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Soluzioni naturali per mitigare l'esposizione a microplastiche alimentari nell'allevamento della trota iridea (*Oncorhynchus mykiss*)

Natural solutions to mitigate dietary microplastic exposure in the culture of rainbow trout (*Oncorhynchus mykiss*)

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RIASSUNTO

Le materie plastiche nella società odierna sono praticamente onnipresenti grazie alle loro numerose proprietà come i bassi costi e l'elevata versatilità. Sfortunatamente, la difficoltà di smaltimento ha reso la plastica uno dei principali inquinanti che si possono trovare nell'ambiente, compresi gli ambienti acquatici (marini e non). Dopo essere stata dispersa, la plastica può trasformarsi in microplastiche (MPs) e una volta entrata nei sistemi idrici, possono essere ingerite/inalate dagli organismi che ci vivono, comprese molte specie ittiche commercialmente importanti, finendo così per presentare un rischio anche per l'essere umano quando vengono consumate. Le specie ittiche d'allevamento non fanno eccezione poiché i sistemi di acquacoltura possono diventare suscettibili alla contaminazione da MPs, derivanti dall'uso di strumenti di plastica come reti, da mangimi per acquacoltura (per esempio la farina di pesce) o direttamente dall'acqua. Esistono molteplici tecniche per rimuovere le MPs dai sistemi idrici e molte di esse sono già applicate negli impianti di trattamento delle acque reflue/potabili. Tuttavia, l'uso di queste tecniche nei sistemi di acquacoltura è scarsamente implementato e, allo stesso tempo, le strategie che mirano specificatamente a mitigare gli effetti delle MPs sui prodotti dell'acquacoltura come l'uso di antiossidanti sono promettenti ma ancora poco utilizzate. In particolare, l'uso di ASX micro-incapsulata per mitigare gli effetti della contaminazione da MPs è stato finora poco esplorato, ulteriori studi su questa nuova tecnica e sugli effetti su diverse specie ittiche sono quindi necessari per capirne le potenzialità.

Cercando di acquisire ulteriori conoscenze su questo metodo innovativo, applicato su una specie comunemente allevata (la trota iridea *Oncorhynchus mykiss*), gli obiettivi di questa tesi sono:

1) la produzione di due diete sperimentali, aggiungendo microsfere fluorescenti di MPs (dimensione 1-5 µm) a una concentrazione di 50 mg/kg, da sole o in combinazione con ASX micro-incapsulata (7 g/kg di microcapsule di ASX corrispondono a 175 mg/kg di ASX), a una dieta base (CTRL) per trota iridea (*Oncorhynchus mykiss*)

2) testare le diete sperimentali per un periodo di 60 giorni

3) valutare il grado di accumulo di MPs nei tessuti bersaglio (muscolo, fegato, intestino) e le risposte fisiologiche con analisi molecolari e istologiche + valutare l'effetto della somministrazione di ASX micro-incapsulata nell'attenuare gli effetti negativi dell'accumulo di MPs sugli organismi.

Nei gruppi alimentati con le diete sperimentali, le MPs sono state assorbite a livello intestinale e hanno raggiunto altri tessuti tramite il sistema circolatorio: in particolare, il fegato è stato l'organo più colpito dall'accumulo di MPs. Il fegato ha infatti agito da "trappola" per le MPs, trattenendole e limitandone la diffusione negli altri tessuti, soffrendo però effetti negativi per la loro presenza (stress ossidativo ed accumulo di lipidi).

Riguardo alla somministrazione di ASX micro-incapsulata i risultati sono promettenti, infatti il gruppo (A50-ASX) nutrito con la dieta contenente sia MPs che ASX ha mostrato un minor accumulo di MPs nei tessuti, minori alterazioni istologiche a livello del fegato ed intestino ed una minore espressione di geni legati allo stress ossidativo rispetto al gruppo nutrito con la dieta contenente solo MPs (A50).

Quindi è deducibile che il ridotto assorbimento di MPs è dovuto all'effetto coagulante dell'amido presente nelle capsule, che ha favorito la creazione di coaguli di MPs troppo grandi per essere assorbiti dall'intestino, mentre l'ASX contenuta nelle capsule ha espletato la sua funzione antiossidante, queste due azioni combinate hanno quindi ridotto assorbimento, alterazioni e stress ossidativo, migliorando il benessere del pesce e dimostrando le potenzialità della somministrazione di ASX micro-incapsulata nel mitigare gli effetti negativi delle contaminazioni da MPs.

SUMMARY

1 INTRODUCTION

1.1 Microplastics in the environment

Plastics include a wide spectrum of synthetic or semi-synthetic materials composed of polymers. A polymer is a compound that is created by interconnecting through chemical reactions four or more monomers, among which some of the most common used to make plastics are ethylene, propylene, and vinyl chloride. Most plastics are produced from fossil fuel-based chemicals like petroleum, however more recent industrial methods can also use compounds like coal, cellulose, and natural gases. Plastics are one of the most widespread and used materials but the huge success and dominance of plastics (mainly starting in the early 20th century) has caused numerous environmental issues. This is especially due because plastics decompose very slowly and tend to persist in the environment. Most plastic produced has not been reused, or is incapable of reuse and, of all the plastic discarded so far, around 14% has been incinerated and less than 10% has been recycled, with the rest mostly ending up in landfills or getting dispersed in all the terrestrial and aquatic ecosystems (Geyer, 2020). Particularly, the marine environment is deeply affected by plastic pollution, with an input of around 12.2 million tonnes of plastics per year (Jerneia, 2018). As previously stated, plastics can persist in the environment for a long time with a rate of degradation depending on many environmental factors that can be abiotic or biotic. Abiotic factors include UV radiation, temperature, physical stress, and oxidation, whilst biotic ones are represented by action of micro-organism like bacteria (Shaji et al, 2024) or some marine fungal strains, such as *Zalerion maritimum*, that have been shown to have the ability to degrade polyethylene (Zeghal et al, 2021). The rate of degradation of plastics in the sea is further reduced in deep water environments where the absence of light, the low temperatures and the relatively stable conditions impede the action of most of the abiotic and biotic factors that contribute to plastic degradation. In addition, deep sea sediments are a likely sink for microplastics (MPs). In fact, MPs from the Atlantic Ocean, Mediterranean Sea and Indian Ocean reached a density in deep-sea sediments up to four times higher (unit per volume) compared to MPs in contaminated sea-surface waters (Woodall et al, 2014).

Regarding MPs, the most currently used and accepted definition is that MPs are fragments of any type of plastic less than 5 mm in length, according to the U.S. National Oceanic and Atmospheric Administration (NOAA) and the European Chemical Agency. MPs can be differentiated in two different categories, primary and secondary ones. Primary microplastics are intentionally created at in their microscopic size, usually for commercial use like in cosmetics. Differently, secondary MPs are created from the breakdown of larger plastic items, like through the action of degrading forces like physical abrasion or UV radiation (Van Cauwenberghe et al, 2015). There are different ways MPs can enter the ocean, most often through urban and industrial wastewater, but they can also originate from fishing activities, the production and transportation of plastic, the poor plastic waste management as well as from waste incineration, exhaust emission, road and tire abrasion and resuspension of dust which can later precipitate into the sea.

Marine MPs are dispersed throughout the water column by wind and currents, with their depth distribution influenced by density (Vermeiren et al., 2016) with the benthic environment exhibiting the highest accumulation of these particles.

MPs can consequently affect the benthic fauna: for example, it was shown that individuals of *Corallium rubrum* exposed to MPs suffered a multitude of impacts from mechanical damage (es. tissue abrasion) to impaired feeding as a consequence of ingestion of microplastics, either directly or indirectly by feeding on contaminated prey (Corinaldesi et al, 2021). Microplastics ingested by planktivorous organisms can be transferred through the food web and reach organisms at higher trophic levels including apex predators. However, by investigating the number of MPs found in organisms of different trophic levels it's suggested that MPs do not bio-magnify, and that organisms at lower trophic levels are more likely to be contaminated by microplastic pollution (Walkinshaw et al, 2020). Nevertheless, it’s important noting that MPs have been found in many species of commercial interest from different trophic chain levels, including Red mullet (*Mullus barbatus*) and European hake (*Merluccius merluccius*) (Giani et al, 2019) as well as large predators like swordfish

(*Xiphias gladius*) and different species of tuna like Bluefin tuna (*Tonnus thynnus*) and Albacore *(Tonnus alalunga*) (Romeo et al, 2015) all sampled in the Mediterranean Sea.

Wild species of commercial interest often contain MPs, raising serious food safety concerns and potential health risks for consumers. This risk extends to farmed fish, including those from mariculture in coastal areas exposed to environmental MPs, and those from inland aquaculture systems, which face various potential sources of MPs contamination. On top of that, aquaculture is becoming more and more important as a source of fish-based food (FAO, 2024) so the need to analyse interactions between MPs and aquaculture systems becomes more necessary than ever.

