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Nanoplastics impair the intestinal health of the juvenile large yellow croaker *Larimichthys crocea*

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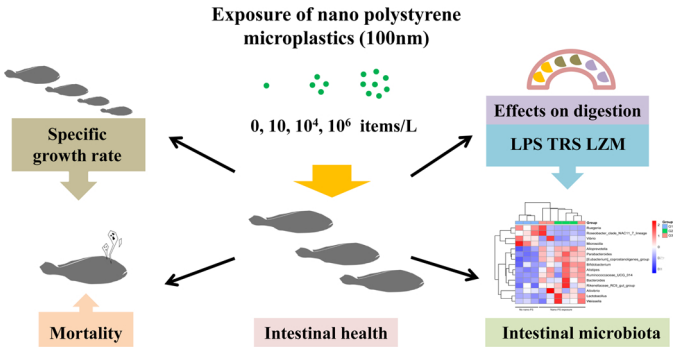
GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics (MPs) have become a severe concern in marine environment worldwide. Micro-polystyrene particles have been proved to accumulate in vivo and caused disorders of digestion, antioxidant system, immunity and intestinal microflora, but little is known about the effects of nano-polystyrene (nano-PS). In order to understand response mechanism of marine fish to nano-PS, the effects of nanoplastics on the intestinal health and growth performance of the juvenile *Larimichthys crocea* were investigated. After 14-d exposure, the reduced digestive enzyme activities indicated that nano-PS had a negative impact on the digestion and absorption of juvenile fish. Moreover, analysis of the intestinal microbiota showed that the proportion of the three-dominant bacterial phyla (Bacteroidetes, Proteobacteria and Firmicutes) in the gut changed significantly, accompanied by a significant increase of potentially pathogenic bacteria (Parabacteroides and Alistipes). In addition, lysozyme activity and specific growth rate (SGR) were significantly reduced, and total mortality of juvenile fish was significantly increased. Overall, nano-PS exposure was harmful for the health of juvenile fish, which might threaten their population in the long term.

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1. Introduction

The global production and application of plastics have reached 348 million tons by 2018 (Plastics Europe, 2018), and the environmental plastic pollution has become a severe concern due to chemical stability, persistence and bioaccumulation (Andrady and Neal, 2009; Barnes et al., 2009). Microplastics (MPs), defined as plastic particles with sizes less than 5 mm come from the degradation of plastic or small particles contained in products like cosmetics and detergents (Kramm et al., 2018; Thompson et al., 2004). MPs never stop the decomposition process and continue to form nanoplastics (NPs), which have the particle size range between 1 nm and 100 nm (Jambeck et al., 2015), or < 1 μm in a wider scope (Tian et al., 2019). The abundance of micro-PS was 0.03 − 0.075 particles/L in East Asian seas (Isobe et al., 2015) and 0.07 ± 0.02 particles/L in Baltic Sea in Europe (Tamminga et al., 2018). As for Louisiana coast in the northern Gulf of Mexico and North Eastern Mediterranean Sea, the abundance of micro-PS was 5.0–18.4 particles/L (Mauro et al., 2017). What's more, the concentrations have reached 4137.3 ± 2461.5 particles/L (Zhao et al., 2014a) and 204.9–4757 particles/L (Nel and Froneman, 2015) in some estuarine and coastline area. Even though plastic particles have been discovered a widespread accumulation in the ocean, little information is available about NPs in the marine environment owning to the limitation of detection techniques (Barnes et al., 2009; Ribeiro et al., 2019; Woodall et al., 2014), the presence of NPs is a concern. NPs of 70 nm could be ingested and accumulated in the body of zebrafish (*Danio rerio*), causing inflammation, oxidative stress and lipid accumulation in fish liver (Lu et al., 2016). Kashiwada (2006) proved that NPs made of latex (polystyrene, 39.4 nm) can be accumulated in gills, testis, liver, viscera and blood of the medaka *Oryzias latipes*, and also showed the transit of the nanoparticles through the blood-brain barrier, implying potential neurotoxicity to the marine fish. Furthermore, the cellular responses induced by polystyrene (nano-PS) have indicated the potential risks to humans (Lehner et al., 2019). The whiteleg shrimp *Litopenaeus vannamei* fed with nano-PS via a simulated marine food chain by the NPs preexposed mussel *Mytilus edulis*, showed changes of microbial activities in the intestine, glutathione S-transferase as well as superoxide dismutase activities. These findings indicated that NPs pollution can directly interfere with nutritional changes in marine living resources, thereby indirectly causing potential health implications for human consumers (Chae et al., 2019).

