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Original Research Article

Dietary nano-selenium alleviated intestinal damage of juvenile grass carp (*Ctenopharyngodon idella*) induced by high-fat diet: Insight from intestinal morphology, tight junction, inflammation, anti-oxidization and intestinal microbiota

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ABSTRACT

In recent years, high-fat diet (HFD) has been widely applied in aquaculture, which reduces the intestinal health of cultured fish. The current study evaluated the protective effects of nano-selenium (nano-Se) on intestinal health of juvenile grass carp (*Ctenopharyngodon idella*) fed with HFD. A total of 135 experimental fish were fed with a regular diet (Con), a HFD (HFD) and a HFD containing nano-Se at 0.6 mg/kg (HSe) for 10 weeks. The results showed that dietary nano-Se significantly improved the survival rate and feed efficiency which were reduced by HFD in juvenile grass carp ($P < 0.05$). Also, nano-Se (0.6 mg/kg) supplement alleviated intestinal damage caused by the HFD, thus maintaining the integrity of the intestine. Moreover, it significantly up-regulated the expression of genes related to tight junction (*ZO-1*, *claudin-3* and *occludin*), anti-oxidization (*GPx4a* and*GPx4b*), and the protein of ZO-1 in the intestine of juvenile grass carp, which were depressed by the HFD ($P < 0.05$). Furthermore, nano-Se supplementation significantly suppressed the expressions of genes related to the inflammation, including inflammatory cytokines (*IL-8*, *IL-1β*, *IFN-γ*, *TNF-α* and *IL-6*), signaling molecules (*TLR4*, *p38 MAPK* and *NF-κB p65*), and protein expression of NF-κB p65 and TNF-α in the intestine of juvenile grass carp which were induced by the HFD ($P < 0.05$). Besides, dietary nano-Se normalized the intestinal microbiota imbalance of juvenile grass carp caused by the HFD through increasing the abundance of the beneficial bacteria, e.g., Fusobacteria. Finally, dietary nano-Se increased the production of short chain fatty acids (SCFA) in the intestine, especially for butyric acid and caproic acid, which were negatively related to the increase of intestinal permeability and inflammation. In summary, supply of nano-Se (0.6 mg/kg) in HFD could effectively alleviate intestinal injury of juvenile grass carp by improving intestinal barrier function and reducing intestinal inflammation and oxidative stress. These positive effects may be due to the regulation of nano-Se on intestinal microbiota and the subsequently increased beneficial SCFA levels.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is one of the most popular farmed fish species in China, with a production of 5.5 million tons in 2019 (Zhang et al., 2020). However, in the last few years, the aquaculture of grass carp has faced more and more challenges, including the adverse effects caused by the increasing application of high-fat diet (HFD) (Tang et al., 2019b; Zhao et al., 2019). Dietary fats play a vital role in fish nutrition and provide essential fatty acids, fat-

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Table 1
The composition of the basal diet, including the regular diet (Con) and the high-fat diet (HFD) (g/kg, air-dry basis).

Item	Group	
	Con	HFD
Ingredients		
Fish meal	120	120
Soybean meal	240	240
Rapeseed meal	100	100
Cottonseed meal	100	100
Wheat flour	222	222
DDGS	50	50
Rice bran	70	70
Soybean oil	8	48
Bentonite	10	10
$Ca(H_2PO_4)_2$	20	20
Microcrystalline cellulose	40	0
Vitamin mixture[1]	10	10
Mineral mixture[2]	10	10
Proximate composition		
Moisture	134	131
Crude protein	300	300
Crude lipid	49	96
Ash	99	95
Basal Se level, mg/kg	0.3	0.3

DDGS = distillers dried grains with solubles.
[1] Vitamin premix provided per kilogram diet: vitamin A, 3,000 IU; vitamin E, 60 IU; vitamin D, 2,000 IU; vitamin C, 200 mg; thiamine, 5 mg; riboflavin, 10 mg; menadione, 10 mg; pyridoxine HCl, 10 mg; cyanocobalamin, 0.02 mg; biotin, 1 mg; calcium pantothenate, 40 mg; folic acid, 5 mg; niacin, 100 mg; inositol, 200 mg. Cellulose was used as a carrier.
[2] The mineral mix contained (g/kg of the total mineral): $KAl(SO_4)_2 \cdot 12H_2O$, 1.59; $CaCO_3$, 181.01; $Ca(H_2PO_4)_2$, 446.01; $CoCl_2 \cdot 6H_2O$, 0.70; $MgSO_4$, 52.16; $MnSO_4 \bullet H_2O$, 0.70; KCl, 165.53; KI, 0.14; $ZnCO_3$, 1.92; NaH_2PO_4, 136.05; Na_2SeO_3, 0.06; $CuSO_4 \bullet 5H_2O$, 0.75; ferric citrate, 13.38.

soluble vitamins, phospholipids and cholesterol needed for normal growth, development and health maintenance of fish (Jobling, 2011). It has been reported that dietary lipids exerted a protein-sparing effect, and the principle was to replace the protein that can be used to produce energy (Du et al., 2006). Therefore, in recent years, HFD has been widely used in aquaculture. However, excessive lipid in the diet often brings many adverse effects. Four percent of lipid in the diet showed the protein-sparing effect, but 6% of lipid in the diet negatively affected growth performance and body composition of juvenile grass carp (Du et al., 2005). The HFD leads to unnecessary liver fat deposition (Wang et al., 2013) and oxidative stress (Huang et al., 2018; Ma et al., 2018), which might consequently affect the health of fish and reduce the yield of fish (Zhao et al., 2019).

Consumption of HFD also causes an increase of intestinal permeabilization, impairs mucosal defenses, and induces intestinal inflammation (Ding et al., 2010; Ma et al., 2018). High-fat diet (15%) fed to Nile tilapia for 8 weeks significantly shortened the length of intestinal villi, reduced the number of goblet cells in intestinal epithelial cells, downregulated the mRNA expressions of tight junction protein, i.e., *occludin* and *claudin*, and induced the expression of intestinal inflammatory factor *IL-1β* (Ma et al., 2018). Furthermore, excessive intake of lipid could affect the diversity of intestinal microflora and lead to an ecological imbalance of intestinal microflora (Al-muzafar and Amin, 2017; Tomas et al., 2016). In addition, an HFD severely affected the composition of the microbiota in mice, characterized by the expansion of Firmicutes (appearance of Erysipelotrichi), Proteobacteria (Desulfovibrionales) and Verrucomicrobia, and decrease of Bacteroidetes and *Candidatus arthromitus* (Tomas et al., 2016). High-fat diet in association with commensal gut microbiota promoted intestinal inflammation in mice (Ding et al., 2010).

Table 2
Primers sequences.

