-related protein 2/3 complex subunit 1A, *arpc1a*; ribosomal protein L13, *rpl13*), after checking their stability of expression through the Bio-Rad CFX Manager 3.1. software. Gene transcript expression variations among experimental groups are reported as relative mRNA abundance (arbitrary units). The software iQ5 optical system version 2.0 (Bio-Rad) was used to process the qPCR, with the addition of GeneEx Macro iQ5 Conversion and GeneEx Macro iQ5 files.

2.12. MPs detection in fish faeces

The characterization of polymer A abundance in faeces was conducted over a 10-day period. Particularly, during the last 10 days of the feeding trial, 30 fish from each experimental group (ten fish per tank) were daily transferred, in the morning, in 30 clean tanks (10 L each; 3 tanks per experimental group) equipped with a double floor (consisting of a 3 mm grid) to allow faeces collection. Fish from each tank were fed their respective experimental diets (at 1.5 % body weight to simulate the half ratio of the daily feed intake). After 8 h the fish were returned to their main tanks and faeces were collected. Three samples of faeces were daily collected for each experimental group, resulting in 30 samples per experimental group at the end of the trial.

Table 2
Sequences, identification numbers (ZFIN ID), and annealing temperature (AT) of primers used in the present study.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	AT (°C)	ZFIN ID
il1b	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	54	040702–2
il10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	56	051111–1
litaf	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAAG	53	040704–23
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	58	050522–503
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	58	990415–91
sod1	GTCGTCTGGCTTGTGGAGTG	TGTCAGCGGGCTAGTGCTT	60	990415–258
sod2	CCGGACTATGTTAAGGCCATCT	ACACTCGGTTGCTCTCTTTTCTCT	60	030131–7742
cat	CCAAGGTCTGGTCCCATAA	GCACATGGGTCCATCTCTCT	60	000210–20
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	59	031007–1
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	60	040116–1

Faeces samples were incubated at 40 °C overnight in an Argo Lab ICN 35 incubator (Argo Lab, Carpi, Italy) for water evaporation. The dried fecal samples were weighted using an OHAUS Explorer analytical balance (Greifensee, Switzerland; accuracy of 0.1 mg) and then subjected to the chemical digestion and MPs quantification previously described in Section 2.9 (Chemical digestion of samples and MPs quantification).

2.13. Statistical analyses

All data were checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test). The data were analysed through one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test, performed using the software package Prism 8 (GraphPad software version 8.0.2, San Diego, CA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Growth and survival

Since the present study focused on adult animals, survival rate was evaluated from month 6 to month 7 and was 100 % in all the experimental groups. Considering growth, no significant differences were evident among fish fed CTRL-diets (258 ± 19 and 259 ± 12 mg) and those fed A diets (263 ± 17, 261 ± 11, 259 ± 13, and 264 ± 15 mg for A50, A50-ASX, A500, and A500-ASX, respectively) or B diets (257 ± 18, 261 ± 16, 262 ± 13, 258 ± 14 mg for B50, B50-ASX, B500, and B500-ASX, respectively).

3.2. MPs coagulation in vitro

The summarized outcomes of the *in vitro* coagulation experiment are detailed in Table 3. No coagulation events were identified in solutions containing only polymer A microbeads (MPs). In solution 50 (50 mg/L of polymer A), no coagulation events were observed with 1 × and 5 × concentrations of astaxanthin (ASX), microencapsulated ASX, empty microcapsules, starch, or Arabic gum. Interestingly, coagulation events were only noted with a 10 × concentration, specifically when ASX microcapsules, empty microcapsules, and starch were introduced, while no coagulation events occurred with ASX or Arabic gum at any concentration.

In solution 500 (500 mg/L of polymer A), coagulation events occurred with microencapsulated ASX, empty microcapsules, and starch at 1 × , 5 × , or 10 × concentrations. Conversely, no coagulation events were observed when ASX or Arabic gum were added at any concentration level. These results served as a valuable guide for determining the diets to be tested during the forthcoming *in vivo* experiment.

Fig. 2 shows examples of: (i) a solution containing only MPs at 500 mg/L with scattered polymer A microbeads (Fig. 1 a); (ii) coagulation events detected in the MPs solutions added with ASX microcapsules, empty microcapsules, and starch at 1 × concentration (Fig. 1 b, c, and d, respectively). The size of the polymer A microbeads coagula ranged from 50 to 83 μm in all the solutions.

3.3. MPs detection in zebrafish tissues

Analysis by confocal microscopy on zebrafish samples (intestine, liver, and muscle) (fish fasted for 24 h) revealed the presence of MPs only in groups fed A-diets (containing MPs 1–5 μm in size) (Fig. 3d–f), while in groups fed CTRL-diets and B-diets no MPs were detected in all tissues analysed (Fig. 3a–c; Fig. 3g–h).