1.2 Microplastics in aquaculture

Aquaculture involves the breeding, rearing, and harvesting of fish, shellfish, algae, and other organisms in all types of water environments (NOAA, 2024). The aquaculture industry has been growing steadily in the past years; in 2022 and for the first time in history, aquaculture surpassed capture fisheries as the main producer of aquatic animals. Global aquaculture production reached an unprecedented 130.9 million tonnes, of which 94.4 million tonnes are aquatic animals, 51% of the total aquatic animal production (185.4 million tonnes) (FAO, 2024). This growth of the aquaculture industry, which is particularly high in developing countries, is likely stimulated by the increase in demand of fish-based products, coupled with the overexploitation of wild fish stocks, making it difficult to satisfy the growing demand with traditional fishing (De Silva, 2012). It's possible to divide aquaculture in different categories and with 2 different approaches, basing the division either on the location of the facilities/systems (inland or in the sea) or by the quantity of human intervention necessary to run the systems. When talking about human intervention (and consequently, the production density of the system), the categories are usually 3: (i) extensive, with the least amount of human interference (even the feeding and stocking is avoided: the system usually provides for itself and routine work is only required during the harvest); (ii) semi-intensive (which may require feeding and stocking depending on the type of system); (iii) intensive, with the highest amount of work

required and the highest production density (Oddson, 2020). In the case of coastal/mariculture systems compared to the inland ones, it's known that coastal systems are some of the most affected by microplastic pollution, being exposed to a wide range of different MPs contamination. The most important type of MPs contamination is usually caused by textile fibres, that can arrive from wastewater treatment plants in the nearby areas through discharge of water, either directly into the sea or into rivers that act as a medium (Akarsu et al, 2020). This puts mariculture systems, that are based on the cultivation of marine organisms in their natural habitat such as estuarine, brackish, and coastal waters (Laird, 2001) to a high risk of exposure to MPs contamination. Analysis done on a Chinese bay where mariculture has been historically practiced have shown high level of MPs in the water (higher than other chinese seas) and in all the sampled fish and oysters in the area. The main causes were the inflowing rivers (coming from an area with high anthropogenic activity), the geographical characteristics of the bay (being a semi closed bay with poor water exchange promotes accumulation of MPs in the area) and the equipment and structures used in the mariculture systems (Zhu et al, 2019). Other studies have shown that a high percentage of MPs composition sampled from seawater (55.7%) and sediment (36.8%) from a bay with a high mariculture presence is coming from various plastic gear used in the mariculture systems in the area. The most common type of microplastic found was polyethylene (PE) foam which matches with the high usage of the component in the systems (Chen et al, 2018). Microplastics were also found in cultured bivalves such as Pacific oyster (*Crassostrea gigas*) and Blue mussel (*Mytlus edulis*) that were grown in open seawater systems like mussel rafts (Van Cauwenberghe and Janssen, 2014). Bivalves are at high risk of microplastic contamination due to their filter-feeding mechanism and since they are often ingested whole, they pose an increased risk of MPs contamination to the consumer (Quaglia et al, 2023). Microplastic contamination in mariculture systems has been also found in shrimp-culturing ponds (Chen et al, 2020), in macroalgae cultures (*Pyropia yezoensis*), as well as in several fish species of commercial interest reared through mariculture (Feng et al, 2020).

Inland aquaculture has not been as deeply analysed for MPs contamination as mariculture. However, it is subjected to several potential MPs sources that can interest the health and quality of farmed fish. First, aquaculture consumes a lot of water, which can be obtained from nearby rivers and lakes, from groundwater and springs or from coastal and marine waters. MPs present in the source water can then be transported to aquaculture systems. In addition, various plastic products are widely used in aquaculture, such as ropes, fishing nets, cages, fences, foam floats, and containers; weathering and wear of those plastic products may also contribute to microplastic pollution. Finally, in the case of open-air systems like aquaculture ponds, atmospheric deposition is another potentially important pathway for the input of MPs. (Wu et al, 2022; Mofijur et al., 2021). The packaging of the aquafeed is one more source of MPs introduction in the aquaculture systems: the feed bags, usually made of single-layer plastic composed of PE and PP, are often damaged during transportation resulting in the generation of tiny plastic particles that can mix into the feed ingredients. Processes in the aquafeed production like crushing, sieving also contribute to contamination by making MPs from both ingredients and the environment become finer in size and larger in quantity. Furthermore, elevated temperatures during the production can lead to the release of considerable amounts of micro-plastics from plastic packaging/containers (Su et al, 2024). MPs contamination may also come from the aquafeed ingredients themselves. Different kind of ingredients can be used for aquafeeds, like from plant sources such as soybean meal, rice bran, wheat bran and wheat four, from terrestrial animals (es. poultry-by product meal) or from fish, also known as fish meal (FM). In a study, MPs were found in all the aforementioned types of aquafeed, poultry by product was the type of ingredient with the highest MPs concentration followed by FM and soybean meal. Polypropylene was the most common polymer found, which is reasonable since it's one of the most used polymers, utilized for the packing, for the creation of synthetic fibres and is even commonly used in agriculture, for example in plastic mulch or irrigation components (Mohsen et al, 2024). Regarding FM, it's a product made usually into powder by cooking, pressing, drying and crushing fish trimmings/byproducts or whole fishes directly, such as sardine, herring and anchovy. It is suggested that the abundance of MPs in fish meal should

be related to the feeding habits and strategies of the marine fish species used for FM production (Wang et al, 2022). When three Malaysian commercial brands of FM were analysed, 366 particles were found, with over 50% being plastic polymers, a quarter being pigment particles, and the dominant form of MPs being fragments, which is usually the most common type found in aquatic ecosystems. The dominant fish species used in the production of the FM was the Indian mackerel (*Rastrelliger kanagurta*) and it's likely that the high MPs presence was linked to the species lifestyle and feeding habits. The Indian mackerel usually lives in coastal waters, where MPs are commonly found due to dense coastal populations along with sewage discharge (which we have seen can provide a direct source of MPs into the marine environment), the species also mainly feed on zooplankton and MPs ingestion by zooplankton has been widely demonstrated. Moreover, some fish meals producers instead of the whole fish prefer to use commonly discarded fish parts such as viscera, head, and gills as the major component (Karbalaei et al, 2020), this may result in a higher concentration of MPs in the aquafeed since both the gills and the digestive system of fish are organs that are inclined to accumulate MPs (Amponsah et al, 2024).

1.3 Effects of microplastics on farmed fish species

Because of their small size and poor biodegradation, MPs can be ingested by organisms and accumulate in various tissues. First thing, it should be noted that the harmful effects of MPs accumulation have been detected even in other non-fish commonly cultured animals: results showed that microplastics ingested by the Manila clam *Ruditapes philippinarum* accumulated in their gills, hepatopancreases and intestines. The accumulation increased the rates of respiration and excretion while significantly decreasing feeding and absorption efficiency (AE), leading ultimately to a slower growth (Jiang et al, 2022). In the edible mussel *Mytilus galloprovincialis* exposure to microplastics was associated with disruption of mussel global homeostasis resulting in the production of stress and immune-related proteins and therefore, a diminution of energy allocated to growth. The expression of stress-related proteins continued after the exposure event suggesting the persistence of

physiological stress and physical damage caused by MPs (Détrée et al, 2018). Accumulation of MPs in the organism causes a variety of harmful effects, like alteration of lipid and carbohydrate metabolism, toxic effects on the immune system, alteration of toxic excretion capabilities and induction of the oxidative stress response. Particularly, several studies have shown that microplastics can change the metabolic state of fish, promote the production of reactive oxygen species (ROS), and then induce oxidative stress response (Wu et al, 2023). Exposure to environmental MPs (0.1 μm polystyrene-MPs) in the red tilapia (*Oreochromis niloticus*) caused the accumulation of MPs in several tissues like gut, gills and liver. Moreover, due to the small size of the plastic particles, MPs were able to enter the circulatory system and reach even the brain. In the brain, the acetylcholinesterase (AChE) activity was inhibited by MPs exposure, with a maximum inhibition rate of 37.7%, suggesting the potential neurotoxicity of MPs to freshwater fish (Ding et al, 2018). When ingested, MPs may also affect the growth of the fish. In a study, to evaluate the potential toxic effects of PVC (polyvinyl chloride) microplastics in freshwater fish larvae, common carp (*Cyprinus carpio*) larvae were exposed to PVC MPs in their diet for a 30- and 60-day period, with various concentrations (10%, 20%, and 30%). The results indicated that MPs initiated histological changes in the liver, induced oxidative damage to the liver, intestine and gills and significantly inhibited weight gain and growth in all the treatment groups. (Xia et al, 2020). Effects on the reproductive systems were confirmed as well, since exposure to MPs could delay gonad maturation, decrease fecundity, and decrease the concentrations of 17β-estradiol (E2) and testosterone (T) in the plasma of female Medaka (*Oryzias melastigma*) (Wang et al, 2019). Microplastics have been reported to absorb pollutants such as persistent organic pollutants, heavy metals, and antibiotics (Rafa et al, 2024). Because of this, ingestion of MPs represents a pathway for fish exposure to potentially hazardous chemicals. Most of them are endocrine disrupters, genotoxic or induce immune depression in fish. In a study, European sea bass (*Dicentrarchus labrax)* juveniles were fed for 60 days with feeds containing polypropylene MPs, either virgin or contaminated with chemical pollutants (like dichlorodiphenyldichloroethylene). The data demonstrated a synergic action of MPs and chemical pollutants to induce an inflammatory-