Gene	Sequences of primers	Accession number
β-actin	Forward: 5′-GACCTGACTGACTACCTCAT-3′ Reverse: 5′-CGAAGTCAAGAGCCACATAG-3′	M25013
ZO-1	Forward: 5′-ACTTTGACCGCCGAAGCT-3′ Reverse: 5′-GAGCAACAGGGTTGATCTTCTC-3′	KF193852.1
Occludin	Forward: 5′-TCCACTGCTGGCTGACTATCCC-3′ Reverse: 5′-GCTCATGCCGAATCTCCACAGG-3′	KF193855
Claudin-3	Forward: 5′-TGGGTTTGCTGCTGCTGTTCTG-3′ Reverse: 5′-GTAGAGCGTGGGGCGGAGTAG-3′	KF193858.1
TLR2	Forward: 5′-AGTCCTTCGCTGAGGGTGGTTC-3′ Reverse: 5′-GATGGGACGGGCTGCTTTCAAG-3′	FJ542042.1
TLR4	Forward: 5′-GCTCAGTCCCGGTTTGTGATGG-3′ Reverse: 5′-ACTCAAAGGGTCCCTGCTCCAC-3′	FJ542043.1
p38 MAPK	Forward: 5′-CTCTCGCGCACCCGTACTTTG-3′ Reverse: 5′-CGTGAGCCGTTTCCACTCTTCG-3′	KM112098
NF-κB p65	Forward: 5′-GAAGAAGGATGTGGGAGATG-3′ Reverse: 5′-TGTTGTCGTAGATGGGCTGAG-3′	KJ526214
IL-8	Forward: 5′-GCTCTACCCTCCTAGCCCTCAC-3′ Reverse: 5′-GGGAGCAGTAGGGTCCAGACAG-3′	JN255694.1
IL-1β	Forward: 5′-CCAAGTGCCACCCCGAATGC-3′ Reverse: 5′-AGGGGAAGAACCATCCGACTCG-3′	JQ692172
IFN-γ	Forward: 5′-ATGATGCTGCTGTGTGGACTTCTG-3′ Reverse: 5′-TCTCGCTTTTGGACCGTCGAAATC-3′	FJ695519.1
TNF-α	Forward: 5′-TGATGGTGTCGAGGAGGAAGGC-3′ Reverse: 5′-TTGAGCGTGAAGCAGACAGCAG-3′	HQ696609
IL-6	Forward: 5′-AGCCAGCTCCAGGTGAGTGAAG-3′ Reverse: 5′-GACGGCTCTGCATGTGTCGATC-3′	KC535507.1
GPx4a	Forward: 5′-ACACATCCTGGCCTTCCCATCC-3′ Reverse: 5′-TCGCCGTTCACGTCAATCTTGC-3′	KU255598
GPx4b	Forward: 5′-AACCGAGGCGGAGATCAAGGAG-3′ Reverse: 5′-TCCCAGAGTCCCCTTGCCTTTG-3′	KU255599
Hif-1α	Forward: 5′-CAAGACCTTCCTTAGCCGTCACAC-3′ Reverse: 5′-CACCGACCTGTTCAGCAGATCATC-3′	AY450269.2

ZO-1 = zonula occludens-1; *TLR* = toll-like receptors; *MAPK* = mitogen-activated protein kinase; *NF-κB* = nuclear factor kappa-B; *IL* = interleukin; *IFN-γ* = interferon γ; *TNF-α* = tumor necrosis factor α; *GPx4* = glutathione peroxidase 4; *Hif-1α* = hypoxia inducible factor-1α.

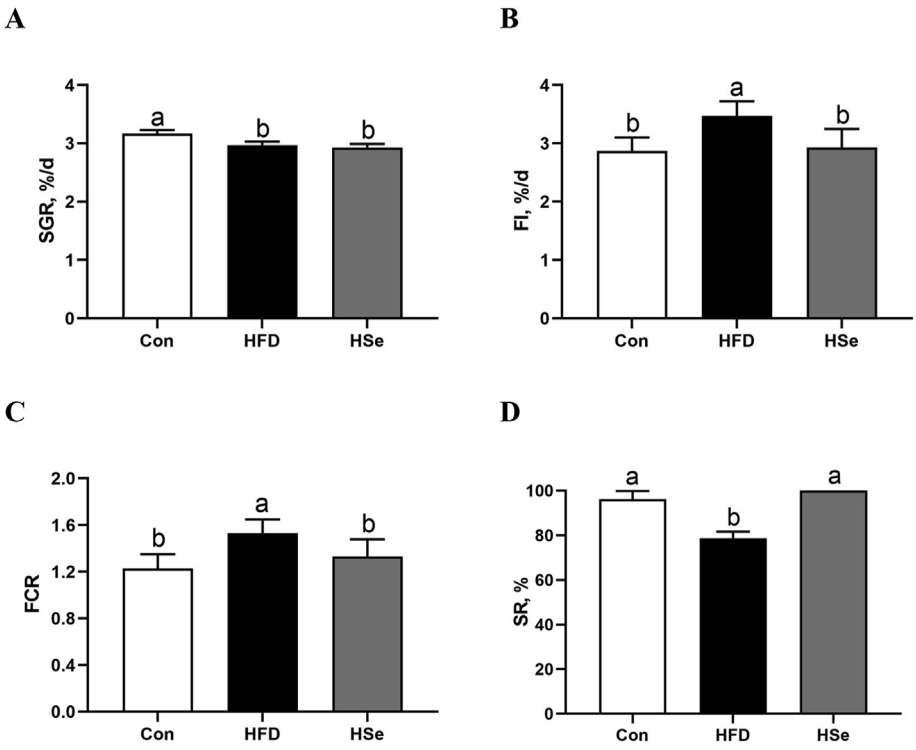


Fig. 1. Effect of dietary nano-Se on growth performance of juvenile grass carp (*Ctenopharyngodon idella*) fed with high-fat diet (HFD). (A) SGR = specific growth rate; (B) FI = feed intake; (C) FCR = feed conversion ratio; (D) SR = survival rate. Values are presented as mean ± SD ($n = 3$). [a, b] Significant differences are indicated by different letters ($P < 0.05$).

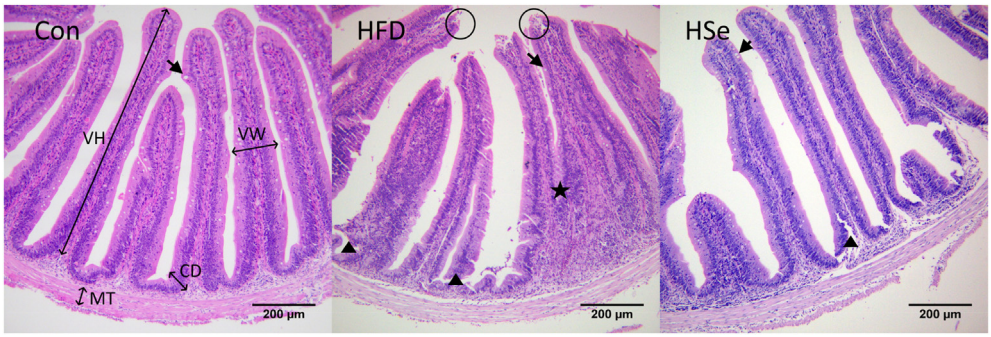


Fig. 2. Effects of dietary nano-Se on intestinal morphology of juvenile grass carp fed with high-fat diet (HFD). VH = villus height; VW = villus width; CD = crypt depth; MT = intestinal epithelial muscle thickness. Arrows represent goblet cells. Circles represent intestinal mucous membrane shedding. Triangles represent intestinal villi fall off. The pentagram represents intestinal villus adhesion. Scale bar, 200 μm.

Dietary supplements have been proven to improve gut health and reduce the negative effects of HFD in fish. Adding berberine to the HFD could mitigate oxidative stress, inhibit apoptosis and enhance disease resistance of fish (Chen et al., 2017). Dietary sodium butyrate could repair or prevent intestinal damage caused by the oxidized oil diet of juvenile common carp (Liu et al., 2014). Selenium, as an essential trace mineral, is vital to fish health. Selenium is an antioxidant and active thyroid hormone production catalyst (Özkan-Yılmaz et al., 2014; Winther et al., 2015). Numerous studies have indicated that selenium deficiency has led to reduced growth, decreased feed intake, damaged cellular oxidative, decreased immunity and increased mortality (Gao et al., 2019; Liu et al., 2018). Adequate selenium supplementation could enhance the immune system of juvenile grass carp, thus reducing the generation of oxidative stress (Liu et al., 2018). Nano-Se has been developed to supplement selenium due to its good water solubility and low toxicity (Yang et al., 2014a). A study showed that nano-Se had a greater impact on the growth performance and even antioxidant defense system of carp than other forms of selenium (Saffari et al., 2016). The protective effects of selenium on the intestinal mucosa and the integrity of intestinal barriers have been reported in animals (Baldwin and Wiley, 2002; Placha et al., 2014; Xu et al., 2018), but its mechanism is still unclear. Besides, our previous studies have reported that the addition of 0.3 and 0.6 mg/kg of nano-selenium alleviated hepatopancreatic injury and improved the survival rate of grass carp, and that the addition of 0.6 mg/kg of nano-selenium was more effective (Liu et al., 2021; Yu et al., 2020). At present, it is not clear whether selenium (including nano-Se) can alleviate the intestinal damage of fish caused by HFD.