3.4. MPs quantification in zebrafish tissues

Table 4 reports the MPs quantification in the intestine, liver, and muscle of zebrafish fed the experimental diets after 24h fasting. No fluorescent MPs microbeads of both polymer A and B were found in the different tissues sampled from fish fed the CTRL-diets.

As regards fish fed A-diets, a dose-dependent increase in polymer A microbeads accumulation was evident in all the tissues analysed. In addition, the liver resulted as the key MPs accumulation organ. Microencapsulated ASX administration caused a significant ($p < 0.05$) reduction of MPs microbeads in A500-ASX respect to A500 group (in all the analysed tissues), while no significant differences were detected comparing MPs microbeads quantification between A50 and A50-ASX groups.

Differently, considering fish fed B-diets, a scattered detection of polymer B microbeads was only evident in the intestine samples, while

Table 3
Results of the *in vitro* coagulation experiment after 6 h.

MPs concentration	Concentration of added components	Solutions (1 mL)					
		MPs only	ASX	MPs + microencapsulated ASX	MPs + empty microcaspules	MPs + starch	MPs + Arabic gum
Solution 50	1 ×	–	–	–	–	–	–
50 mg/L of polymer	5 ×	–	–	–	–	–	–
A	10 ×	–	-	+	+	+	–
Solution 500	1 ×	–	-	+	+	+	–
500 mg/L of polymer	5 ×	–	-	+	+	+	–
A	10 ×	–	-	+	+	+	–

All the solutions were tested in triplicate. + detection of coagulation events; - absence of coagulation events. MPs = polymer A microbeads.

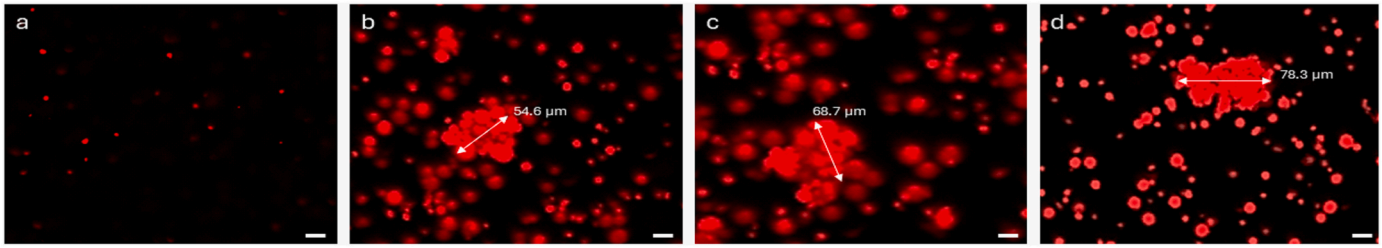

Fig. 2. Examples of coagulation events of polymer A microbeads detected in the *in vitro* experiment. Representative images of: (**a**) solution containing only MPs at 500 mg/L; (**b**) MPs solution (500 mg/L) added with microencapsulated ASX (1 × concentration); (**c**) MPs solution (500 mg/L) added with empty microcapsules (1 × concentration); (**d**) MPs solution (500 mg/L) added with starch (1 × concentration). Scale bars: 20 μm.