Gut microbiota has played an irreplaceable role of the organism growth and human health, and the ingestion of NPs might impact the microbial community (Aron-Wisnewsky and Clement, 2016; Jin et al., 2017). Previous studies have indicated the comprehensive involvement of gut microbes in many biological processes, such as antioxidant defense and immune modulation under various environmental stresses in organisms (Evariste et al., 2019; Liu et al., 2019; Zeraati et al., 2019). Jin et al. (2018) have observed both the microbiota dysbiosis and inflammation in the gut of adult zebrafish induced by polystyrene MPs and NPs. Qiao et al. (2019b) have found MPs induced significant metabolomic alterations and gut microbiome perturbations and suggested the metabolic and microbial alterations were associated with oxidative stress, inflammation and lipid metabolism. The intake and accumulation of NPs mainly couple with the digestive process of organisms. Digestive system plays important roles for nutrient intake and absorption under the assistance of related digestive enzymes, which have been considered as indicators of digestive ability and physiological toxicity (Gu et al., 2019; Kong et al., 2019; Rabei et al., 2018). Previous studies have provided innovative perspectives of gut microbe's analysis under the exposure of plastic particles. Evidences have also been provided to illustrate the relationship between microbial changes and inflammation, oxidative stress (Qiao et al., 2019b). More information is supposed to be provided to illustrate the dynamic changes in the digestive system including digestive enzymes, gut microbes and the mutually interdependent influences between these indicators.

The large yellow croaker *Larimichthys crocea* is one of the most productive commercially marine fish in China and farmed in cage, which is exposed to the offshore artificial aquaculture areas. Studies have proved that human activities and aquaculture have significantly increased the regional concentrations of MPs in such areas (Jabeen et al., 2017; Li et al., 2015; Teng et al., 2019), inferring the necessity of studies on NPs for the large yellow croaker. Fish intestine plays pivotal function in the maintenance of digestion/absorption of nutritious minerals and immune defense (Nicholson et al., 2012). Given the importance of gut health, this study aims to understand the effects of nano-PS on gut digestion, immune-related enzymes, compositions of gut microbes and growth of the large yellow croaker *L. crocea*. In addition, the relationship between these biological indicators was analyzed in order to reveal the regulation mechanism of juvenile fish under nano-PS exposure.

2. Materials and methods

2.1. Characterization of nano-PS

Nano-PS (green fluorescent microspheres, particle size: 100 nm; concentration: 1.8189×10^{13} items/mL; excitation and emission peaks: 488 nm and 518 nm) was purchased from Tianjin BaseLine ChromTech Research Centre, Tianjin, China. The polystyrene plastic used for the experiment was verified by a micro-Fourier Transform Infrared (μ-FT-IR) spectroscope (Nicolet iN10 MX, Thermo-Fisher Scientific). Compared with the database provided by Thermo-Fisher, the spectral matching degree was 93 % (Fig. S1A). A stock solution (1×10^4 items/L) of nano-PS in ultrapure water was prepared and then sonicated 30 min before the assay. Particle shape and morphology were characterized by Scanning Electronic Microscopy (SEM, HitachiJSM-7500 F) (Fig. S1B).

2.2. Animal culture and experimental design

About 3000 healthy large yellow croaker juveniles with similar size obtained from the Institute of Marine and Fisheries Research of Ningbo (China) were acclimated to laboratory conditions for two weeks in aerated seawater prior to being exposed to nano-PS. Formulated diets containing roughly 48 % crude protein, 6% crude lipid, 3% crude fiber and 2.1 % amino acids were used. Two grams of the feed pellets (diameter < 0.2 mm) were added per tank (5% of the individual weight) twice a day. The juvenile fishes were kept with a 14:10 h light: dark cycle. The bottom of the tanks was cleaned and half of the seawater was renewed daily. After acclimation, juvenile fish (30 dph, initial body weight: 0.19 ± 0.06 g and length: 2.4 ± 0.3 cm) were randomly divided into 12 fiberglass tanks (100 L) and exposed to four different concentrations [0, 10 items/L ($5.50*10^{-12}$ mg/L), 10^4 items/L ($5.50*10^{-9}$ mg/L), 10^6 items/L ($5.50*10^{-7}$ mg/L)] of nano-PS. Each treatment consisted of three replicate tanks containing 200 fish each. During 14-day exposure, juvenile fish living in aerated seawater (dissolved oxygen: 7.2 ± 0.3 mg/L; temperature: 22.0 ± 0.1 °C; pH: 8.1 ± 0.1 salinity: 28 ± 0.5) were fed with artificial food the same as acclimation. To maintain nano-PS concentrations in each treatment, seawater containing relevant nano-PS was fully renewed per day.