Intestinal flora is involved in nutrient harvest, energy regulation, intestinal barrier and inflammation (Lin et al., 2014). For example, in mice fed with HFD, the bacterium *Akkermansia muciniphila* improves the integrity of the intestinal barrier and changes the metabolism of adipose tissue, thus preventing obesity and inflammation (Everard et al., 2013). Moreover, short chain fatty acids (SCFA), which are major products of dietary fibres fermentation by gut microbiota in

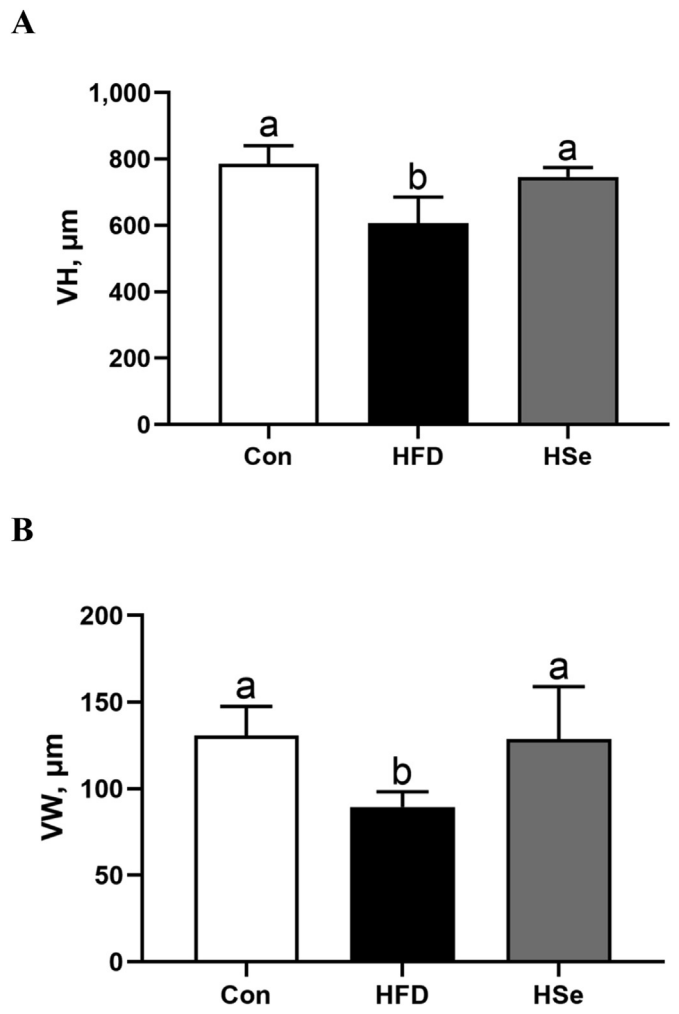


Fig. 3. Effects of dietary nano-Se on intestinal morphological parameters of juvenile grass carp fed with high-fat diet (HFD). (A) VH = villus height; (B) VW = villus width. Values are presented as mean ± SD. [a, b] Significant differences are indicated by different letters ($P < 0.05$).

the large intestine, play an essential role in maintaining intestinal barrier function (Chen et al., 2017). Studies indicate that the gut microbiota of poultry can be changed by selenium nanoparticles in feed (Gangadoo et al., 2018), and the improvement of dietary selenium yeast (SY) on goat colon fermentation mode increases total SCFA concentration (Benazir et al., 2016). However, it is not clear whether selenium (including nano-Se) can regulate the intestinal microbiota and their metabolites SCFA in fish and consequently alleviate intestinal damage caused by HFD.

Hence, in this study, the protective effects of nano-Se supplementation on intestinal morphology and intestinal integrity of juvenile grass carp that are fed with HFD were evaluated. Then the mRNA expression levels of genes related to tight junction (zonula occludens-1 [*ZO-1*], *claudin-3* and *occludin*), inflammation (*IL-8*, *IL-1β*, interferon γ [*IFN-γ*], tumor necrosis factor-α [*TNF-α*], *IL-6*, toll-like receptor 4 [*TLR4*], *p38 MAPK*, *NF-κB p65*) and anti-oxidization (glutathione peroxidase 4a [*GPx4a*] and *GPx4b*), and the proteins expression levels of ZO-1, NF-κB p65 and TNF-α in the intestine were detected. Further, the intestinal microbiota and the concentration of SCFA were further determined, aiming to explore the possible mechanism of adding nano-Se to alleviate intestinal damage induced by HFD.

2. Materials and methods

2.1. Animal ethics

All experimental procedures were carried out in accordance with the Guidelines for Experimental Animals by the Animal Care and Use Committee of Northwest A&F University, China.

2.2. Experimental design

The regular diet (Con), the HFD, and the HFD with added nano-Se (0.6 mg/kg) (HSe) were prepared. The compositions of the basal diet are shown in Table 1. The crude fat content of the regular diet and HFD were 49 and 96 g/kg, respectively (Li et al., 2016). The addition level of nano-Se was according to our previous study (Liu et al., 2021). The nano-Se (>99% purity) was purchased from Guangzhou Bosar Biochemical Technology Research Co., Ltd (Guangzhou, Guangdong Province, China).

2.3. Fish management and feeding

Healthy juvenile grass carp were purchased from a fishing ground in Ankang City, Shanxi Province, China. Before the start of the experiment, the experimental fish were cultured in a circulating water system. After 2 weeks acclimatization, a total of 135 juvenile grass carp individuals, with the size of 11.85 ± 0.10 g, were randomly divided into 9 tanks. Every 3 tanks (triplicate) were randomly assigned to one kind of experimental diet, and the feeding trial lasted 10 weeks. During the feeding trial, the experimental fish were fed artificially 3 times a day (08:30, 12:30 and 16:30), and the feeding amount was recorded. The water environment was as follows: water temperature, 28 ± 1 °C; dissolved oxygen concentration >6.0 mg/L; pH, 7.0 ± 0.5. Water quality parameters were monitored daily to ensure the stability of the water environment.

2.4. Sample collection

At the end of the trial, the fish were anaesthetized with MS-222 (90 mg/L) and weighed and sampled 24 h after the last feeding. Three fish were randomly taken from each tank, and their intestines were quickly separated on the ice. The same parts of the intestines of different fish were cut carefully and immediately place in 4% neutral formaldehyde for histopathological examination (about 1 to 2 cm lengths). The intestinal contents were gently scraped with tweezers and placed in a 1.5-mL DNase-/RNase-free centrifuge tube. These samples were transferred to liquid nitrogen and then stored at −80 °C. The rest of the intestinal tissues were quickly wrapped in tin foil and put into a 2-mL freezing tube, frozen in liquid nitrogen and transferred to −80 °C for storage until further analysis.