like response in distal intestine of sea bass, made evident by indicators like upregulation of cytokine il-6, tnf-α gene expression, increase of lymphocytes and intestinal dysbiosis (Montero et al, 2022). Pollutants carried by MPs may also affect the aquaculture systems in indirect ways, studies have shown that pollutants like MPs and TBBPA (tetrabromobisphenol A) significantly suppressed the motility and the growth of microalgae *Platymonas subcordiformis* and *Dicrateria zhanjiangensis,* this was likely caused by the imposed significant oxidative stress and the disruption of photosynthesis and glycolysis caused by the pollutants. In addition to being primary producers of the marine ecosystem, these two microalgae species are also major feed species for the aquaculture of shellfish and finfish, therefore ecological balance and food availability for cultured fish may end up being affected as well (Zhang et al, 2022). MPs may also damage aquaculture systems by also altering the microbial communities. It was effectively shown that MPs may increase the abundance of antibiotic resistance genes (ARGs) in aquaculture environments resulting in increased potential risks of reduced effectiveness for antibiotics. Analysis done on recirculating aquaculture system farms (RAS) have revealed on samples of MPs a higher abundance of ARGs compared to samples of water; microbial community diversity of MPs was higher than that of water, too. The results suggested that MPs might be an important reservoir of antibiotic resistance genes in RAS (Lu et al, 2019). However, in general, a lot of studies focus their attention on the effects of MPs when they get ingested through the diet and MPs contamination by fish feed, since it's potentially one of the most important contamination routes in aquaculture (Zhou et al, 2019). A primary significant impact from MPs ingestion is observed even during the fish larval stage. In this phase, fish may mistakenly feed in plastic particles, confusing them with their natural zooplankton prey (Ma et al., 2020) and this can result in gastrointestinal blockages and reduced predatory behaviour due to a false sense of satiety. Particular attention should be paid to the size of the plastic particles since it affects how they travel and accumulate in the organism and what kind of damage they deal. In a study by Cattaneo et al., 2023, zebrafish (*Danio rerio*) was exposed to two types of dietary MPs: polymer A comprised of plastic microbeads with a size ranging 1-5 μm and polymer B with microbeads of size range 40-47 μm. The results of the study

showed that in the tissues (intestine, liver, muscle) of fish where polymer B (40-47 μm) was administered through the diet next to no MPs were found, likely because MPs of this size are too big to be absorbed by the intestine and simply transit in the gut lumen without being assimilated. Shortened mucosal folds and an increase of goblet cells were observed indicating an abrasive effect of MPs at an intestinal level and as a consequence an increase in lubrification to facilitate the passage of MPs in the intestine. Other studies with MPs of even bigger size (100-400 μm) confirmed the limited impact of greater size plastic particles, usually limited to abrasive and disturbance of the extrinsic mucus barrier properties (Ašmonaitė et al, 2018). The study by Cattaneo et al., 2023 also showed the accumulation of polymer A (1-5 μm) in the intestine, liver and muscle tissue of zebrafish. This was possible because smaller size particles were able to be internalised by the fish intestinal folds likely through endocytosis or pinocytosis processes, then through blood circulation they can reach different tissues (Liu et al, 2021, Guerrera et al, 2021). However, the liver is able to act as a sort of trap for MPs, working as an "accumulation organ" and limiting MPs translocations to other tissues, like muscle or adipose tissue. While even the intestine doesn't end up too much affected by the MPs passage, acting simply as a "transit organ", the MPs accumulation in the liver has repercussions as demonstrated by the upregulation of genes involved in the oxidative stress response (sod.1, sod.2, and cat) obtained from molecular analysis on liver samples), all signalling oxidative stress on the liver tissues (Cattaneo et al., 2023). Oxidative stress is defined as a disturbed balance between oxidation and antioxidant systems, and the production of reactive oxygen species (ROS) reflects an organism's capacity to deal with such stresses. In general, superoxide dismutase (SOD), catalase (CAT) are considered as enzymes of the first line of defence that directly eliminate ROS, therefore being good markers to indicate oxidative stress response. Even other processes like lipid peroxidation are involved in oxidative stress events (Lyu et al, 2020). Other studies in which zebrafish (*Danio rerio*) was exposed to different sizes MPs exhibited similar results, with only the smaller size plastic particles being able to accumulate in the liver and causing oxidative stress response (Du et al, 2023). This happened even when the MPs particles where not administered through the diet but

environmentally, indicating a strong link between the MPs size and the transfer in the fish tissues (Lu et al, 2016). Furthermore, the are multiple other studies that demonstrated how MPs tend to accumulate in the liver and cause oxidative stress as one of the most common disorders, in different species as well, like in Large yellow croaker (*Larimichthys crocea*) (Li et al, 2021) and rainbow trout (*Oncorhynchus mykiss*) (Hollerova et al, 2023).

In a study conducted on Gilthead seabream (Sparus aurata), the highest levels of oxidative damage, as a result of MPs ingestion, were found in the blood and, not surprisingly, in the liver. The recovery time for the upregulated genes was also tested, while some of them required approximately 60 days others, such as glutathione-S-transferase (another common gene upregulated during oxidative stress events), required more extended periods, around 120 days (Capó et al., 2022). Even with the liver acting as natural barrier against MPs contamination in the other tissues, a small percentage of MPs may still reach the muscle. For example, it was demonstrated in European seabass (*Dicentrarchus labrax*), a common commercial species, that MPs provided in the diet (size range of 1-5 μm) were able to translocate to the fillet. Even if the levels of translocation in the fillet were low compared to the high levels of ingestion in the study, it still shifts the focus of MPs contamination onto potential risks for human health (Zeytin et al., 2020). Therefore, techniques to mitigate the MPs contamination/impact in aquaculture systems are recommended.

1.4 Methods for MPs removal and mitigation strategies

To handle the problem of microplastic pollution in the aquaculture systems, multiple solutions have been proposed with the scientific community continuously trying to find new advancements. The removal of MPs before they enter the aquaculture systems is often suggested, like supplementary treatments of water coming from water treatment plants aimed to reduce MPs specifically (Iheanacho et al, 2023). The replacement of the plastics in the equipment, gear and tools used in aquaculture with natural materials was also suggested, for example materials such as coconut shell, bamboo stick, and dry activated charcoal can be used as alternative biofilters in place of the popular commercial plastic

ones (Mnyoro et al., 2022). Many types of techniques have been developed to remove MPs from the water, including filtration, adsorption/magnetic removal, coagulation, chemical oxidation and biodegradation (Gao et al, 2022). MPs removal by coagulation is a process that aims to make the microplastics sediment or to floc together obtaining larger masses that can be more easily removed and is already commonly used in drinking water treatment plants, thanks to the low costs and operational simplicity. It's carried out by three different mechanisms: charge neutralization, adsorption bridging and sweep flocculation. The process usually works by combining two or more mechanisms and utilizes different kinds of coagulants that can be inorganic, organic (polymer based) or natural/biological (Tang et al, 2022). Inorganic coagulants are usually metals with iron and aluminum-based ones being the most commonly used (Reza et al, 2024). These inorganic coagulants may reach high removal efficiency (Rajala et al, 2020), but they are not biodegradable and persist in water unless specifically treated, creating toxic sludges and requiring a high cost for the treatment of the effluent water (Alazaiza et al, 2022). Differently, natural coagulants instead have high biodegradability, non-toxic and non-corrosive properties and the sludge that they generate is lower in volume, generally biodegradable and can be potentially used as fertilizer. Because of these advantages, materials like microbial polysaccharides, starches, cellulose derivatives, chitosan, glues, plant-based materials, and alginate, are being increasingly utilized in the treatment of water/wastewater (Girish et al, 2023). In particular, flocculants derived from starch precursor are receiving a lot of attention in the field of water treatment due to their significant advantages of environmental friendliness and biodegradability coupled with widespread availability and low costs (Asharuddin et al, 2021). Another type of strategy to deal with MPs is trying to mitigate their effects once they reach the organisms. For example, it was shown that the inclusion of vitamin D in the diet was able to alleviate the damage caused by exposure to polystyrene microplastics in zebrafish to the lipid metabolism, reducing oxidative damage and lipid accumulation (Li et al, 2023). The positive effects of vitamins (mainly vitamin C) combined with probiotics to mitigate MPs exposure were shown also in other species, like Nile Tilapia (Hayati et al, 2023). Another way to mitigate MPs