2.3. Sample collection

At the end of exposure, 18 individuals with similar size in each tank were selected to dissect the entire intestine. To get enough sample size and reduce differences between individuals, three juvenile fish intestines washed with sterile PBS were all placed in a 2 mL RNase/DNase free cryogenic vial (Corning, 430,659-ND). Each intestinal sample was rapidly frozen in liquid nitrogen and then stored at -80°C until biochemical analysis and molecular experiment. All protocols were approved by the Institutional Animal Care and Use Committee of Second

Institute of Oceanography, Ministry of Natural Resources, China.

2.4. Total mortality and specific growth rate (SGR)

The dead juvenile *L. crocea* were counted in each tank twice a day and then removed. Finally, the mortality in each tank was calculated after 14-d exposure. Ten juveniles were randomly sampled and weighed in each replicate before the experiment and the average was taken as the initial weight (W_0). After 14-d (t) exposure,10 juveniles were randomly taken from each tank and weighed, and the average was taken as the terminal weight (W_t). SGR was computed by the following equation (Li et al., 2018a): SGR (%/d) = (lnw_t - lnw_0) *100/t.

2.5. Determination of enzyme activity

Juvenile fish intestines accurately weighed were homogenized (1:9 w/v) in ice-cold Stroke-physiological saline solution 0.89 % buffer and the homogenate was centrifuged at 2500 *g*, 4℃ for 10 min to collect the supernatant for the measurement of enzyme activities (amylase, lipase, trypsin and lysozyme) using commercial kits (Nanjing Jiancheng, Bioengineering Institute, Nanjing, China). Protein content was quantified by Bradford (1976) method using bovine serum albumin as a standard. All experimental methods and formulas were strictly in accordance with the instruction of manufacturer.

2.6. Microbial community composition analysis

The total DNA of the juvenile fish intestinal contents was extracted (n = 3) using a QIAamp DNA mini kit (QIAGEN, Germany). Agarose gel electrophoresis and DNA concentration (Nanodrop 2000, ThermoScientific) detection were carried out to ensure that the DNA quality met the requirements. PCR amplification was performed using the V3–V4 region-specific primers 343 F (5′-TACGGRAGGCAGCAG-3′) and 798R (5′-AGGGTATCTAATCCT-3′) of 16S rRNA according to the sequencing requirements of Illumina Miseq (Shanghai OE Biotechnology Co., Ltd). The raw data obtained by Illumina MiSeq sequencing was de-interleaved and spliced, The Usearch clustering method was used for all high-quality sequences to determine the Operational Taxonomic Units (OTUs) using a similarity threshold of 97 %. Subsequently, PyNAST was used to compare the abundance sequence in the OTUs classification with the GreenGenes database, and then the species classification annotation was performed with a confidence threshold of 80 %, and the alpha diversity and community abundance were further analyzed in the end.

2.7. Statistical analysis

The tested parameters were expressed as mean ± SD and data were analyzed for distribution normality and homogeneity of variance with the Kolmogorov-Smirnov test and Levene's test, respectively. One-way ANOVA with SPSS 25 (SPSS Inc., Chicago, IL, USA) software was used to evaluate the differences among different groups. Significances were indicated by $P < 0.05$. SPSS 25 (SPSS Inc., Chicago, IL, USA) was also used to analyze the correlations between s total mortality and other parameters. Diagrams were created by using GraphPad Prism. Heatmap was constructed showing 15 genera with significant differences in abundance between samples by R software (2.9.0) based on the relative abundance of species at genus level in OTU table. Principal Coordinate Analysis (PCoA) was performed to compare the changes of bacterial community between samples based on the unweighted UniFrac distances. The PICRUSt was used to predict the functional profiling of the intestinal microbiota with high cost performance that uses evolutionary modeling to predict metagenomes from 16S data and a reference genome database.

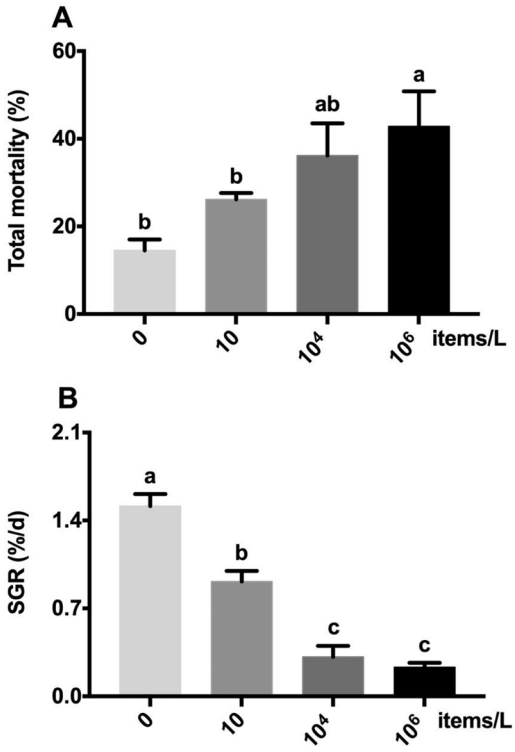

Fig. 1. Total mortality (A) and specific growth ratio (SGR, B) of juvenile *L. crocea* exposed to four concentrations (0, 10, 10^4, 10^6 items/L) of nano-PS for 14 days. Different letters indicate significant differences among nano-PS concentrations ($P < 0.05$).