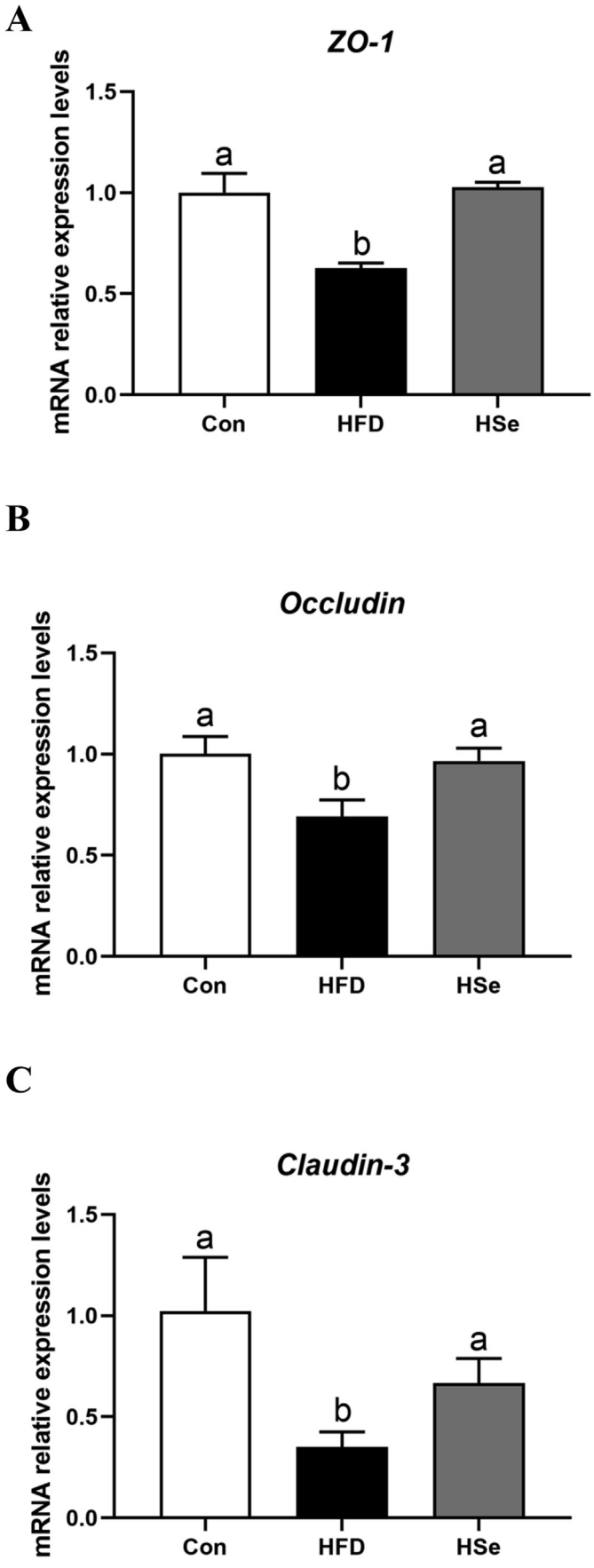
The growth performance parameters were calculated as follows.

Specific growth rate (SGR, %/day) = 100 × (ln Final weight − ln Initial weight)/Days

Feed intake (FI, %/day) = 100 × {Amount of feed intake/[(Final weight + Initial weight)/2]}/Days

Feed conversion rate (FCR) = Amount of feed intake/Body weight gain

Survival rate (SR, %) = 100 × Final number of fish/Initial number of fish



2.5. Intestinal histological analysis

After paraffin embedding, the collected intestinal segments were cut into slices about 5 μm thick. They were then stained with hematoxylin and eosin (H&E) and observed under an optical microscope (Meng et al., 2017). Then, the height (VH) and width (VW) of intestinal villi in different groups were measured by ImageJ. VH was measured from the tip of the villi to the mouth of the crypt and VW was measured at the midpoint of each villus.

2.6. Gene expression analysis

Gene sequences were found from the National Center for Biotechnology Information (NCBI) and primers were designed. The sequences of primers used in this paper are shown in Table 2. Samples were taken from the −80 °C refrigerator, and 50 to100 mg of tissues were used to extract RNA. The RNA extraction reagent: AG RNAex Pro Reagent from Accurate Biotechnology (Hunan) Co., Ltd; the concentration and purity of RNA were determined by spectrophotometry (NanoDrop 1000, Thermo Scientific). Reverse transcription reagent: HiScript II Q Select RT SuperMix for qPCR (+ gDNA wiper), reverse transcriptase was prepared according to the instructions; qPCR reagent: ChamQ SYBR qPCR Master Mix. The reaction mixture for PCR consisted of 5 μL 2 × SYBR Green PCR Master Mix, 1 μL of synthesized cDNA, 1.5 μL each specific primer to a final volume of 10 μL. The amplification was carried out in a real-time PCR detection system (CFX96, Bio-Rad) and the PCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 62 °C for 30 s, and melting temperature from 65 to 95 °C. The Ct value was obtained, and the relative changes of each target gene expression between the experimental groups were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The amplification efficiency of each pair of primers ranged from 97% to 102%.

2.7. Western blot

Protein extraction was performed with a protein extraction kit (RIPA buffer, Solarbio, China) and the content was determined with a BCA protein assay kit (Solarbio, China). The protein marker used in this experiment was sourced from ThermoFisher Scientific (26634). Proteins were separated with 8% to 10% sodium dodecyl sulfate gels (40 μg per sample), and wet transferred and blotted on polyvinylidene fluoride membrane (YA1701, 0.45 μm, Solarbio, China). After blocking with 5% skimmed milk powder for 1.5 h at room temperature in TBST, they were washed 4 times with TBST for 8 min. The following antibodies were used in blocking buffer overnight at 4 °C: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the control protein of total protein, rabbit anti-GAPDH (1:2,000, Servicebio, China), anti−NF−κB p65 (1:800, Wanlei Bio., China), anti-TNF-α (1:800, Wanlei Bio., China) and anti-ZO-1 (1:800, Wanlei Bio., China). Samples were then washed with TBST 4 times, for 8 min, incubated with goat anti-rabbit IgG (H + L) HRP (Biosharp, China) (1:2,000) in TBST for 1 h, then washed 4 times with TBST for 8 min each time. The membrane was then incubated in ECL Super Sensitive Kit (DiNing, China) for 30 s and exposed to X-ray film. ImageJ was used to analyze the gray level of

Fig. 4. Effects of dietary nano-Se on mRNA expression of intestinal tight junction protein in juvenile grass carp fed with high-fat diet (HFD). (A) *ZO-1*; (B) *occludin*; (C) *claudin-3*. Values are presented as mean ± SD ($n = 3$). [a, b] Significant differences are indicated by different letters ($P < 0.05$). *ZO-1* = zonula occludens-1.

the image. The relative expression level was calculated based on the ratio of the gray value of the target protein to the GAPDH protein.

2.8. Intestinal contents DNA extraction and sequencing

To evaluate the effect of nano-Se on the composition of gut microbiota, the V4–V5 region of bacterial 16 S rDNA was amplified and sequenced by Illumina MiSeq platform. Frozen intestinal contents were used to detect the richness of intestinal flora in different experimental groups. The experimental steps were mainly divided into the following aspects: the extraction of genetic DNA, PCR amplification, Miseq library construction and computer sequencing by Illumina MiSeq. Finally, the sequences of the template DNA fragment were obtained based on the fluorescence signal.

2.9. Short chain fatty acid content tested by GC–MS

About 200 mg of intestinal contents were taken to measure the content of SCFA. Seven kinds of SCFA were tested by targeted detection based on the gas chromatography-mass spectrometer (GC–MS) detection platform, according to the method of Furuhashi et al. (2018). The 7 kinds of SCFA are acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and caproic acid.