exposure with dietary inclusions could be by using antioxidants to deal with the oxidative stress caused by the MPs. Some antioxidants such as ferulic acid (Yu et al, 2020), selenium (Daowood et al, 2021), but also different kinds of vitamins (es. vitamin C), amino acids and carotenoids (Ciji et al, 2021) including astaxanthin (Elbahnaswy et al, 2024) are already commonly used in aquaculture to deal with oxidative stress, albeit coming from other sources. Astaxanthin (ASX) is a carotenoid that is already well known in the aquaculture industry for its key role in the pigmentation of some aquatic animals, including rainbow trout (*Oncorhyncus mykiss*), Atlantic salmon (*Salmo salar*), and several ornamental fish and crustacean species. Once integrated in the diet, ASX deposition in the flesh results in a decrease in lightness and an increase in redness and yellowness in the fillet (Rahman et al, 2016) making the final product more appreciable to the buyer. Additionally, ASX supplementation may increase reproductive performance (Ahmadi et al, 2006) and gamete quality (Tizkar et al, 2015), can enhance growth performance and survival rates (Hansen et al, 2016) and boost disease and stress resistance thanks to its antioxidant properties (Liu et al, 2016). A distinction should be made when talking about ASX, considering mainly how it's produced. We talk about natural astaxanthin when it's obtained by extraction from sources like microalgae es. *Haematococcus pluvialis* (the most common natural source) (Mota et al, 2021), *Chlorella zofingiensis, Chlorococcum* sp., other microorganism like yeasts and bacteria or even higher plants (Stachowiak and Szulc, 2021). Synthetic astaxanthin is instead obtained by synthesis from petrochemicals and is the type that is most commonly used as a feed ingredient, primarily to pigment the flesh of salmonids in aquaculture. Synthetic ASX has proven to be way less effective compared to natural ASX when it comes to antioxidant capabilities (Capelli et al, 2014) but is still the most common used type in aquaculture for two main reasons. The first is that natural ASX is extremely more costly to produce compared to its synthetic counterpart (Panis and Carreon, 2016). The second one is that natural ASX has a limited application because of its poor water solubility and low stability making it not generally suitable for most industrial processes. Therefore, to take advantage of natural ASX antioxidant properties methods to mitigate such problems become a necessity (Yao et al, 2023). In this regard, encapsulation

technology can be very effective for enhancing the solubility, stability and bioavailability of natural ASX. Many different encapsulation methods currently exist: oil in water (O/W) emulsions, high pressure homogenization, lipid nanodispersions, membrane and microchannel emulsification and different drying methods like spray or freeze drying. (Khalib and Barrow, 2018). For the last method, after being dried ASX gets turned into power, then different materials can be used to create microcapsules to contain it. A series of wall materials that can be used to create the microcapsules include chitosan (Higuera-Ciapara et al, 2004), maltodextrin, starch (Sharayei et al, 2024) or arabic gum (Pan-Utai et al, 2021) and more. Among the materials used to produce microcapsules, starch has recently gained attention due to its MPs coagulation properties (Gao et al, 2023). This opens up interesting possibilities to couple the use of natural astaxanthin with a nature-based microencapsulation technology (i.e. using starch as a wall material) able to preserve the antioxidant molecule and, in turn, coagulate MPs particles in the fish gut. This technique may turn out to be unexpectedly effective also because of the fact that the coagulating properties of starch are enhanced in acidic conditions, like the ones in carnivorous fish stomachs (Girish et al, 2023; Asharuddin et al, 2021) Very recent studies highlighted interesting results using microencapsulated ASX to reduce the adverse effects of dietary MPs in both freshwater, zebrafish (*Danio rerio),* and salt water, European seabass (*Dicentrarchus labrax*), fish species. Particularly, in both of studies, the antioxidant properties of astaxanthin were able to mitigate the dietary MPs-induced oxidative stress response, whilst the starch present in the wall matrix of the microcapsules exerted a coagulation effect on the MPs in the fish gut, reducing their absorption and consequent translocation to other tissues (Zarantoniello et al, 2024) (Cattaneo et al, 2024). This technique was comparatively less effective in zebrafish because of the lack of an acidic stomach in the species but was still overall successful when utilized with higher MPs quantities in the diet. The potential of this innovative method is therefore very high and should be studied and experimented upon further, with particular attention in testing it in other species with different lifestyles and feeding habits.

2. AIM OF THE THESIS

Plastics in today society are pretty much ubiquitous thanks to their many properties like the low costs and the high versatility. Unfortunately, the difficulty in disposal has made plastic one of the main pollutants that can be found in the environment, including marine ones. Plastic after being dispersed can turn into microplastics and once they enter the water systems, they can be uptaken by organisms, including many commercially important fish species and thus ending up posing a risk to the human consumer as well. Farmed fish species are no exception since aquaculture systems can become susceptible to MPs contamination, coming from the usage of plastic tools like nets, from aquafeeds (fish meal in particular) or directly from the water. Multiple techniques exist to remove the MPs from water systems with many of them already being applied in waste/drinking water treatment plants. However, the usage of these techniques in aquaculture systems has been barely implemented and at the same time, strategies specifically aiming to mitigate the effects of MPs on aquaculture products like the usage of antioxidants are promising but still underutilized. In particular, the usage of microencapsulated ASX to mitigate the effects of MPs contamination has been barely explored currently, further studies on this new technique and on the effects on different fish species are therefore necessary and can provide new interesting insights.

By seeking to gain further knowledge on this innovative method, the aims of this thesis are:

1) the production of two experimental diets, adding fluorescents MPs microbeads (size 1-5 μm) at a concentration of 50 mg/kg, alone or in combination with microencapsulated ASX (7 g/kg of ASX microcapsules corresponds to 175 mg/kg of ASX), to a base (CTRL) diet for rainbow trout (*Oncorhynchus mykiss*)

2) testing the experimental diets during a 2-month feeding trial

3) evaluating the degree of MPs accumulation in target tissues (muscle, liver, intestine) and the physiological responses with molecular and histological analysis + evaluating the effect of administered microencapsulated ASX in mitigating the negative effects of MPs accumulation in the organisms

3. MATERIALS AND METHODS

3.1. Experimental model

Rainbow trout (*Oncorhynchus mykiss*) is a species of the family Salmonidae native to North America and Asia. It inhabits the coastal waters and the tributaries of the Pacific Ocean with the native range starting from the Russian peninsula Kamchatka and reaching the northern parts of Mexico, crossing through the Aleutian Islands. Rainbow trout usually inhabit and spawn in small to moderately large, well-oxygenated, shallow rivers with gravel bottoms or also in deep and cool lakes, while still requiring access to gravelly-bottomed streams to have a self-sustaining population. Some subspecies of rainbow trout, mainly the coastal rainbow trout (*O. m. irideus*) and the Columbia River redband trout (*O. m. gairdneri)*, may also present anadromous populations, commonly called steelheads. Steelheads, like salmons, return to their hatching grounds to spawn. They are iteroparous so they can make several spawning trips from fresh to saltwater during their lifetime. Juvenile steelheads may remain up to three years into the river before migrating to the sea. Steelhead and lake dwelling trout may reach much greater sizes compared to the ones that only live in rivers (9 kg compared to 2.5 kg max). The coloration also changes, with trout living in streams usually showing a blue-green coloration with heavy black spotting over the length of the body and a reddish stripe along the lateral line (only presents in adults and especially showing on breeding males) while lake and anadromous forms are usually silverier in color with the reddish stripe mostly gone. Rainbow trout are predators, their diet includes insects (aquatic or accidentally fallen terrestrial ones), fish eggs, small fish, crayfish and other crustaceans. Rainbow trout have been cultured since 1870 mainly to restock wild populations in the USA. Shortly after commercial farming also started, increasing dramatically worldwide after the 1950s. The largest producer is Chile (mainly marine-grown), but inland production of rainbow trout to supply domestic markets has increased in countries such as Italy, France, Germany, Denmark, and Spain. Usually, in rainbow trout farms, gametes are released during the fall and winter season and females produce around 200 eggs per kg of weight. For an optimized gamete production, the dry method of fertilisation without admixture of water is the most common

approach. Eggs are removed manually from females by applying pressure from the pelvic fins to the vent area or by air spawning. Males are stripped in the same way as females, collecting milt in a bowl, then mixing it with the eggs (milt from more than one male is used, generally 0.5 – 1.5 mL for 0.5 L of eggs). Water is added to activate the sperm and cause the eggs to increase in size by about 20% by filling the perivitelline space between the chorion and yolk; a process known as "water-hardening". Eggs are then moved in incubators, the most used ones being hatching throughs, to maximise the chance of survival of the embryo. The hatching throughs have eggs placed in wire baskets or screened trays with water passing through the tray at 10 L/min. As the eggs hatch (usually after 30/32 days), the fry drop through the mesh to a bottom trough while impurities and unhatched eggs remain on the tray and can be easily removed (this is important to avoid fungal infections). Fry do not start eating until 2/3 of the yolk sack is re-absorbed (which completely happens in a 2 to 6 weeks periods). Fries are initially reared in fiberglass or concrete tanks (1 to 25 m^2) and after 12 weeks they are moved in bigger tanks, separating them by size to avoid cannibalism. Rainbow trout other than being considered a good tasting fish are also very popular among anglers as a game fish. This, coupled with the high availability from farming, has made rainbow trout one of the most used fish for restocking streams and lakes in many parts of the worlds, introducing the species far beyond is native range. This has caused problems to the native fish populations either for direct competition but also for the introduction of diseases like whirling or redmouth disease.