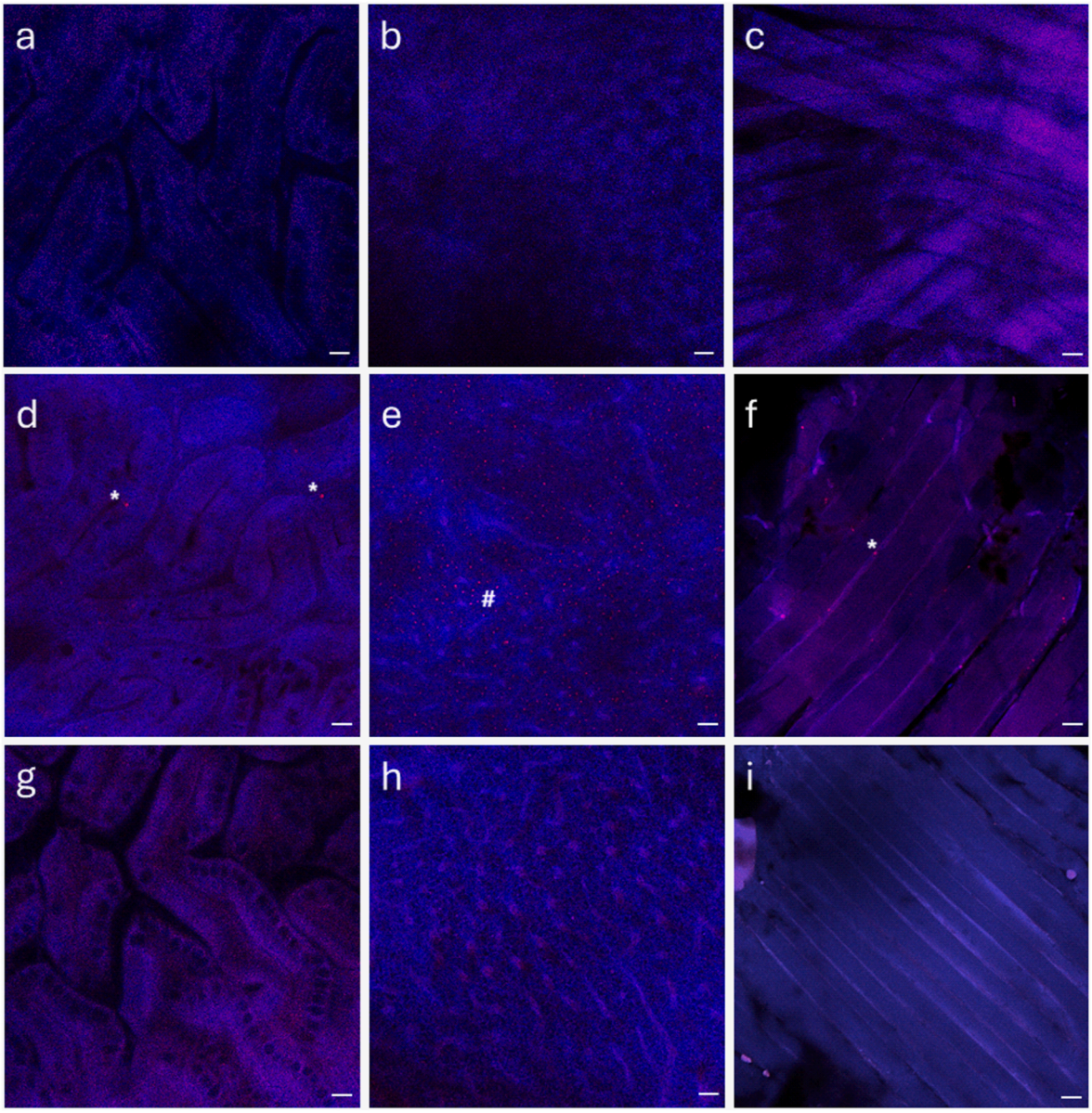

Fig. 3. Confocal microscopy. Representative images for zebrafish fed (**a-c**) CTRL-diets, (**d-f**) A-diets, (**g-h**) B-diets. (**a**,**d**,**g**) intestine; (**b**,**e**,**h**) hepatic parenchyma; (**c**, **f**,**i**) skeletal muscle. Scale bars = 20 μm. Asterisks indicate isolate MPs; # indicate groups of MPs.

in liver and muscle samples no microbeads were detected.

3.5. Histology

No pathological alterations or signs of inflammation were evident in both intestine and liver of fish from all the experimental groups (Fig. 4).

Table 4
MPs quantification (number of microbeads/mg of tissue) in the intestine, liver, and muscle of adult zebrafish fed experimental diets.

Polymer A (1–5 μm)						
	CTRL	CTRL-ASX	A50	A50-ASX	A500	A500-ASX
Intestine	0	0	2.9 ± 0.3 [a]	2.6 ± 0.9 [a]	170.9 ± 20.6 [c]	20.5 ± 2.5 [b]
Liver	0	0	5.5 ± 1.7 [a]	5.5 ± 2.1 [a]	821.1 ± 95.5 [b]	12.2 ± 3.0 [a]
Muscle	0	0	2.0 ± 0.2 [a]	1.9 ± 0.9 [a]	48.0 ± 4.3 [b]	3.2 ± 1.8 [a]
Polymer B (40–47 μm)						
	CTRL	**CTRL-ASX**	**B50**	**B50-ASX**	**B500**	**B500-ASX**
Intestine	0	0	1.6 ± 0.3 [a]	1.5 ± 0.3 [a]	1.8 ± 0.3 [a]	1.6 ± 0.4 [a]
Liver	0	0	0	0	0	0
Muscle	0	0	0	0	0	0

Data are reported as mean ± SD ($n = 15$). a,b Within each line, different letters denote statistically significant differences ($p < 0.05$) among the experimental group.