2.10. Statistical analysis

All data were statistically analyzed with SPSS 20.0 software (SPSS). A one-way ANOVA and then a least significant difference (LSD) post hoc test to analyze the data. Prior to applying ANOVA, the mean square error of all data was tested by the Levene test, and the normal distribution was tested by the Kolmogorov–Smirnov test. The significant difference level was set at $P < 0.05$. Furthermore, GraphPad Prism 8.0 was used for mapping.

3. Results

3.1. Growth and FCR

The effects of dietary nano-Se on SGR, FI, FCR and SR of juvenile grass carp are presented in Fig. 1A, B, C and D. High-fat diet significantly decreased SGR and SR of juvenile grass carp and increased FI and FCR compared with the regular diet ($P < 0.05$). Nevertheless, nano-Se supplementation significantly increased the SR but decreased the FCR of juvenile grass carp in comparison with the HFD ($P < 0.05$). However, no significant changes were observed in the SR and FCR of juvenile grass carp in the HSe and Con groups ($P > 0.05$).

3.2. Intestinal morphology

By observing the microstructure of the intestine, the effects of the supplement of nano-Se on the intestinal morphology and function in juvenile grass carp were evaluated (Figs. 2 and 3). Compared with the control group, the intestinal villi in the HFD group were structurally disordered and showed intestinal villi atrophy, intestinal villi adhesion and intestinal villi shedding. In contrast, the addition of nano-Se in HFD showed that the above intestinal injury could be effectively alleviated (Fig. 2). Also, the intestinal epithelial cells of the HSe and control groups were arranged closely and orderly compared with HFD group, and there were apparent gaps (Fig. 2). Compared with the regular diet, HFD markedly decreased the intestinal villus height (VH) and width (VW) of juvenile grass carp. However, dietary nano-Se resulted in taller ($P < 0.05$) VH as well as longer ($P < 0.05$) VW in comparison with the HFD group (Fig. 3A and B).

3.3. The expressions of the tight junction, inflammation and anti-oxidization related genes and proteins

First, as shown in Fig. 4, HFD significantly reduced the expression of tight junction protein genes of the intestine, including *ZO-1* (Fig. 4A), *occludin* (Fig. 4B) and *claudin-3* (Fig. 4C) ($P < 0.05$). Moreover, dietary nano-Se significantly increased the mRNA expression of *ZO-1*, *occludin* and *claudin-3* compared with HFD ($P < 0.05$). Compared with the regular diet, protein expression level of ZO-1 in the intestine significantly decreased in the HFD group. However, dietary nano-Se significantly enhanced the protein expression level of ZO-1 (Fig. 5, $P < 0.05$).

Secondly, as presented in Fig. 6, the mRNA expression levels of *TLR4* (Fig. 6B), *p38 MAPK* (Fig. 6C), *NF-κB p65* (Fig. 6D), *IL-8* (Fig. 6E), *IL-1β* (Fig. 6F), *IFN-γ* (Fig. 6G) and *TNF-α* (Fig. 6H) elevated significantly due to the HFD ($P < 0.05$). However, the mRNA levels of *TLR4*, *p38 MAPK*, *NF-κB p65*, *IL-8*, *IL-1β*, *IFN-γ*, *TNF-α* and *IL-6* (Fig. 6I) were significantly reduced owing to the nano-Se supplement ($P < 0.05$). Meanwhile, as shown in Fig. 7, the protein expression levels of NF-κB p65 and TNF-α significantly increased in the HFD group, and significantly decreased in the HSe group compared with Control ($P < 0.05$).

Furthermore, HFD significantly increased the mRNA levels of *Hif-1α* (Fig. 8) compared with the regular diet ($P < 0.05$). But nano-se supplementation markedly reduced the mRNA expression level of *Hif-1α* compared with that in the HFD group ($P < 0.05$).

Finally, as presented in Fig. 9, the mRNA expression levels of *GPx4a* and *GPx4b* significantly ($P < 0.05$) decreased in the intestine of juvenile grass carp that were fed with HFD. But, nano-Se

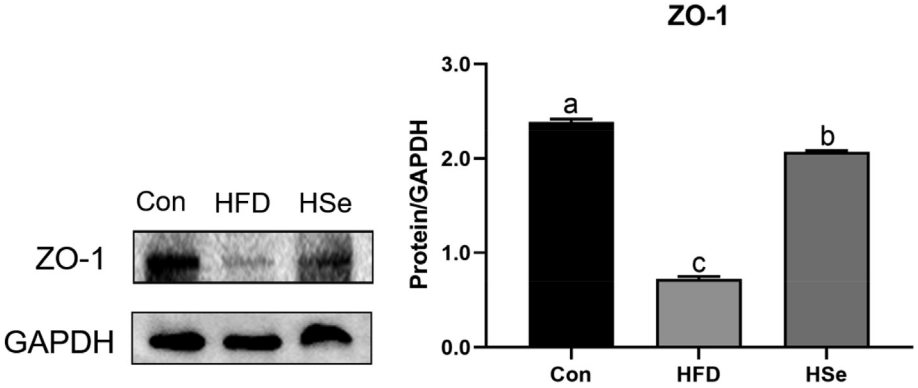


Fig. 5. Effects of dietary nano-Se on the protein expression of intestinal tight junction protein (ZO-1) in juvenile grass carp fed with high-fat diet (HFD). Values are presented as mean ± SD ($n = 3$). [a, b] Significant differences are indicated by different letters ($P < 0.05$).

supplementation in the HFD group markedly induced the mRNA expressions of the *GPx4a* and *GPx4b* of the intestine in juvenile grass carp.