3. Results

3.1. Total mortality and specific growth rate (SGR)

During 14-d exposure, total mortality of juvenile fish increased significantly ($p < 0.05$) with increasing nano-PS concentrations. The maximum total mortality was up to 42.8 % in the high concentration treatment (10^6 items/L) (Fig. 1A). Except for the high-concentration exposure treatment, SGR showed a concentration-dependent decrease. In addition, there were no differences of SGR between high and medium concentration treatments (Fig. 1B).

3.2. Intestinal digestive and immune stress induced by nano-PS exposure

Intestinal digestive enzyme and immune-related enzyme activities were detected to access intestinal digestive and immune stress. In general, lipase, trypsin and lysozyme activities were decreased significantly ($p < 0.05$) after 14-d nano-PS exposure, no differences were found among different nano-PS treatments, except for trypsin activity in low concentration (10 items/L nano-PS) treatment, which showed no difference compared to the control group (Fig. 2A, B, D). Nano-PS had no effect on amylase activity (Fig. 2C). Significant correlations between total mortality and SGR, lysozyme, trypsin, and lipase activities were summarized in Table S1.

3.3. Diversity indices of bacterial community in the gut

The indicators (OTUs and Chao1) related to richness showed a significant downward trend, except for OTUs in the 10 items/L nano-PS treatment (Table 1). However, there were no significant differences in Shannon index between treatments at different concentrations (Table 1).

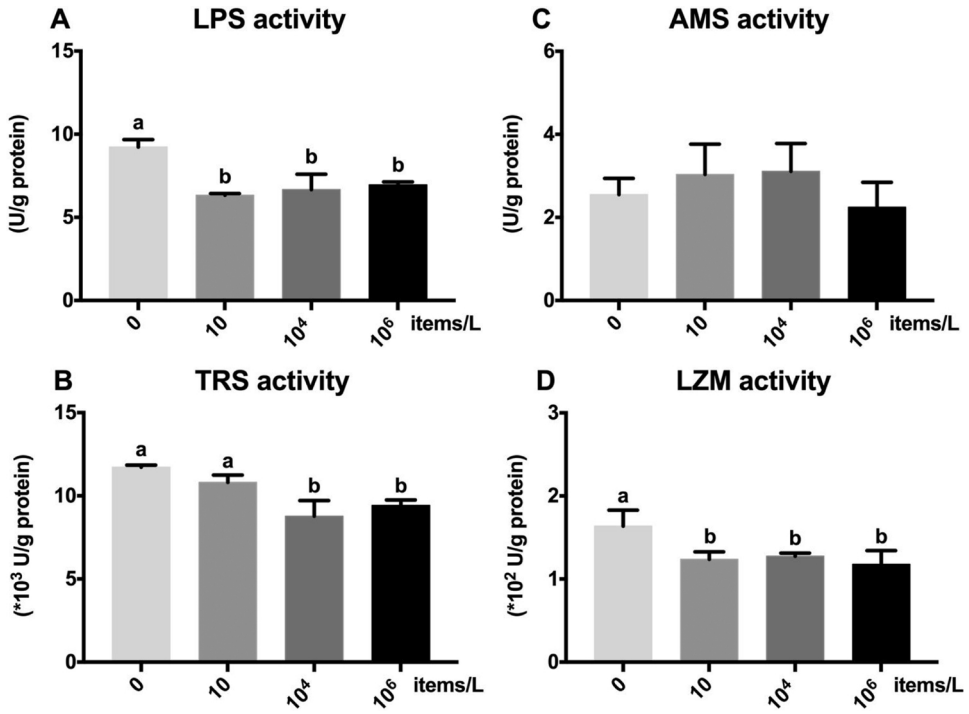

Fig. 2. Lipase (LPS) (A), trypsin (TRS) (B), amylase (AMS) (C) and lysozyme (LZM) (D) activities in intestine of juvenile *L. crocea* exposed to four concentrations (0, 10, 10^4, 10^6 items/L) of nano-PS for 14 days. Different letters indicate significant differences among nano-PS concentrations ($P < 0.05$).