Table 5 summarize the results of the histological indexes analysed in the adult zebrafish intestines. Considering the histological indexes evaluated in the intestine, no significant differences were detected among the experimental groups fed A-diets and those fed CTRL ones in terms of mucosal fold height. However, all groups fed A-diets, regardless ASX administration, showed a slight increase in Ab + goblet cells relative abundance compared to those fed the CTRL ones. Differently, as regards fish fed B-diets, a significant ($p < 0.05$) reduction in mucosal folds height was evident only in fish from B50 and B500 groups. On the contrary, fish fed B-diets including ASX showed a regular height of mucosal folds, comparable to that detected in CTRL groups. In addition, a slight increase in the relative abundance of Ab + goblet cells was shown by all the groups fed B-diets compared to those fed the CTRL ones, particularly pronounced in B500 group.

3.6. Real-time PCR

Intestine. Considering the relative expression of genes involved in the immune response (*il1b*, *il10*, and *litaf*), no significant differences were evident among the experimental groups fed the A-diets and those fed the CTRL ones (Fig. 5a–c). A similar *scenario* was detected for groups that were fed the B-diets compared to the CTRL ones (Fig. 5d–f).

Liver. Considering the oxidative stress response in fish fed A-diets, both A50 and A500 groups showed a significantly ($p < 0.05$) higher *sod1*, *sod2*, and *cat* gene expression compared to the CTRL groups. ASX administration was able to reduce *sod1*, *sod2*, and *cat* gene expression only in group A50-ASX, which showed comparable values to CTRL groups, while no effect was observed in group A500-ASX (Fig. 5g–i). As regards fish fed B-diets, a significant ($p < 0.05$) upregulation of *sod1* was observed in B50 and B500 groups compared to the CTRL groups and B50-ASX one, with B500-ASX showing an intermediate value (Fig. 5 j). Differently, no significant differences were evident among the experimental groups fed the B-diets and those fed the CTRL ones in terms of both *sod2* (Fig. 5 k) and *cat* (Fig. 5 l) relative gene expression.