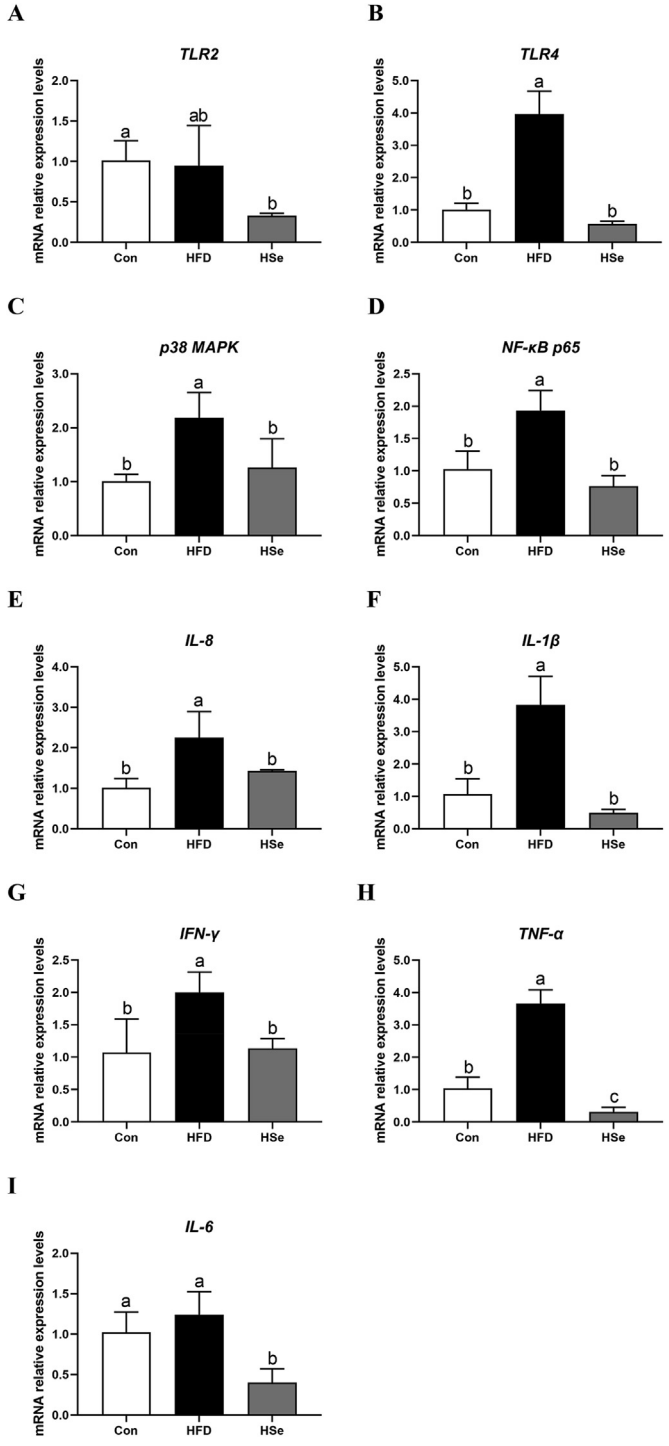


Fig. 6. Effects of dietary nano-Se on mRNA expression of intestinal inflammatory factors in juvenile grass carp fed with high-fat diet (HFD). (A) *TLR2*; (B) *TLR4*; (C) *p38 MAPK*; (D) *NF-κB p65*; (E) *IL-8*; (F) *IL-1β*; (G) *IFN-γ*; (H) *TNF-α*; (I) *IL-6*. Values are presented as mean ± SD ($n = 3$). [a, b, c] Significant differences are indicated by different letters ($P < 0.05$). HSe = nano-Se group; *TLR* = toll-like receptors; *MAPK* = mitogen-activated protein kinase; *NF-κB* = nuclear factor kappa-B; *IL* = interleukin; *IFN-γ* = interferon γ; *TNF-α* = tumor necrosis factor α.

3.4. Intestinal microbiota

The V4 and V5 regions of the bacterial 16 S rDNA were amplified and sequenced on the Illumina MiSeq platform to evaluate the impact of nano-Se on the composition of gut microbiota. After the quality screening, each of the 9 samples (3 duplicate samples for each group) received at least a good sequence (clean tags) of 70,932. Clean tags were clustered (or denoised) to generate operational taxonomic unit (OTU), and a total of 720 OTU were observed in all samples. As shown in Fig. 10A, B and C, compared with group C, the observed species and the Chao-1 index reflecting species richness and diversity were reduced in group H, and the Shannon index also decreased. In contrast, the richness and diversity of the gut microbiota of juvenile grass carp fed with HFD added with nano-Se (0.6 mg/kg) were enhanced (Fig. 10A, B and C). In addition, non-metric multidimensional scaling (NMDS) was used to analyze the beta diversity shown in Fig. 10D. Nano-Se group (HSe) clustered separately from the HFD group, and was similar in their microbiota profiles to the Con group.

The composition of gut microbiota at the phylum and genus levels in juvenile grass carp in Con, HFD and HSe groups are presented in Fig. 11A and B. Most of the gut microbiota belongs to Proteobacteria (31.03%, 21.31% and 16.14%), Fusobacteria (10.12%, 3.49% and 34.46%), Actinobacteria (16.87%, 12.12% and 11.89%), Chloroflexi (18.28%, 19.02% and 3.03%), Planctomycetes (11.29%, 11.06% and 10.13%), Bacteroidetes (1.07%, 19.42% and 11.29%), Firmicutes (4.80%, 12.03% and 9.70%), Chlamydiae (3.61%, 1.12% and 1.21%), Verrucomicrobia (2.62%, 0.28% and 2.07%) in Con, HFD, and HSe groups, respectively.

On the other hand, at the genus level, HFD decreased the percentage of *Cetobacterium*, *Leifsonia* and *Pirellula* compared with the regular diet. In contrast, the percentage of *Bacteroides*, *Gemmobacter*, *Planctomyces* was higher than that in the Con group, and all these variations above were reversed by the dietary nano-Se intervention (Fig. 11B).

3.5. Concentration of short chain fatty acid

As presented in Fig. 12, among the 6 kinds of SCFA detected in all 3 groups, acetic acid, propionic acid and butyric acid had the highest concentrations. High-fat diet reduced the concentration of 6 kinds of SCFA compared with the regular diet. However, nano-Se supplementation in the HFD increased acetic acid, isobutyric acid, butyric acid, isovaleric acid and caproic acid concentration levels and reduced the concentration level of propanoic acid.

3.6. Correlation between the abundance of gut microbiota and SCFA levels

A heatmap of Spearman's correlation between the abundance of gut microbiota (at the genus level) and SCFA levels is presented in Fig. 13. *Cetobacterium* was significantly positively correlated with isobutyric acid and caproic acid, but *Planctomyces* and *Alpinimonas* were opposite ($P < 0.01$). The abundance of *Pirellula*, *Nocardioides*, *Meganema*, *Luteolibacter* was significantly positively correlated with the butyric acid.

4. Discussion

4.1. Nano-Se supplementation decreased mortality rate and FCR of juvenile grass carp fed with high-fat diet

A previous study found that the growth reaction increased with the increase of dietary lipid level until the optimal demand level was reached, and after that it decreased as the lipid level in the diet increased (Meng et al., 2018). In the present study, HFD significantly

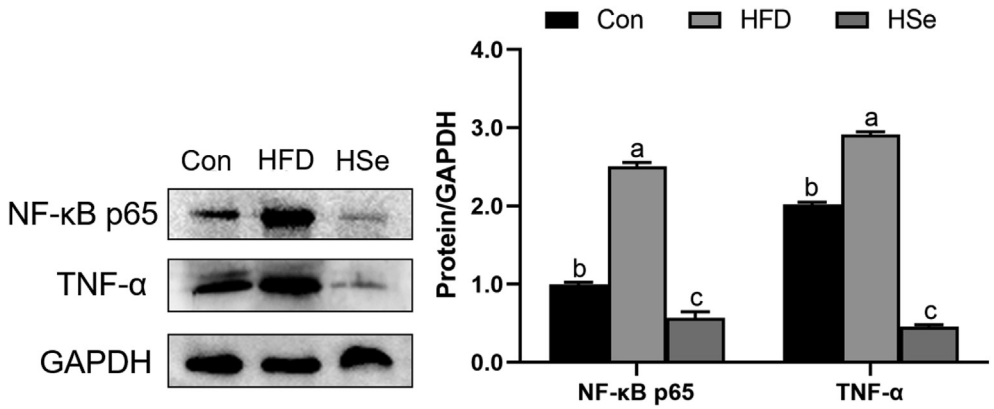


Fig. 7. Effects of dietary nano-Se on proteins expression of intestinal inflammatory factors in juvenile grass carp fed with high-fat diet (HFD). Values are presented as mean ± SD (n = 3). [a, b, c] Significant differences are indicated by different letters (P < 0.05). HSe = nano-Se group; NF-κB = nuclear factor kappa-B; TNF-α = tumor necrosis factor α.