3.2. Ethics

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

3.3. MPs features

Amino formaldehyde polymer (FMV-1.3) fluorescent microbeads with a size range of 1-5 μm and characterized by an emission peak of 636 nm and when excited at 584 nm, were purchased from Cospheric LLC (Goleta, CA, USA). Before being included in the experimental diets preparation, MPs fluorescent microbeads, hydrophobic in their pristine state, were resuspended in a 0.1% tween-80 solution as a surfactant (Merck KGaA) and then rinsed with deionized water for three times according to Cattaneo et al. 2023.

3.4. Astaxanthin and microcapsules composition

Natural ASX (AstaReal® L10, Nacka Sweden) was microencapsulated by the STM Aquatrade S.r.l. (Ancona, Italy) using a new technology called Co.M.E. (Coating Made Easy), developed within a private project called +POP (Powder on Pellets; STM Aquatrade S.r.l). The special matrix of the microcapsules wall was composed by gum arabic (55%), starch (22%) and, in a smaller fraction, of cellulose, sodium ascorbate, and vitamin E, listed in decreasing order according to their relative abundance. The procedure of microcapsules' preparation cannot be fully disclosed due to intellectual property protection. The encapsulation process ensures both the protection of the carried molecule (ASX) as well as the maximum deliverability to the feed. In fact, this technology promotes perfect adhesion between the dry microcapsules to any aquafeed thanks to specific physical and chemical interactions. Due to their chemical properties, the microcapsules released their contents within approximately 90 seconds after entering the water. All products used to prepare the microcapsules are safe for humans and animals as reported from the technical data sheet and the Material Safety Data Sheet (MSDS). The provision of 1 g of microcapsules filled with ASX (ASX microcapsules) corresponds to a net transfer of 25 ppm of astaxanthin to the feed (equivalent to the 2.5% of the microcapsule composition.

3.5. MPs coagulation *in vitro*

Coagulation tests were carried out by preparing stock solutions at pH 3.7 (for the stomach) (Jung et al, 2023) and pH 7.7 (for the intestine) (Fard et al, 2007) to mimic the stomach and intestinal conditions of rainbow trout after feed intake. Coagulation experiments were performed according to the method determined by Zarantoniello et al. 2024. The results obtained are not presented in the current study since they are similar to the results of the MPs coagulation tests on European seabass performed by Zarantoniello et al. 2024.

3.6. Experimental diets

All the diets were formulated and prepared starting from identical batches of individual ingredients. A control diet (named CTRL, free of fluorescent MPs microbeads) was formulated to match the proximate composition of a commercial diet intended for rainbow trout. The MPs diet, that will be called A50 was prepared by including the fluorescent MPs microbeads in the control mixture diet at a concentration of 50 mg/kg feed. Before being included in the preparation of the MPs-containing diets, fluorescent microbeads, hydrophobic in their pristine state, were resuspended in a tween-80 (Merck KGaA) solution (0.1%) as a surfactant and subsequently washed three times using deionized water, according to the company's technical support suggestion.

All powdered ingredients used to produce the test diets were well mixed (GastroNorm 30C1PN, ItaliaGroup Corporate Srl, Ponte nelle Alpi, Italy) for 20 min, adding oil and water into the blend to achieve the appropriate consistency suitable for pelleting. Water was then used to include fluorescent MPs microbeads in the mixture. Using a 3 mm die meat grinder, pellets were obtained and dried at 37 °C for 48 h in a ventilated heater and then vacuum-stored.

To confirm the absence of eventual contamination of fluorescent MPs microbeads in the control diet (CTRL), three subsamples were checked through a confocal microscope (Nikon A1R; Nikon Corporation, Tokyo, Japan). In addition, three subsamples were subjected to chemical digestion

followed by filtration on fiberglass filters with 0.7 µm-pores (Whatman GF/A, Merck KGaA). The filters were dried at room temperature and analysed through a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) to confirm the absence of fluorescence MPs microbeads.

Starting from the above-described diets (CTRL and A50), the following ones were prepared as follows: (i) CTRL-ASX diet: 7 g/kg feed of ASX microcapsules (corresponding to 175 mg/kg of AX) were added to the CTRL diet; (ii) A50-ASX diet: 50 mg/kg feed of fluorescent MPs microbeads and 7 g/kg feed of ASX microcapsules (corresponding to 175 mg/kg of AX) were added to the CTRL diet.

To produce these diets, 1 kg batch of CTRL or A50 diets and 7 g of microencapsulated ASX were transferred to glass airtight jars and then vigorously mixed to allow them to uniformly cover the feed pellets.

The effective presence of MPs and/or ASX microcapsules in the experimental diets was checked by analysing feed subsamples (in triplicate for each diet) by a confocal microscope (Nikon A1R; Nikon Corporation) or a stereomicroscope (Leica, Wetzlar, Germany) equipped with a camera (Invenio 10SCIII, DeltaPix; Smoerum, Denmark), respectively. The aim of this study was to exclusively assess the effects of voluntarily added fluorescent MPs microbeads during MPs diet preparation, excluding the potential intrinsic microplastic contamination of the ingredients used. It was assumed that this level of contamination was consistent among all the tested diets since the basic mixture was composed of the same ingredients from the same batches. Therefore, any effect observed in fish was uniquely attributable to the added fluorescent MPs microbeads.

Finally, the dietary ASX content determination was performed on 3 subsamples of CTRL-ASX and A50-ASX diets analysed at the beginning and at the conclusion of the feeding trial, according to Du et al. (2016). An average concentration of 172 ± 6 mg/kg for both diets was detected at both sampling times.

3.7. Experimental design

Rainbow trout juveniles, provided by Eredi Rossi (Rieti, Italy), were acclimated to the experimental conditions for two weeks in a single 1000 L tank equipped with mechanical, biological, and UV filtration (Panaque s.r.l., Viterbo, Italy). Water parameters were as follows: temperature, 17.0 ± 0.5 °C; ammonia and nitrite < 0.05 mg/L; nitrate < 10 mg/L. At the end of the acclimation period, fish were collected and randomly assigned to four experimental groups (60 fish per group, 20 fish per tank) fed for 60 days as follows: (i) CTRL group: fish fed the CTRL diet; (ii) CTRL-ASX group: fish fed the CTRL-ASX diet; (iii) A50 group: fish fed the A50 diet; (iv) A50-ASX group: fish fed the A50-ASX diet.

During the whole trial, fish were subjected to a natural photoperiod (12L/12D) and water in the tanks was maintained at constant temperature (12.0 ± 0.2 °C). Fish were hand-fed the experimental diets with a daily feeding rate corresponding to 2% of the total fish body weight (divided in two equal amounts, one given in the morning and one in the afternoon). During the trial, rainbow trout 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 (60 days), after 24h fasting, all fish were collected and euthanised (lethal dose of MS222; 0.3 g/L). Liver, intestine, and muscle were dissected and immediately stored for the further analysis.

3.8. Survival rate and zootechnical performance

At the end of the 60-day treatment period, all the fish from each tank (60 per experimental group) were weighted with an OHAUS Explorer analytical balance (Greifensee, Switzerland). For each tank, survival rate (SR), relative growth rate (RGR) and specific growth rate (SGR), were calculated as follows:

SR (%) = (final number of fish / initial number of fish) × 100

RGR (%) = [(FBW - IBW) / IBW] × 100

SGR (% day $^{-1}$) = [(ln FBW – ln IBW) / days of trial] × 100

where FBW and IBW are final and initial body weights, respectively.

3.9. Samples' chemical digestion and MPs quantification

Samples of muscle, liver, and intestine were collected from 5 fish per tank (a total of 15 fish per experimental group). All the samples were weighted and then digested through a 10% KOH solution according to Cattaneo et al., 2023. The solution was added to each sample (1:10 w/v ratio) in glass tubes; samples were then incubated for 48 hours at 40 °C. After 48 hours, the digestate was filtered through GF/F Whatman® fibreglass filter papers (Merck KGaA, Darmstadt, Germany) with pore size of 0.7 μm using a vacuum pump. The filters were dried at room temperature and stocked in glass Petri dishes until the quantitative analysis. The 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 manually counted using the ZEN Blue 2.3 software (Zeiss) and the acquisition of images was made by the Axiocam 503 digital camera (Zeiss).

3.10. Histological analyses

For each tank, liver and intestine samples obtained from 5 fish (15 fish per experimental group) were fixed in Bouin's solution at 4 °C for 24 hours and then stocked at 4 °C in a 70% ethanol solution. Samples were dehydrated using 80, 95, and 100 % ethanol solution, washed with xylene, and finally embedded in paraffin (Bio-Optica, Milano, Italy). Paraffin blocks were cut to obtain sections of 5 μm thickness using a Leica RM RTS microtome (Leica, Nussloch, Germany). Sections were then stained and examined using a Zeiss Axio Imager.A2 microscope equipped with an Axiocam 105 combined color digital camera (Zeiss, Oberkochen, Germany). Intestine sections were stained with (i) Mayer hematoxylin and eosin Y (Merck KGaA; H&E) to assess mucosal folds height, supranuclear vacuolization of enterocytes, and eventual presence of inflammatory infiltration in the submucosa; (ii) Alcian Blue (Bio-Optica) to quantify the relative abundance of Alcian blue positive (Ab+) goblet cells on a 500 μm^2 surface area. The score assignment criteria for the above-mentioned parameters

are reported in Table 1. The image analysis was performed using the ZEN 2.3 software (Zeiss, Oberkochen, Germany).