As regards the stress response in fish fed A-diets, a significant ($p <$

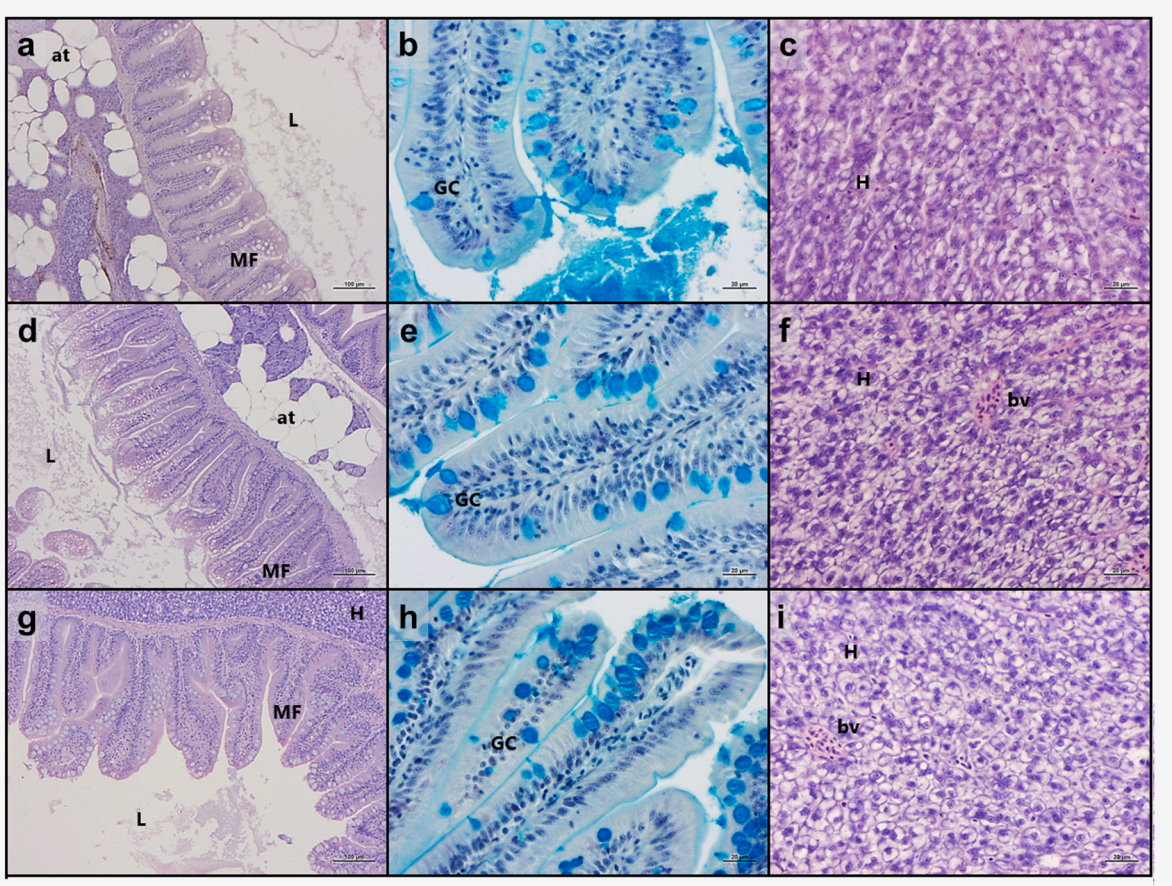

Fig. 4. Histological evaluation of intestine and liver. Example of histomorphology of zebrafish fed (**a-c**) CTRL-diets, (**d-f**) A-diets, (**g-h**) B-diets. (**a**,**d**,**g**) intestine, scale bars = 100 μm; (**b**,**e**,**h**) details of Ab + goblet cells, scale bars = 20 μm; (**c**,**f**,**i**) hepatic parenchyma, scale bars = 20 μm. Abbreviations: at, perivisceral adipose tissue; L, gut lumen; MF, intestinal mucosal folds; H, hepatocytes; GC, goblet cells; bv, blood vessels.

Table 5
Histological indexes measured in the intestine of adult zebrafish fed the experimental diets.

Polymer A (1–5 μm)						
	CTRL	CTRL-ASX	A50	A50-ASX	A500	A500-ASX
Mucosal folds height (μm)	427.8 ± 12.9 [a]	430.4 ± 14.5 [a]	425.2 ± 11.2 [a]	426.3 ± 2.3 [a]	424.0 ± 14.6 [a]	427.5 ± 12.9 [a]
Ab + goblet cells' relative abundance	+	+	++	++	++	++
Polymer B (40–47 μm)						
	CTRL	CTRL-ASX	B50	B50-ASX	B500	B500-ASX
Mucosal folds height (μm)	427.8 ± 12.9 [a]	430.4 ± 14.5 [a]	391.2 ± 8.7 [b]	420.5 ± 14.1 [a]	390.9 ± 14.3 [b]	428.8 ± 21.4 [a]
Ab + goblet cells' relative abundance	+	+	++	++	+++	++

Data of mucosal folds height are reported as mean ± SD ($n = 15$). a,b Different letters denote statistically significant differences ($p < 0.05$) among the experimental group. Ab + goblet cells: + = scarce; ++ = diffused; + + + = highly abundant.

0.05) upregulation of both *nr3c1* (Fig. 5 m) and *hsp70.1* (Fig. 5 n) was detected in A50 and A500 groups compared to both the CTRL groups and the A50-ASX and A500-ASX ones, which did not show significant differences among them. Differently, no significant differences were evident among groups fed B-diets and those fed the CTRL-diets in terms of both *nr3c1* (Fig. 5 o) and *hsp70.1* (Fig. 5 p) relative gene expression.

3.7. MPs detection in zebrafish faeces

No MPs microbeads were detected in samples of faeces from CTRL and CTRL-ASX groups. Considering fish fed A-diets, no significant differences were highlighted between A50 and A50-ASX groups (16.9 ± 2.4 and 17.3 ± 3.1 polymer A microbeads/μg), while a three-fold significant ($p < 0.05$) increase in polymer A microbeads abundance was observed in faeces from A500-ASX group compared to those from A500 one (189.6 ± 11.8 and 638.7 ± 14.1 polymer A microbeads/μg).

Differently, no significant differences were detected in polymer B microbeads abundance among samples of faeces from B50, B50-ASX, B500, and B500-ASX groups (4.1 ± 1.1, 3.7 ± 1.2, 31.6 ± 9.2, and 38.4 ± 7.7 polymer B microbeads/mg).