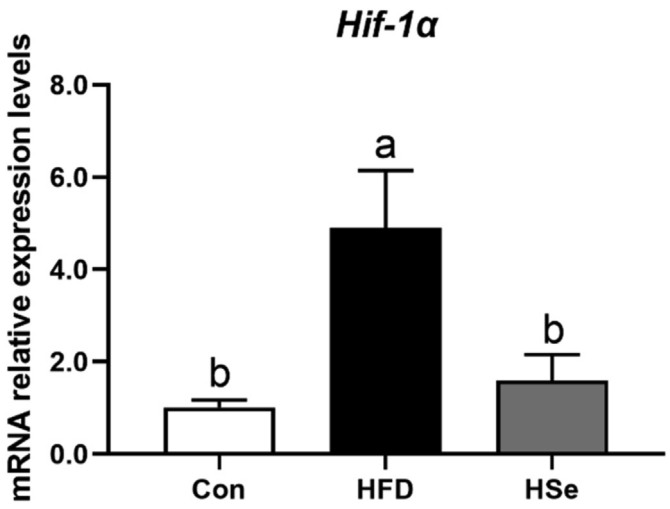


Fig. 8. Effects of dietary nano-Se on mRNA expression of *Hif-1α* in the intestine of juvenile grass carp fed with high-fat diet (HFD). Values are presented as mean ± SD (n = 3). [a, b] Significant differences are indicated by different letters (P < 0.05). HSe = nano-Se group; *Hif-1α* = hypoxia inducible factor-1α.

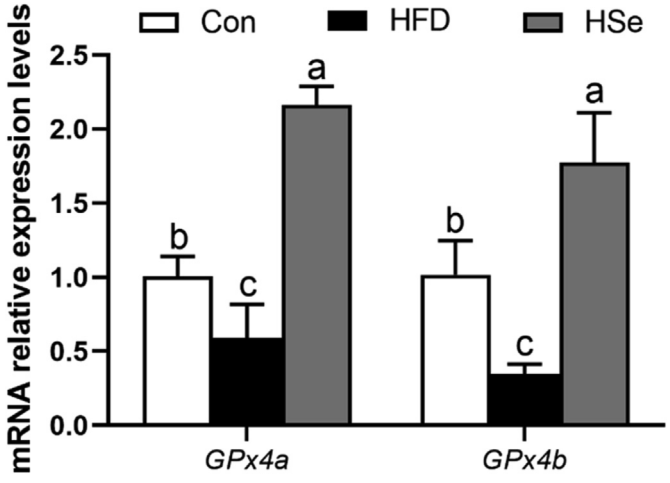


Fig. 9. Effects of dietary nano-Se on expression of *GPx4a* and *GPx4b* in the intestine of juvenile grass carp fed with high-fat diet (HFD). Values are presented as mean ± SD (n = 3). [a, b, c] Significant differences are indicated by different letters (P < 0.05). HSe = nano-Se group; *GPx4* = glutathione peroxidase 4.

decreased the SGR and the SR of juvenile grass carp and increased the FCR compared with the regular diet, indicating the adverse effects of excessive fat intake. Liu et al. (2018) reported that the optimal level of dietary selenium (0.83 mg/kg) significantly improved the growth performance of grass carp. But in the present study, the addition of nano-Se (0.6 mg/kg) in the HFD did not reveal a significant increase in the SGR of the juvenile grass carp. The different level of dietary Se or lipid may explain such diverse results.

Interestingly, nano-Se supplementation significantly increased the SR of the juvenile grass carp fed with HFD, and significantly decreased the FCR of the juvenile grass carp fed with HFD. The results were similar to the study in which a significant dose-dependent improvement in FCR and SR were observed in Meagre (*Argyrosomus regius*) fed different levels of Se-yeast (Mansour et al., 2017). The above results indicated that dietary supplementation of nano-Se could significantly increase the yield and reduce the production cost of juvenile grass carp fed with HFD by decreasing mortality and improving the feed utilization efficiency of the juvenile grass carp.

4.2. Dietary nano-Se alleviated HFD induced histopathological damage in intestine of juvenile grass carp

The intestinal epithelial barrier consists of epithelial cells, tightly coupled proteins, and intestinal secretions that prevent luminal substances and antigens from passing through the paracellular space (Yan and Ajuwon, 2017). Goblet cells synthesize secretory mucin glycoproteins and bioactive molecules to form the intestinal mucin layer, which form the front line of natural host defense (Cornick et al., 2015; Kim and Ho, 2010). The indicators for evaluating a healthy intestine include goblet cell count, intestinal villus height (VH), villus width (VW) and so on (Kuebutornye et al., 2020). Many studies have reported that HFD decreased the health status of the intestine (Ding et al., 2010; Meng et al., 2018). The present study also observed that the intestine of juvenile grass carp fed with HFD showed structural disorder and a decrease in VH and VW, which might consequently result in the increase of the FCR.

However, nano-Se supplementation significantly reduced intestinal villi damage and enhanced the morphological parameters of the intestine (VH and VW), thus improving the integrity of the intestinal mucosa. This positive effect has been reported by Tang et al. (2019a), in which dietary selenium alleviated the intestinal epithelial cell injury in mammals induced by heat stress. The increase in the structural integrity of intestinal villi reduces the colonization rate of pathogenic microorganisms, thus increasing

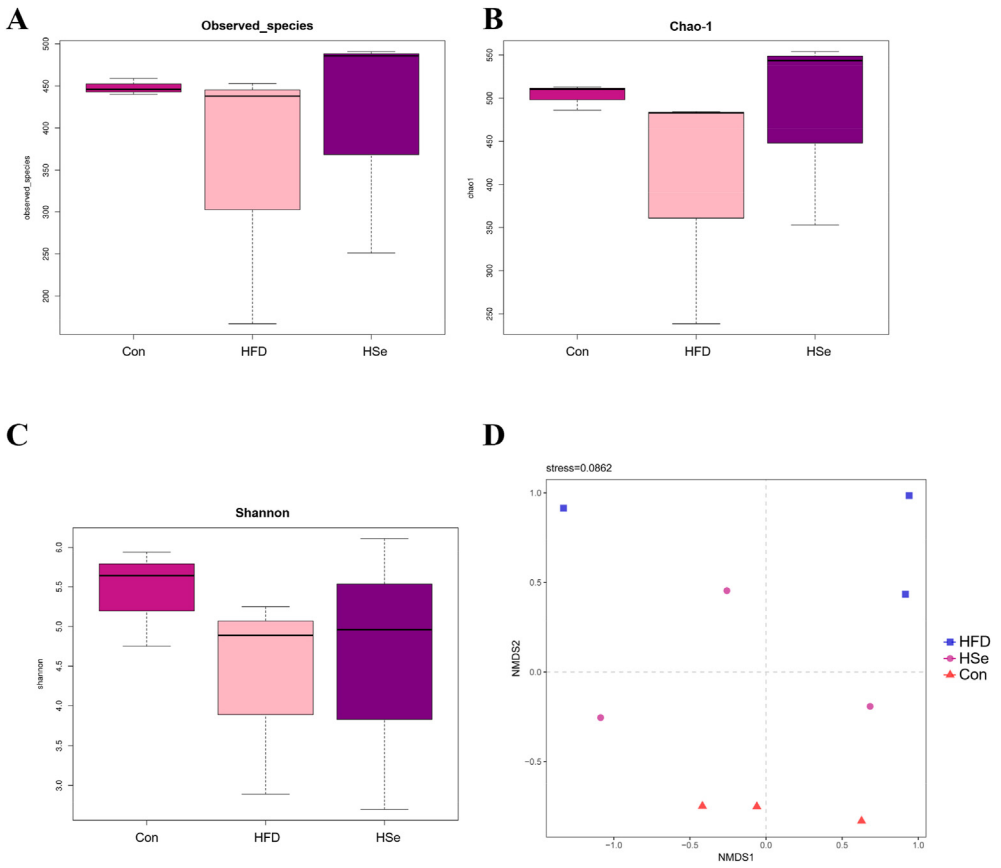


Fig. 10. Effects of dietary nano-Se on alpha and beta diversity of gut microbiota in juvenile grass carp fed with high-fat diet (HFD). Alpha diversity was evaluated by index of (A) observed species, (B) Chao-1 and (C) Shannon. (D) Beta diversity was evaluated by non-metric multidimensional scaling (NMDS) ($n = 3$).

the disease resistance of fish (Xia et al., 2019). Therefore, the mortality rate of juvenile grass carp fed with HFD that were supplemented with nano-Se was observed. Moreover, an increase in VH and VM led to the increase of the intestinal absorption surface (Kuebutornye et al., 2020), which could consequently increase the feed utilization efficiency. Hence, the increased FCR of juvenile grass carp in the diet of juvenile grass carp fed with nano-Se was observed.