Table 1. Score assignment criteria for presence of supranuclear vacuoles in the enterocytes, Ab+ goblet cells' relative abundance (on a 500 μm^2 surface area), and lymphocyte infiltration at submucosal level in the intestine.

Parameter	Score	Description
Supranuclear vacuoles	-	Absent
	+	Scattered
	++	Diffused
	+++	Highly abundant
Goblet cell relative abundance	+	Scattered cells
	++	Diffused and widely spread
	+++	Highly abundant and tightly packed cells
Inflammatory events	+	Present
	-	Absent

Liver sections were stained with Mayer's hematoxylin and eosin Y (Merck KGaA, Darmstadt, Germany) to assess hepatocyte morphology and the degree of fat accumulation through a quantitative analysis. Blood vessels and bile ducts were excluded from quantitative analysis since they are considered non-assessable areas. Results regarding fat accumulation were expressed as the percentage of area occupied by fat on the total hepatic parenchyma analyzed in the respective section. Image analysis was performed using the ZEN 2.3 software (Zeiss, Oberkochen, Germany), while the percentage of fat fraction was determined with ImageJ.

3.11. Molecular analyses

Intestine and liver samples of 3 fish per tank (9 fish per experimental group) were dissected and then stored at -80 °C. Total RNA extraction from the samples was performed using RNAzol^{TM} reagent (Merck KGaA) following manufacturer's instructions. The final RNA concentration was obtained by a Nanophotometer P-Class (Implen, München, Germany), while the RNA integrity was checked by the 28S and 18S ribosomal RNA bands staining (GelRed ™) on 1% agarose gel. The cDNA synthesis was performed with the iScript ™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) using 1 µg of total RNA.

Real-time qPCR were performed in an iQ5 iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using a 96-well plate. For each sample, 1 µL of 1:10-diluted cDNA, 5 µL of fluorescent intercalating agent (2× concentrated iQ ™ Sybr Green, Bio-Rad, Hercules, CA, USA), 0.3 µM of forward primer and 0.3 µM of reverse primer were mixed. The thermal profile was set as follows: 3 min at 95 °C and then 45 cycles of 20 s at 95 °C, 20 s at the specific annealing temperature of each primer (reported in Table 2), and 20 s at 72 °C. For each primer, the annealing temperature was optimized with a temperature gradient assay. The verification of the primer specificities was evaluated through the absence of primer–dimer formation and dissociation curves. Moreover, the efficiencies for each pair of primers were 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 fluorescence was checked at the end of each cycle, and one single peak was detected for each qPCR product in the melting curve analyses. Two no template controls (NTCs) were added in each run to guarantee absence of contamination (no peaks detected for the NTC in each reaction). Amplification products were sequenced, and homology was verified.

Relative quantification of the expression of: (i) genes involved in immune response (interleukin-1β, *il1b*; interleukin-10, *il10*; tumor necrosis factor alpha, *tnfa*) was performed on intestine samples; (ii) genes involved in oxidative stress response (superoxide dismutase 1, *sod1*; superoxide dismutase 2, *sod2*; catalase, *cat*) was performed on liver samples. Beta-actin (*β-actin*) and 60S ribosomal protein (*60s*) were used as housekeeping genes (hk) in both tissues. The primers sequences are summarized in Table 2. The mRNA levels of target genes analysed were calculated using the geometric mean of the two reference genes after demonstrating that they were stably expressed through applications implemented in the Bio-Rad CFX Manager 3.1. software. Gene transcript expression alterations 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.

Table 2. Sequences and identification numbers of primers used in the current study.

Gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)	ZFIN ID
il1 (b)	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	040702-2
il10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	051111-1
tnfα	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAA	050317-1
sod1	GTCGTCTGGCTTGTGGAGTG	TGTCAGCGGGCTAGTGCTT	990415-258
sod2	CCGGACTATGTTAAGGCCAT	ACACTCGGTTGCTCTCTTTTCT	030131-7742
cat	CCAAGGTCTGGTCCCATAA	GCACATGGGTCCATCTCTCT	000210-20
60S (hk)	TTCCTGTCACGACATACAAA	GTAAGCAGAAATTGCACCATC	DT044641.1
B-actin (hk)	AGACCACCTTCAACTCCATC	AGAGGTGATCTCCTTCTGCAT	AJ537421

hk: housekeeping genes

3.13. Statistical analysis

The tanks were used as the experimental unit for data related to survival rate and zootechnical performance, while fish were considered the experimental unit for all the remaining analyses. All data were checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). Data on MPs quantification, histological analysis, and relative quantification of gene expression were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. Significance was set at $p < 0.05$, and results were expressed as mean ± standard deviation (SD). The statistical software package Prism-8 (GraphPad Software, San Diego, CA, USA) was used for the data analysis.

4. RESULTS

4.1. Survival rate and growth rates

At the end of the feeding trial, the fish survival rate (SR) was 100% in all the experimental groups. SGR and RGR values of the experimental and control groups are presented in Table 3.

Table 3. SGR and RGR of rainbow trout juveniles fed the experimental diets

	CTRL	**CTRL-ASX**	**A50**	**A50-ASX**
SGR (% day-1)	3.2 ± 0.6	3.4 ± 0.5 a	3.3 ± 0.6	3.5 ± 0.4
RGR (%)	627.8 ± 217.1	687.7 ± 219.1 a	687.0 ± 245.0	759.4 ± 192.4

Values are shown as mean ± SD (n=60). Abbreviations: SGR, specific growth rate; RGR, relative growth rate.

4.2. MPs Quantification

MPs amounts determined in the muscle, intestine and liver of rainbow trout are presented in Table 4. Fluorescent MPs microbeads were not detected in both CTRL and CTRL-ASX. Considering fish fed MPs contaminated diets, A50-ASX group was characterized by a significantly ($p < 0.05$) lower MPs abundance in both intestine and liver samples compared to the A50 one. Differently, no significant differences were detected between A50 and A50-ASX groups in muscle samples.

Table 4. MPs quantification (number of microbeads / g) in the intestine, liver, and muscle of rainbow trout juveniles fed the experimental diets.

	CTRL	**CTRL-ASX**	**A50**	**A50-ASX**
Intestine	0	0	9.1 ± 2.1 a	3.3 ± 0.9 b
Liver	0	0	40.0 ± 4.5 a	10.9 ± 1.4 b
Muscle	0	0	1.0 ± 0.9 a	0.5 ± 0.3 a

Values are shown as mean ± standard deviation (SD) (n = 15 for each tissue). a-b Within each line, different letters denote statistically significant differences among the experimental group

4.3. Histology

No pathological alterations or inflammatory events were observed in the intestine of fish from all the experimental groups. The proper architecture of the organs was not altered by different dietary treatments. Considering the histological indexes evaluated in the intestine, no significant differences were found among the experimental groups in terms of mucosal fold height. Differently, A50 and

A50-ASX groups were characterized by an increase in the enterocytes' supranuclear vacuolization and the relative abundance of Ab+ goblet cells compared to both CTRL and CTRL-ASX ones. The results of the histological indexes analyzed in rainbow trout intestines are presented in Table 5.

Table 5. Histological indexes measured in the intestine of rainbow trout juveniles fed experimental diets

	CTRL	CTRL-ASX	A50	A50-ASX
Mucosal fold height	225.5 ± 40.4	225.8 ± 41.2	241.3 ± 41.6	226.9 ± 32.7
Supranuclear vacuoles	+	+	+ + +	+ +
Ab+ goblet cells	+ +	+ +	+ + +	+ + +
Inflammatory events	-	-	-	-

Data of mucosal folds height are reported as mean ± SD.