4. Discussion

Contamination of fish feeds by MPs can severely impact fish health status since particles under 20 μm in size can be absorbed by the gastrointestinal tract of fish and translocate to different tissues, among which liver represents the main site of accumulation (Compa et al., 2024; Ma et al., 2021). The MPs retaining in the liver parenchyma can led to adverse effects, especially oxidative stress (Aiguo et al., 2022). Since the short-term environmental elimination of MPs is not possible, strategies to mitigate MPs-related issues on fish are of primary interest. In this context, the present study aimed to assess the potential synergistic benefits of natural ASX encapsulated in natural-based microcapsules on zebrafish fed diets containing MPs of different sizes and at concentrations, over the whole life cycle. Results showed that microencapsulated ASX was able to counteract the negative effects caused by both bigger MPs, that simply transited in the fish gut, and smaller ones, which were absorbed at intestinal level and consequently translocated to different organs and tissue. Particularly, the effects of the two dietary MPs tested on adult zebrafish in the present study are in line with the results previously obtained in zebrafish larvae and juveniles (Cattaneo et al., 2023). In fact, large dietary polymer B microbeads (40–47 μm) were not absorbed at intestinal level and only caused a reduction in the fish mucosal fold height and an increase in Ab + goblet cells relative abundance. These results suggested that the long-term provision of the bigger polymer used in the present study caused an abrasive action and an increased lubrication at intestinal level, without inducing episodes of inflammation, as also confirmed by the expression of genes involved in immune response and accordingly to previous studies (Ašmonaitė et al., 2018; Espinosa et al., 2019). In fact, severe alterations in the intestinal architecture are generally caused by the gut transit of bigger particles, with a range size between 200 and 1000 μm (Varó et al., 2021). In contrast, the dietary polymer A microbeads (size range 1–5 μm) were absorbed at the intestinal level and were capable of reaching other organs and tissues. These findings align with other research on fish, indicating that only MPs smaller than 20 μm can cross the intestinal epithelial layer to access the liver (Lu et al., 2016; Qiao et al., 2019a; Su et al., 2019) and, to some extent, the muscle (Di Giacinto et al., 2023; Zeytin et al., 2020). Particularly, in fish liver, one of the most detrimental effects associated to MPs accumulation is oxidative stress (Capó et al., 2021; Cattaneo et al., 2023; Espinosa et al., 2019; Iheanacho et al., 2023; Xia et al., 2020; Xiao et al., 2023; Yedier et al., 2023). Accordingly, in the present study, fish fed diets containing only polymer A microbeads at both concentrations (A50 and A500 diets) showed a significant upregulation of *sod1*, *sod2*, and *cat*. This result is consistent with the higher expression of the same genes involved in the oxidative stress response analysed in European seabass fed diet containing the same polymer A microbeads used in the present study at 50 mg/kg feed concentration, over a 60-day feeding trial (Zarantoniello et al., 2024). In addition, several studies related the MPs accumulation in fish liver with a consequent activation of a response to oxidative stress (Liu et al., 2022; Romano et al., 2020; Xie et al., 2021), further confirming the need to find potential strategies to mitigate the adverse effects of MPs accumulation and to possibly prevent their absorption by fish.

Despite the extensive use of astaxanthin (ASX) in aquaculture (Kanwugu et al., 2021; Nakano and Wiegertjes, 2020), limited research has explored the potential of this compound as a viable solution to counteract the detrimental impacts of microplastic assimilation in fish. Studies conducted by Deng et al. (2023) and Huang et al. (2023a,b)have touched upon this aspect and, to the best of our knowledge, only one study directly correlated the provision of dietary microencapsulated ASX with the adverse effect of MPs with a range size of 1–5 μm, obtaining promising results (Zarantoniello et al., 2024). The present study further demonstrated that the provision of microencapsulated ASX had positive effects on fish exposed to the same dietary MPs (polymer A, 1–5 μm) and, additionally, on those exposed to bigger polymers (polymer B, 40–47 μm) which differently affect fish health status.

Specifically, in fish fed diets containing polymer B, ASX administration was able to counteract the mucosal folds height reduction and Ab + goblet cells increase, making the results comparable to those observed in zebrafish fed CTRL diets. This outcome underscores the significant role of ASX in preserving intestinal mucosal integrity and aligns with previous research demonstrating its capacity to restore mucosal fold height and goblet cell abundance to physiological levels in fish subjected to stressors. Particularly, treatment with ASX was shown to restore mucosal fold morphology and goblet cell counts after 1.5% ethanol exposure in goldfish (*Carassius auratus*; specific ASX dosage not specified) or diazinon (0.11 mg/L) in rainbow trout (*Oncorhynchus mykiss*) (Alesci et al., 2021; Shabanzadeh et al., 2023).

Differently, in fish fed diets containing polymer A, a dual beneficial effect of the microencapsulated ASX was evidenced. Particularly, the observed decreasing trend of *sod1* along with the significant lower expression of *sod2* and *cat* in A50-ASX group respect to A50, with values comparable to both the control groups, can be correlated to the effectiveness of natural ASX, preserved through the microencapsulation