3.4. Functional prediction of the intestinal microbiota

Bacterial gene functions were predicted by using PICRUSt. Comparing the control group and nano-PS treatments, KEGG pathways with significant difference were divided into two categories at KEGG level 1: "Metabolism" and "Organismal Systems" (Table 2). Pathways involved in biosynthesis of other secondary metabolites significantly increased in nano-PS exposure groups, whereas circulatory system significantly decreased (Table 2).

3.5. Intestinal microbial community structures at phylum and genus taxonomic level

The results indicated that Bacteroidetes, Proteobacteria and Firmicutes are the three-major bacterial phyla in intestine of juvenile *L. crocea* (Fig. 3). Compared with the control group (no nano-PS), the relative abundance of Bacteroidetes (at 10 and 10^4 items/L nano-PS) and Firmicutes (at 10 items L^{-1} nano-PS) were significantly increased (Fig. S2A). On the contrary, the relative abundance of Proteobacteria was significantly reduced at 10 and 10^4 items/L nano-PS treatments (Fig. S2A).

The top 15 abundant genera were selected for comparative analysis (Fig. 4). At genus taxonomic level, Ruegeria, Vibrio and Microscilla were less abundant under nano-PS exposure, however, the richness of Alloprevotella, Parabacteroides, Bifidobacterium, Alistipes, Bacteroides, Aliivibrio, Lactobacillus and Weissella was increased. Despite this, only three bacterial genera were found to have significant differences. Lactobacillus showed a significant increase ($p < 0.05$) between the low concentration treatment and the control group (Fig. S2B). In addition, the relative abundance of Parabacteroides (at 10 and 10^4 items/L) and Alistipes (at 10^4 items/L) were also increased significantly ($P < 0.05$) due to the exposure of nano-PS (Fig. S2B).

PCoA analysis revealed that nano-PS shaped the bacterial community composition by separating the nano-PS exposure treatments from the no nano-PS group by PC1, which contributed to 63.11 % of the variance (Fig. 5).

4. Discussion

Plastic fragments ultimately can be converted into smaller-sized particles as micron even nanometer scale, and toxicological studies have turned to explore their toxicity and lethal mechanisms after discovering that plastic particle (Thompson et al., 2004; Gigault et al., 2018). Due to the properties and adsorption behavior of the NPs as well as the limitations of the detection technology, currently there have been no effective methods existing for the reliable detection of NPs in samples (Mendenhall, 2018). Worse still, the smaller the plastic is, the stronger the toxic effect is (Browne et al., 2008). Studies have indicated that the presence of marine plastic debris might be depressing overall fisheries productivity, therefore reducing available fish biomass (Mendenhall, 2018). Studies have showed that particles would accumulate in the skin, gill and gut of fish (Feng et al., 2019), indicating that particles can be ingested by fish through the digestive system theoretically. A few studies have highlighted the gut microbiota dysbiosis and the intestinal toxicity under particle exposure (Chen et al., 2018; Qiao et al., 2019b), and thus NPs are likely to disorder the intestinal microflora. Based on this speculation, it is meaningful to further explore the health of marine fish in the presence of NPs.

The intestine as the main digestive, absorbing, immune and defensive organ of the fish, plays an important role in preventing the invasion of pathogens (You et al., 2019). More and more studies have showed that MPs or NPs mainly accumulated in the intestine of various marine

Table 1
One-way ANOVA summary on effects of nano-PS on diversity indices of bacterial community in the gut (OTUs, Chao1, Shannon). Different letters within the same column indicate significant differences ($p < 0.05$). G1, G2 and G3 represent experimental treatments 0, 10 and 10^4 items/L of nano-PS respectively.

Group	OTUs	Chao1	Shannon
G1	897 ± 40[a]	1046 ± 47[a]	7.13 ± 0.21
G2	814 ± 65[a]	904 ± 93[b]	7.07 ± 0.12
G3	790 ± 22[b]	897 ± 25[b]	6.85 ± 0.31

Table 2
Relative abundance of predicted functions. KEGG level 1 and level 2, as well as level 3 are listed. G1, G2 and G3 represent experimental treatments 0, 10 and 10^4 items/L of nano-PS respectively.

KEGG level	KEGG pathway	G1 (%)	G2 (%)	G3 (%)	p-value
1	**Metabolism**				
2	Biosynthesis of Other Secondary Metabolites	0.922	1.023	0.969	**0.039**
3	Penicillin and cephalosporin biosynthesis	0.051	0.042	0.039	**0.039**
3	Beta-Lactam resistance	0.031	0.036	0.030	**0.039**
1	**Organismal Systems**				
2	Circulatory System	0.042	0.013	0.026	**0.039**
3	Cardiac muscle contraction	0.042	0.013	0.026	**0.039**