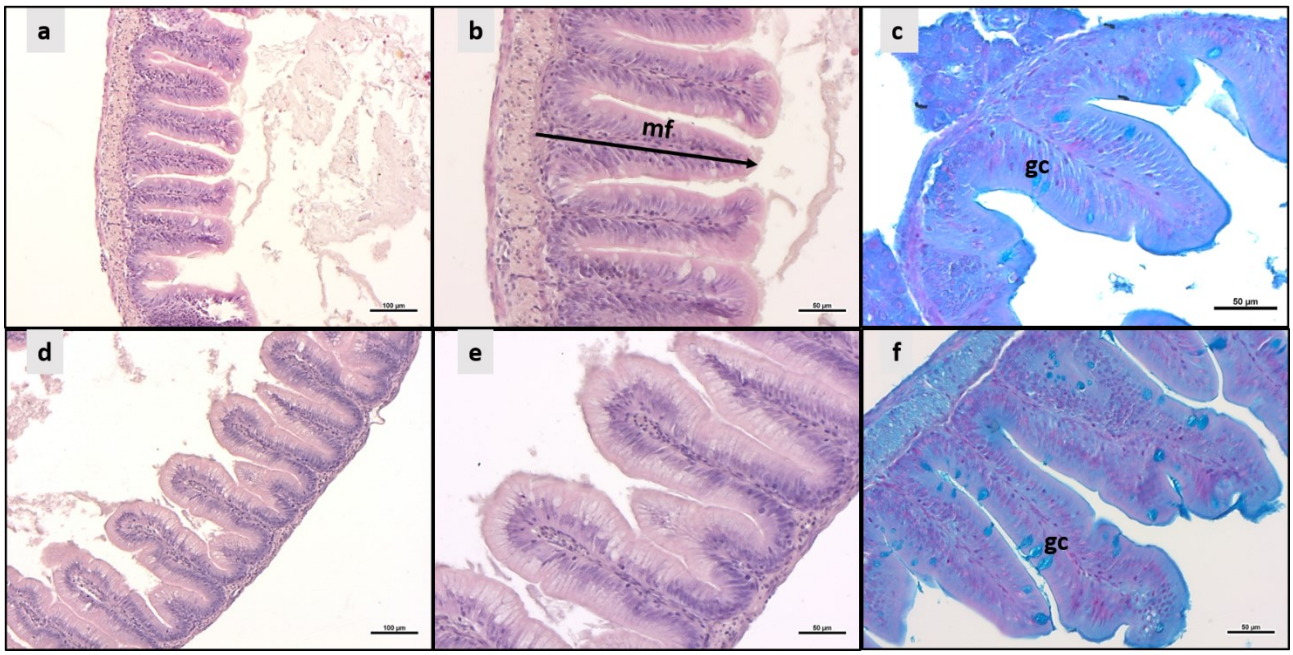

Figure 1. Example of histomorphology of intestine from rainbow trout. (a-c) mucosal architecture of fish fed CTRL diet; (d-f) details of mucosal architecture of fish fed A50 diet. Abbreviations: mf, intestinal mucosal folds; gc, goblet cells. Staining: (a,b,d,e) Mayer's Hematoxylin and Eosin Y; (c,f) Alcian Blue. Scale bars: (a,d) 100 µm; (b,c,e,f) 50 µm.

Considering the liver, all experimental groups showed the physiological structure of the hepatic parenchyma, and no signs of pathological alteration were detected. Fish from all experimental groups were characterized by a moderately fatty liver parenchyma and the widespread presence of hepatocytes with fat-filled cytoplasm (Figure 2). Considering the percentage of fat fraction, A50 group was characterized by a significantly ($p < 0.05$) higher value compared to the other experimental groups (Table 6).

Table 6. Fat fraction of liver from rainbow trout. a-b Different letters denote statistically significant differences among the experimental group.

	CTRL	**CTRL-ASX**	**A50**	**A50-ASX**
Fat fraction (%)	47.81 ± 5.97 a	44.29 ± 4.54 a	55.66 ± 8.35 b	43.26 ± 5.06 a

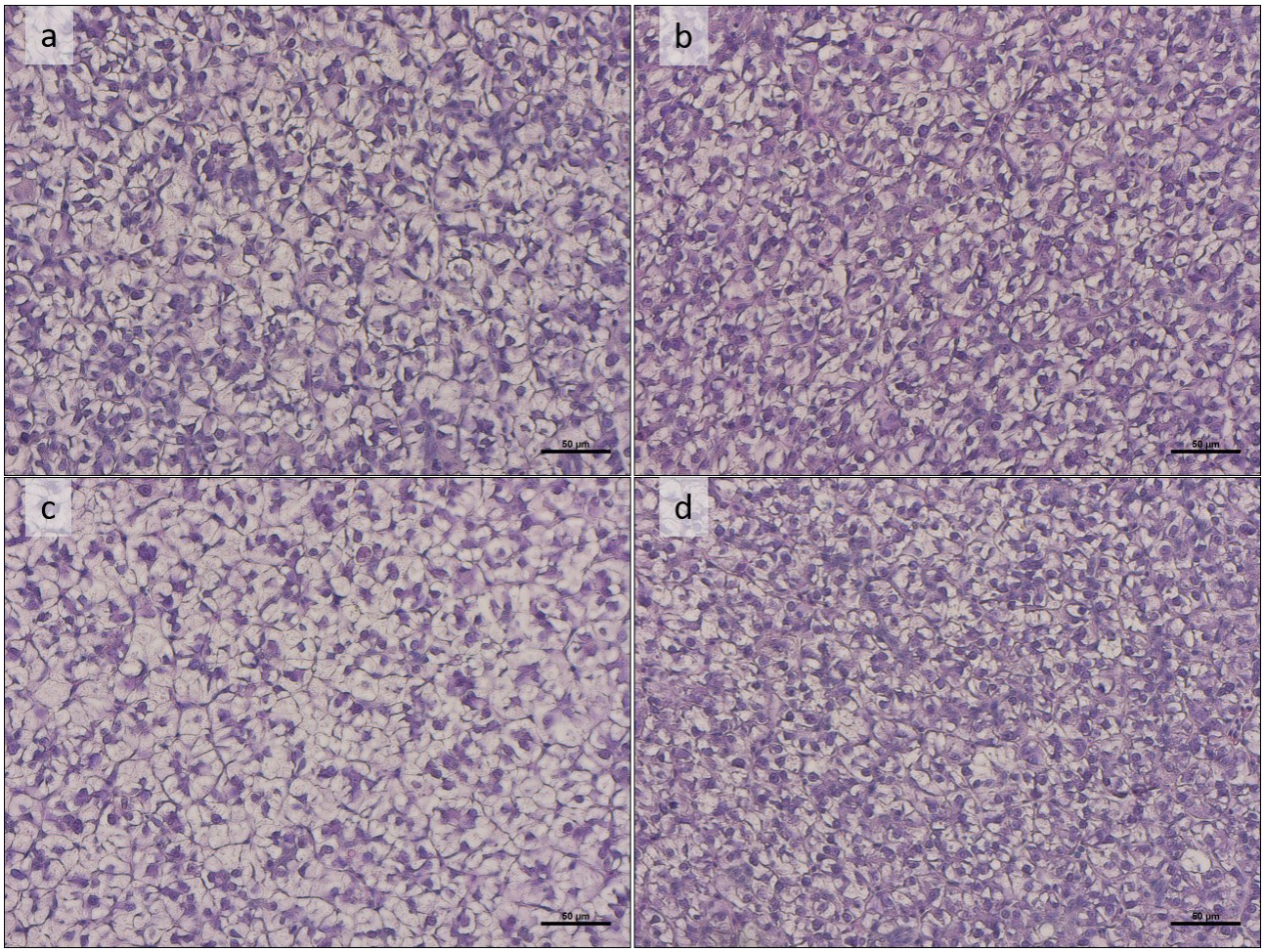

Figure 2. Example of histomorphology of liver from rainbow trout fed the experimental diets. (a) CTRL; (b) CTRL-ASX; (c) A50; (d) A50-ASX. Scale bars: 50 µm.

4.4. Real-time PCR

Oxidative stress response. Considering the expression of genes involved in oxidative stress response (*sod1, sod2 and cat*), the A50 group was characterized by a significantly ($p < 0.05$) higher value compared to the other experimental groups (Figure 3a-c).

Immune response. Considering the expression of genes involved in immune response (*il1b, il10, tnfα*; Figure 3d-f), no significant differences were detected among the experimental groups

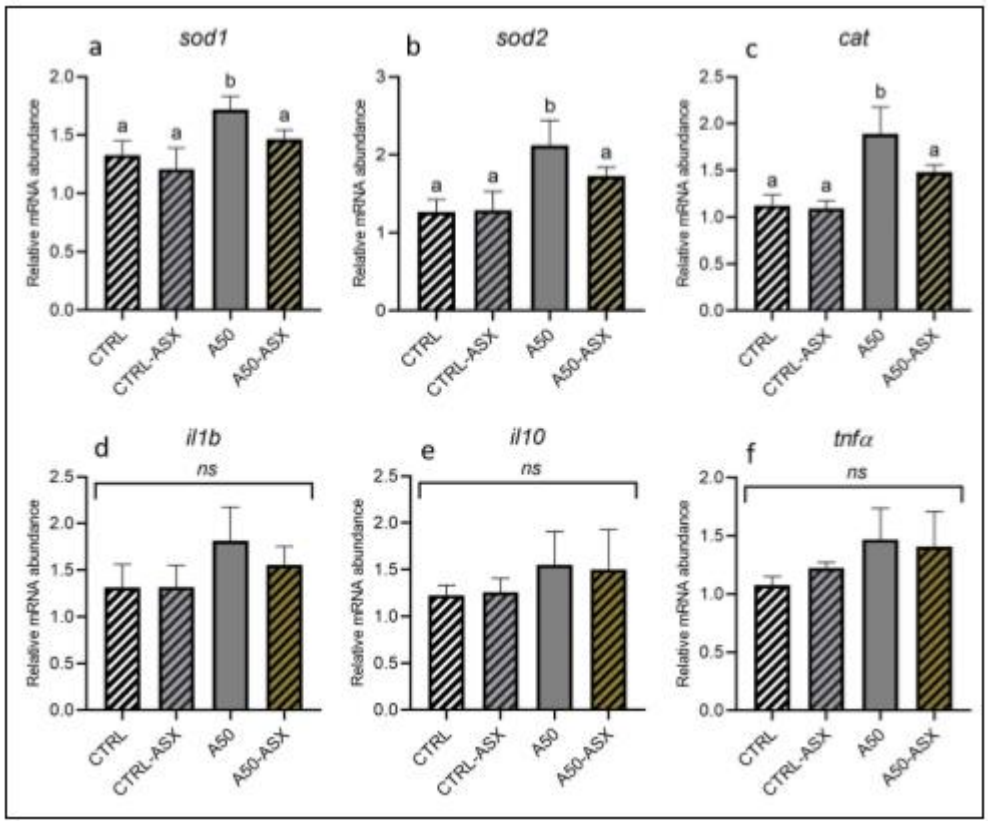

Figure 3. Relative mRNA abundance of genes involved in the oxidative stress response analyzed in liver (a-c) and immune response analyzed in the intestine (d-f) of rainbow trout juveniles fed the experimental diet. Data are presented as mean ± standard deviation (n=9). ns, no significant differences ($p > 0.05$).