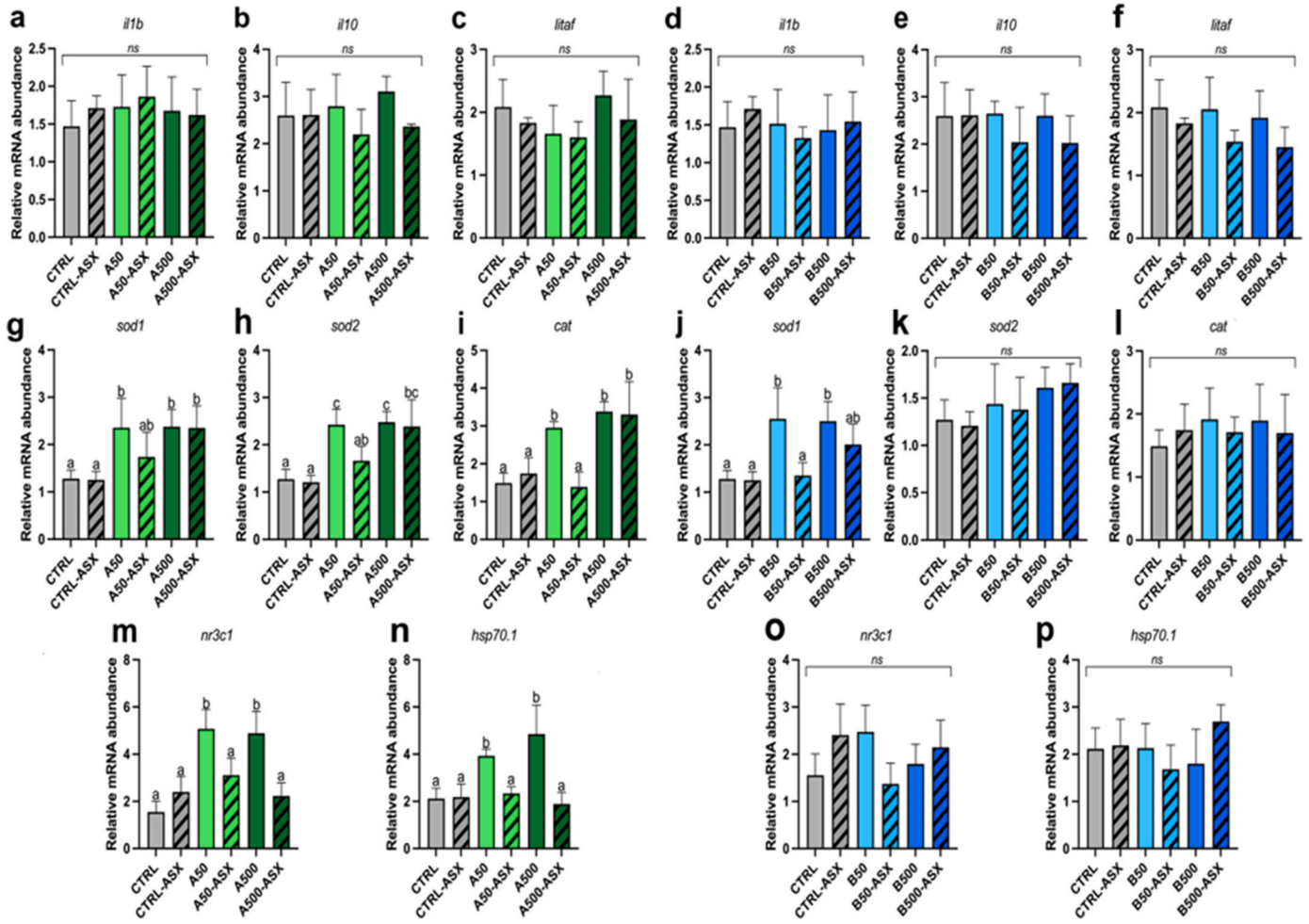

Fig. 5. Real-time qPCR results. Relative mRNA abundance of genes involved in immune response (*il1b*, *il10*, *litaf*) analysed in the intestine and involved oxidative stress response (*sod1*, *sod2*, *cat*) and stress response (*nr3c1* and *hsp70.1*) analysed in the liver of adult zebrafish fed the experimental diets. (**a-c**; **g-i**; **m**,**n**) groups fed A-diets (A50, A50-ASX, A500, A500-ASX) compared to those fed the CTRL ones (CTRL and CTRL-ASX); (**d-f**; **j-l**; **o**,**p**) groups fed B-diets (B50, B50-ASX, B500, B500-ASX) compared to those fed the CTRL ones (CTRL and CTRL-ASX). Data are reported as mean ± SD ($n = 5$). $^{a-c}$ Different letters denote statistically significant differences among the experimental group. *ns*, no significant differences among the experimental groups ($p > 0.05$).