organisms from different trophic level (Arossa et al., 2019; Browne et al., 2008; Qiao et al., 2019a). Thus, intestinal status can be an important target for biotoxicity of NPs. Sequences are usually divided into different OTUs based on a 97 % similarity threshold, and an OTU is usually considered as a biological species. Bacteria was assigned to OTUs for suitably measuring the highly diverse bacterial communities (Hill et al., 2003). The Chao1 and Shannon indices represent the richness and diversity of the bacterial community respectively, of which the values are in direct proportion to the amount of species and the diversity of the microbiota respectively (Li et al., 2018b). The variation of intestinal bacterial community richness reflects the physiological conditions of individual. In addition, small particles did cause changes in the microbiome structure in several animal species (Merrifield et al., 2013; Wilding et al., 2016; Xia et al., 2017). Our study found that the intake of nano-PS caused no changes of Shannon indices, implying the stability in gut microbiota diversity. In spite of this, the decreased of Chao 1 indices and change of bacterial relative abundance showed NPs affect the OTUs of fish gut microbiota by changing the bacterial community richness. This may alter the dominant species of gut microbiota, disrupting the stability of intestinal bacterial community. Some studies have showed that overgrown shrimp and obese children presented a more complex gut bacterial interspecies interaction (Riva et al., 2017; Xiong et al., 2016). Moreover, the gut microbiota diversity could be used as ideal an indicator to evaluate intestinal inflammatory response (Ott et al., 2004). Therefore, the effects of nano-PS on OTUs of fish gut microbiota may indicate that the presence of nano-PS might be harmful to growth of fish and imply the mild inflammatory response to nano-PS. The slight decrease of Shannon indices probably explained the slight disorder of intestinal bacterial community caused by nano-PS, but the potential threat could not be ignored. From another perspective, the abundance of the microbiota was significantly reduced, indicating that the presence of nano-PS might be may be the reason for the decrease of SGR in juvenile *L. crocea*.

The Bacteroidetes and Firmicutes generally showed an increasing trend, whereas Proteobacteria was in a downward trend after 14-d nano-PS exposure. Li et al. (2017) pointed out that the growth of large yellow croaker is closely related to Proteobacteria. Moreover, obese individuals present predominance of Firmicutes and relative low proportion of Bacteroidetes (Roh and Seki, 2013). The low abundance of Bacteroidetes promoted the growth of other bacterial populations, in addition, Firmicutes could be more effective for getting energy from the diet (Ley et al., 2006). our results discovered that the Proteobacteria was significantly decreased at 10 and 10^4 items L^{-1} nano-PS treatments, and the Bacteroidetes dominated the gut of juvenile *L. crocea* and increased significantly after exposure to nano-PS, indicating that nano-PS might have a negative impact on the growth of juvenile *L. crocea*. Based on various results from different studies, several mechanisms have been proposed to explain the effects of MPs or NPs on the growth of organisms, including reduced feeding activities, extra energetic costs for the extensive digestion of MPs, and immune regulation caused by MPs leading to higher energy expenditure (Besseling et al., 2012; Pettit et al., 1981; Wright et al., 2013). Firmicutes in our results presented a markedly elevated situation, probably in response to the excessive growth of Bacteroidetes, providing more energy to other bacterial populations and encouraging the bacteria abundance. PICRUSt analysis showed a significant increase in Biosynthesis of Other Secondary Metabolites pathway, probably because juveniles initiated self-regulation mechanisms to cope with MP intake. Eventually, we found that the SGR in the nano-PS treatments were significantly lower compared to the control group, especially in the high- and medium-concentration treatments, suggesting that nano-PS can inhibit the growth of juvenile *L. crocea*.

Lactobacillus affiliated to the Firmicutes is generally considered to be closely related to the health of humans and animals, and has the function of preventing and treating diseases of the digestive system (Chaves et al., 2017; Liu et al., 2020; Santarmaki et al., 2017). The content of Alistipes (affiliated to the Bacteroidetes) in patients with intestinal stress syndrome was often increased (Hsiao et al., 2013). Studies have found that Alistipes can affect the utilization of tryptophan and destroyed the balance of the 5-hydroxytryptamine system in the intestine, which is involved in the regulation of various physiological functions and pathological states (Ait-Belgnaoui et al., 2014). The significant increase in Alistipes abundance was observed under the exposure of 10^4 items L^{-1} nano-PS, indicating the sensibilities of intestinal flora to the environmental change, so as to make adaptive adjustment and regulate the energy metabolism. Parabacteroides affiliated to the Bacteroidetes were inversely related to the mRNA levels of the tight junction proteins Occludin and ZO-1 associated with the intestinal barrier, and might aggravate intestinal infection by increasing