5. DISCUSSION

The presence of microplastics in the feed is a common route of contamination for farmed fish species (Siddique et al, 2023). Since the complete removal of MPs from contamination sources like fish feed is notoriously difficult, techniques to mitigate the negative effects of MPs that reach farmed fish are the focus of many recent studies. The present study aimed to evaluate the role of dietary implementation of microencapsulated ASX in counteracting both MPs accumulation and side-effects in rainbow trout (*Oncorhynchus mykiss*).

In this study, survival rate and growth performances did not change among the experimental groups, consistently with previous studies. In fact, it has been demonstrated that the assumption of dietary MPs did not impair fish growth in Gilthead seabream (*Sparus aurata*) (Jovanović et al, 2018) or in Stickleback (Bunge et al, 2022), as well as zebrafish (*Danio rerio*) (Cattaneo et al, 2024) and European sea bass (*Dicentrarchus labrax*) (Zarantoniello et al, 2024) subjected to the same dietary MPs used in the present study.

In addition, all the groups fed diets containing MPs did not show signs of inflammation at intestinal level. This was highlighted by both the histological indexes and the relative expression of immune response markers (*il1b*, *il10*, and *tnfa*) analysed, suggesting that the transit of small MPs, like those used in the present study, did not alter the intestinal structure or its functionality, as already demonstrated in different fish species (Cattaneo et al, 2023; Ašmonaite et al, 2018). In fact, severe intestine alterations have been evidenced in fish exposed to MPs with a bigger range size (between 200 and 1000 μm) (Varó et al, 2021). However, it should be noted that both A50 and A50-ASX groups showed an increase in goblet cells relative abundance compared to the control ones. This result can be possibly explained by the fact that MPs can cause an impairment in mucus barrier properties (Ašmonaitė et al. 2018), leading to an increase of goblet cells number to counteract this effect. Additionally, the administration of 50 mg/kg of dietary MPs with a range size of 1-5 μm led to the same effects previously observed in both zebrafish and European seabass exposed to the same fluorescent microbeads used in the present study (Cattaneo et al., 2024; Zarantoniello et al., 2024).

In fact, in the present study, dietary MPs were found with the highest concentration in the liver, followed by the intestine and finally in the muscle in both A50 and A50-ASX group. This result is in accord with previous studies that evidenced how dietary MPs microbeads with the same range size (1 to 5 μm) used in the present study can be absorbed at intestinal level and, through the bloodstream, can be translocated to other organs, mainly in the liver (Cattaneo et al., 2023; Cattaneo et al., 2024) (Zarantoniello et al., 2024 (Lu et al., 2016). Considering the absorption, it has been demonstrated that enterocytes' supranuclear vacuoles are involved in the internalization of dietary MPs which follow, through pinocytosis, the same route of absorption of intact nutrient molecules (Liu et al, 2021; Rennick et al, 2021). Accordingly, in the present study, the density of supranuclear vacuoles was found to be significantly higher in A50 and A50-ASX groups compared to the control ones.

After absorption, MPs are translocated to the liver which acts as an important organ for retaining this contaminant, leading to a notable accumulation within the tissue (Romano et al, 2020; Kim et al, 2021). This accumulation prevents the MPs translocation to other organs but causes adverse effects on the liver's welfare. Particularly, in the present study, fish from A50 group were characterized by a significantly higher hepatic lipid accumulation compared to those from the other experimental groups. This is consistent with other studies that showed that MPs translocated to the liver can lead to impairments in the lipid signalling pathways, leading to lipid accumulation and possibly steatosis (Del Piano et al, 2024). In addition, as previously demonstrated by previous studies (Cattaneo et al., 2023; Zarantoniello et al., 2024; Compa et al, 2024), one of the most detrimental effects associated to MPs fish exposure is oxidative stress, which further results in impairment of cellular processes that may consequently affect the overall health and quality of farmed fish over the long term (Lesser, 2006; Hoseinifar et al, 2020; Menon et al, 2023). Accordingly, in the present study, a significantly higher expression of genes related to the oxidative stress response (*sod1, sod2* and *cat*) was evidenced in the liver samples from the A50 group.

Interestingly, fish from A50-ASX exhibited an expression of *sod1, sod2* and *cat* that was comparable to that observed in both control groups. This result can be explained by the provision of a strong

antioxidant molecule, like natural astaxanthin, that has been demonstrated to be effective in mitigating MPs-induced oxidative stress (Cattaneo et al., 2024; Zarantoniello et al., 2024). Additionally, the oxidative stress mitigation in this group can be also addressed to a lower MPs abundance in the liver that possibly led to milder adverse effects, as also demonstrated by the absence of significant differences in hepatic lipid accumulation in respect to the control groups. In fact, as demonstrated in previous studies, (Cattaneo et al., 2024; Zarantoniello et al., 2024), the microencapsulation technology used to provide and preserve the natural ASX played a significant role in reducing the MPs absorption at intestinal level and thus reducing the number of MPs translocated to other organs, including liver. Particularly, the wall matrix of the microcapsules was mainly composed of starch, which is known to have coagulation properties for MPs, particularly enhanced in acidic conditions like those observed in fish stomach (Girish et al., 2023; Fakhri et al., 2023; Zarantoniello et al., 2024). Like evidenced in previous studies, the microcapsules, after releasing their content, were able to coagulate MPs microbeads at gut level, forming coagula that were too large to be absorbed by the enterocytes. This result was further confirmed by the lower enterocytes' supranuclear vacuolization in A50-ASX group compared to A50 one. Previous studies that used this technology in response to the dietary contamination with fluorescent MPs microbeads of 1-5 μm in range size confirmed this result. In European seabass juveniles, the microencapsulation technology was able to significatively reduce the absorption of MPs included in the diet at 50 mg/kg concentration over a 2-month feeding trial, like in the present study. Differently, in zebrafish, the same technology was not effective in preventing the absorption of dietary MPs when included at 50 mg/kg concentration, but it obtained promising results when a higher dietary MPs concentration was used (500 mg/kg). This difference is related to the different gut environment observed in the fish species considered. The coagulating properties of starch, such as the action of bridging and enmeshing exercised on MPs, are favored in acidic conditions, like the ones that can be found in carnivorous fish stomachs (Asharuddin et al, 2021; Girish et al., 2023). Zebrafish instead, like other cyprinids, is a stomach-less fish lacking a strong acid digestion (Wallace et al, 2005). Consequently, it can be hypothesized that in zebrafish fed

with the 50 mg/kg MPs and ASX diet, the low MPs concentration in the digestive tract and the absence of a high acidic environment, were insufficient to trigger coagulation processes. Therefore, the success of our study can also be attributed to the fact that rainbow trout, like European seabass is a carnivorous fish possessing an acid stomach, where the coagulating agents present in the encapsulation technology of ASX were able to work more optimally. Considering that most farmed fish in aquaculture are also carnivores, the results obtained are also of great importance in terms of aquaculture. Furthermore, in the case of rainbow trout, ASX is already commonly administered in cultured individuals for the pigmentation of the fillet, obtaining a final product more appreciable to the buyer (Rahman et al, 2016). This new technology therefore has high potential in rainbow trout farming, adding to the already employed pigmentation treatments an extra utility against MPs contamination.

6. CONCLUSION

The adverse effects of dietary MPs were clearly demonstrated in the present study, with the results showing expression of genes related to the oxidative stress response in the liver and the alteration at the hepatic level in the A50 group. The liver was revealed as the major site of microplastic accumulation. The administration of microencapsulated ASX was able to mitigate these effects in the A50-ASX group combining the properties of both ASX and the wall matrix of the microcapsules. In particular, ASX with its natural properties was able to mitigate the oxidative stress at the hepatic level, while the materials comprising the walls of the microcapsules reduced the absorption of MPs at the intestinal level, enabling the formation of MPs coagula too large to be absorbed. Therefore, this study demonstrated the effectiveness of this technique, that works especially well in carnivorous fish like rainbow trout, thanks to the acidic conditions of the stomach favouring the coagulating properties of the components of the microcapsules' wall matrix. This technique is thus very promising for the aquaculture industry and new studies on different species and conditions should be carried out to fully explore the potential of microencapsulated ASX to counteract the negative effects of MPs contamination.

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