technology, in mitigating the oxidative stress caused by polymer A microbeads accumulation in the liver. This result is consistent with numerous previous studies that highlighted a an oxidative stress reduction in fish treated with ASX (Li et al., 2020; Wang et al., 2023; Xie et al., 2020) and especially with a recent research which evidenced a clear reduction of *sod1*, *sod2*, and *cat* in European seabass juveniles fed diet containing the same polymer A microbeads (at a concentration of 50 mg/kg feed) implemented with microencapsulated ASX (Zarantoniello et al., 2024). However, in the present study, this effect was evident when comparing A50 and A50-ASX groups which were fed diets containing a MPs concentration similar to that found in the environment (Auta et al., 2017; Zhao et al., 2014). In contrast, the absence of significant differences between A500 and A500-ASX groups in the expression of genes involved in oxidative stress response can be related to an excessively high MPs concentration in this experimental diets and/or inadequate dietary ASX supplementation levels respect to the dietary MPs provided, as proposed by Huangfu et al. (2013) and Zhang et al. (2013). However, it should be pointed out that the administered dietary microencapsulated ASX level was able to significantly down-regulate the expression of *nr3c1* and *hsp70.1* in both A50-ASX and A500-ASX groups, suggesting an important role of this antioxidant molecule in fish stress response regulation. This finding is reinforced by prior research in mammals, and birds, which has also highlighted the pivotal role of ASX in stress regulation (Priyadarshini, 2018; Tolba et al., 2020).

In addition to the beneficial effects on oxidative stress response and stress modulation and, the current study revealed a compelling and promising outcome regarding the use of microincapsulated ASX to counteract the MPs accumulation in fish. Notably, samples from the intestine, muscle, and liver of the A500-ASX group exhibited a significant reduction in MPs accumulation compared to the A500 group. The *in vitro* investigation on MPs coagulation conducted in the current study highlighted that empty microcapsules, microencapsulated ASX and starch, all administered at the same concentration as in the zebrafish feed, effectively coagulated polymer A microbeads at a concentration of 500 mg/L. Given that only MPs smaller than 20 μm can be absorbed at the intestinal level (Lu et al., 2016; Qiao et al., 2019a; Su et al., 2019), the present study proposes that the starch present in the microcapsules employed caused the coagulation of polymer A microbeads in the digestive tract of fish from the A500-ASX group. This coagulation process allowed the formation of MPs coagula too large to be absorbed at the intestinal level, consequently leading to a substantial reduction of MPs accumulation in the tissues and organs from fish fed A500-ASX diet compared to those fed the A500 one. Data obtained from faeces analyses further supported this hypothesis since the A500-ASX group exhibited a significantly higher number of MPs in the faecal samples compared to the A500 group. The interesting outcome observed in the A500-ASX group regarding MPs accumulation was not mirrored in the analysis of

the A50 and A50-ASX groups, as they displayed comparable results in terms of MPs accumulation. This result is in contrast to what observed by Zarantoniello et al. (2024) in which the concurrent provision of 7 g/kg feed of microencapsulated ASX and 50 mg/kg feed of polymer A microbeads over a 60-day feeding trial led to a significant reduction of MPs intestinal absorption and, thus, accumulation in European seabass target tissues (intestine, liver, blood, muscle, and adipose tissue). This difference can be related to the absence of a strong acid digestion in zebrafish which is a stomach-less fish (Flores et al., 2020). In fact, the molecular properties of starch in bridging and enmeshing MPs are favoured by acidic conditions like those observed in carnivorous fish stomachs (Girish et al., 2023; Mohd Asharuddin et al., 2021; Teh et al., 2014). Consequently, it can be hypothesized that in fish fed A50-ASX diet, the low MPs concentration in the digestive tract and the absence of a high acidic environment, were insufficient to trigger coagulation processes. This hypothesis is further supported by the comparable abundance of MPs detected in the faecal samples collected from fish fed A50 and A50-ASX diets. Accordingly, the *in vitro* study showed that to induce coagulation events in the less concentrated MPs solution (solution 50 - 50 mg/L of polymer A), significantly higher concentrations of microcapsulated ASX, empty microcapsules, or starch were required. Taken together, these results suggested that the effectiveness of the microcapsule's wall matrix in forming MPs coagula in the fish gut tract, is strictly dependent to several factors, including MPs dietary concentration, time of exposure, and type of fish digestive physiology.

5. Conclusions

The current study showed that by incorporating antioxidant molecules like ASX and natural organic coagulants into aquafeeds, fish consuming diets containing MPs exhibited a reduction in their retention and a decreased oxidative stress response. These findings are still pioneering and demonstrated that the effectiveness of this technology is subjected to a number of variables including fish gut environment, degree of MPs contamination, dietary concentration of microencapsulated ASX, and time of exposure. Further research is necessary to refine this approach taking into account the importance of developing a sustainable, natural-based tool to ensure fish overall health status in an ever-expanding sector like aquaculture.

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CRediT authorship contribution statement

N. Cattaneo: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **M. Zarantoniello:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **F. Conti:** Validation, Formal analysis. **A. Tavano:** Formal analysis. **A. Frontini:** Writing – review & editing, Validation, Methodology. **I. Sener:** Writing – review & editing, Formal analysis. **G. Cardinaletti:** Writing – review & editing, Formal analysis. **I. Olivotto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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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Data availability

Data will be made available on request.

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