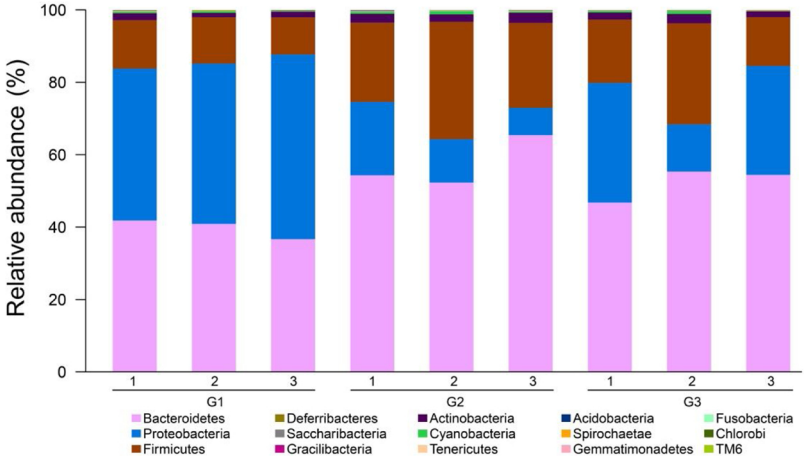

Fig. 3. Composition of intestinal bacterial community of juvenile *L. crocea* at phylum taxonomic level. Only the top 15 most abundant (Based on relative abundance) bacterial phyla was shown. Three replicates are labelled with the numbers 1, 2 and 3. G1, G2 and G3 represent experimental treatments 0, 10 and 10^4 items/L of nano-PS respectively.

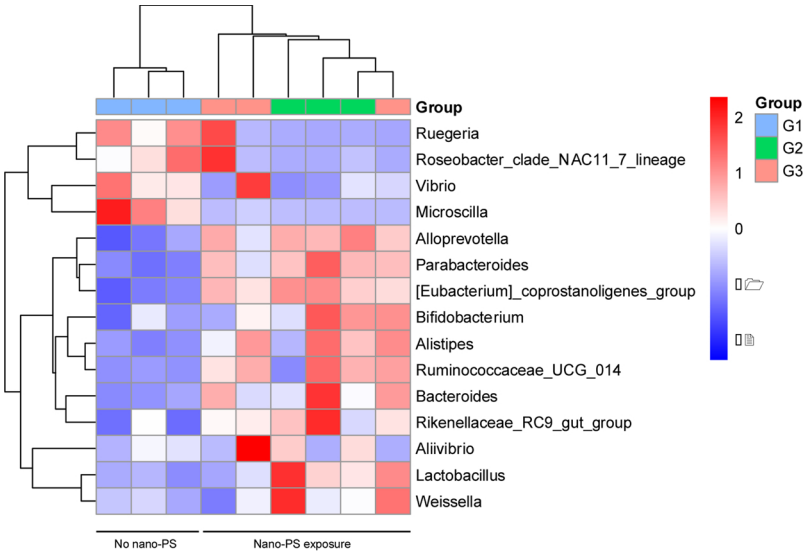

Fig. 4. Heatmap revealed the top 15 bacterial genera (%) of juvenile *L. crocea*. Red colours indicate higher abundance; blue colours indicate lower abundance. G1, G2 and G3 represent experimental treatments 0, 10 and 10^4 items/L of nano-PS respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

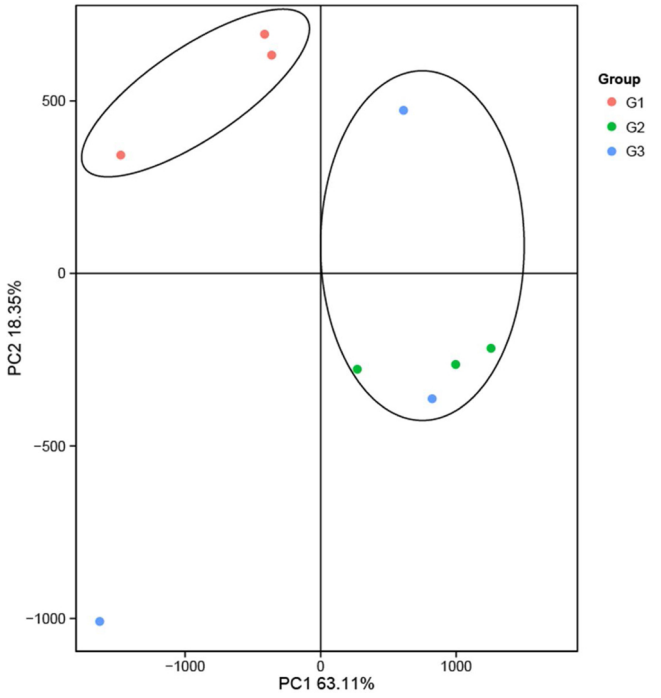

Fig. 5. Principal components analysis of juvenile *L. crocea*. G1, G2 and G3 represent experimental treatments 0, 10 and 10^4 items /L of nano-PS respectively.

intestinal permeability (Lee et al., 2015). In this study, both a significant decrease in lysozyme (LZM) and increase in Parabacteroides abundance showed the intestinal permeability changes. Assumption based on the intestinal permeability has been widely discussed among the previous studies. A study on the biofilm permeation of nano-PS particles using coarse-grained molecular simulations showed that nano-PS changed the membrane bilayer structure and penetrated into the lipid membrane, indicating that nano-PS may seriously affect the activity of membrane proteins and cellular functions (Rossi et al., 2014). Based on this theory and combined with the significantly decreased KEGG pathway involved in circulatory system, we speculated that the intestinal mucosa of juvenile fish may be damaged, resulting in a change in the proportion of the three-dominant bacterial phylum and an imbalance of beneficial bacteria and potential pathogens in the intestine. The digestive system might be disordered due to the intake of nano-PS, which was fully compatible with the reduction of intestinal digestive enzyme activities (e.g., lipase and trypsin). The number of potential pathogens in the intestine was increased under nano-PS, which was not conducive to the health of juvenile *L. crocea*. Moreover, the increase in Lactobacillus in the gut might be to counter this threat. Previous studies have shown that growth inhibition caused by MPs or NPs might be related to changes in the intestinal microbiome, because various components of the microbiome play a basic role in digestion (Douglas, 1998), reproduction (Lavelle et al., 1995) and development (Visôtto et al., 2009). Therefore, the changed gut microbiome after ingestion of NPs may reduce the absorption of nutrient and the efficiency of food utilization by juvenile *L. crocea*, and ultimately lead to a negative impact on individual growth. Surprisingly, the activity of amylase was not affected by nano-PS during the exposure, the commercial feed with no starch was mainly rich in crude protein and crude fat may be the reason. PCoA analysis also revealed that nano-PS is an important factor in shaping microbial communities in the intestine of juvenile *L. crocea* between the NP treatments and the control group.

Total mortality of juvenile fish increased significantly with the increase of nano-PS concentration. Furthermore, elevated mortality might be linked to reduced lysozyme activity. Lysozyme, a proteolytic enzyme participating in various immune responses and resisting the invasion of foreign pathogenic bacteria or viruses (Luo et al., 2018). It was showed that the digestive enzyme activities and lysozyme activity were affected only under high MPs (10^6 particles/L) and revealed the enzyme regulation ability (Wang et al., 2019). It was found that exposure to NPs decreased heart rate of zebrafish at low concentrations without dose-effect implied the possibility of unconspicuous dose-effect (Pitt et al., 2018). In addition, gastric intestinal tract of fish may be also an important site for NPs excretion (Zhao et al., 2014b). It was found that plastic particles would be partly rejected by bivalve molluscs (Ward et al., 2019). Therefore, an increase in microplastics exposure does not necessarily lead to a significant increase in the accumulative amount, and a dose-effect of NPs on the biological responses may not be identified. In intestine of juvenile *L. crocea*, the potential pathogens were increased, on the contrary, the activity of lysozyme was significantly reduced under the nano-PS exposure of all NP treatments. An important reason might be that the intestine and intestinal homeostasis of juvenile fish were destroyed because of nano-PS, leading to an impaired role of lysozyme participating in immune responses eventually. Moreover, significant negative correlations between total mortality and SGR, lysozyme, trypsin, and lipase activities were found, presumably lysozyme did not effectively participate in immune response and low digestion and absorption led to a significant reduction in SGR. Possibly disturbance of the physiological processes of juvenile fish exposed to nano-

PS prevented them from adapting to the environment, which might be responsible for the high total mortality.

5. Conclusion

In short, the intestine of juvenile *L. crocea* showed an unhealthy state under NP exposure. Juvenile fish tried to increase intestinal probiotics to cope with the growth of potential pathogenic bacteria. However, reduced immune and digestive enzyme activities reduced the growth performance of *L. crocea*, and high total mortality revealed a collapse in the self-regulating mechanism of juvenile fish. Our research provided some new insights in assessing the impact of NPs on juvenile fish health.

CRediT authorship contribution statement

Huaxin Gu: Methodology, Investigation, Writing - original draft. **Shixiu Wang:** Conceptualization, Methodology, Resources. **Xinghuo Wang:** Software, Formal analysis. **Xiang Yu:** Investigation, Writing - review & editing. **Menghong Hu:** Investigation, Formal analysis, Software. **Wei Huang:** Project administration, Writing - review & editing. **Youji Wang:** Conceptualization, Resources, Supervision.

Declaration of Competing Interest

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.122773.

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