4.3. Nano-Se supplement modulated the mRNA expression levels of genes associated with the tight junction, inflammation and oxidative stress in the intestine of juvenile grass carp fed with HFD

Tight junction is a complex dynamic structure composed of the transmembrane protein occludin, junctional adhesion molecule, claudin family members, and linker proteins such as ZO-1 (Dokladny et al., 2016; Zhang and Guo, 2009). Decreased expression of intestinal tight junction protein interrupted the tight junction barrier and led to increased intestinal permeability (He et al., 2019). Studies have shown that HFD could down-regulate the expression of tight junction proteins, increase intestinal permeability, and consequently promote the diffusion of LPS, which plays a key role in inducing intestinal and systemic inflammatory responses (Kawano et al., 2016; Murakami et al., 2016; He et al., 2019). In this study, HFD significantly suppressed the mRNA expression of epithelial tight junction protein genes (*ZO-1*, *occludin* and *claudin-3*) and the protein expression of ZO-1, leading to the increase in intestinal permeability of juvenile grass carp. Previous studies have confirmed that selenium treatment can increase relocation to endothelial cell–cell junctions of the tight-junction proteins occludin and ZO-1 (He and Xu, 2017; Pan et al., 2018). The current research also observed that the mRNA expressions of *ZO-1*, *occludin* and *claudin-3* and the protein expression of ZO-1 in the intestine of juvenile grass carp were significantly up-regulated by the dietary nano-Se, which indicated that the addition of nano-Se prevented the increase of intestinal permeability caused by HFD.

Dysfunction of the intestinal barrier is often associated with inflammatory bowel disease (McGuckin et al., 2009; Salim and Soderholm, 2011). Toll-like receptors (TLR) are involved in the recognition of invading pathogens and trigger inflammatory responses, including the production of pro-inflammatory cytokines (Shih et al., 2018). Molecular mechanisms that stimulate inflammatory responses through TLR involve activation of intracellular signaling pathways, including MAPK and NF-κB (Goral and Kovacs, 2005; Hotamisligil, 2006; Han et al., 2019; Kim et al., 2012). It has been reported that increased expression of *TLR4* gene in intestinal epithelial cells leads to a change in tight connectivity permeability and an increase in intestinal inflammation (Serre et al., 2010). High-fat diet can cause intestinal inflammation and increased *TNF-α*, *IL-1*, and *IL*-6 mRNA expression levels in mice (Cani et al., 2008; Moran-Ramos et al., 2017). The current study also found that HFD significantly induced the expression of intestinal inflammation-related genes, including *TLR4*, *p38MAPK*, *NF-κB p65*, *IL-8*, *IL-1β*, *IFN-γ* and *TNF-α* ($P < 0.05$), and also significantly induced the proteins expression of NF-κB p65 and TNF-α, which might lead to the increase of intestinal permeability and damage ($P < 0.05$).

Studies have reported that selenium may inhibit the activation of NF-κB (Duntas, 2009; Liu et al., 2016), and the intake of a low-

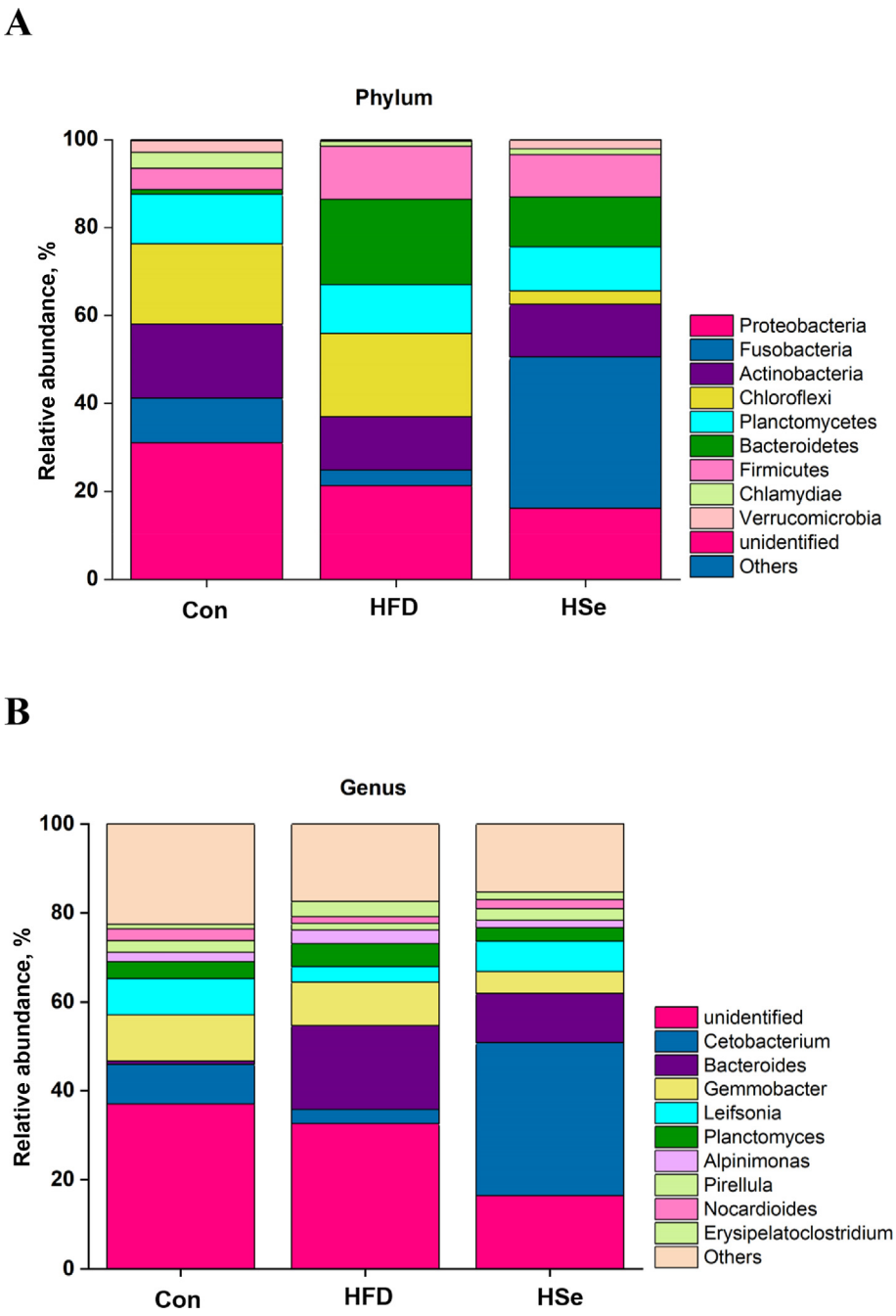


Fig. 11. Effects of dietary nano-Se on the species composition of gut microbiota in juvenile grass carp fed with high-fat diet (HFD) at (A) phylum and (B) genus levels ($n = 3$).

selenium diet was often accompanied by the increase of *IL-6*, *IL-1β*, and *TNF-α* (Tsuji et al., 2015; Zhou et al., 2014), thus aggravating gastrointestinal inflammatory lesions (Gao et al., 2016; Liu et al., 2016). In this study, a marked reduction in the expression of *TLR4* in the intestine of juvenile grass carp supplied with HFD containing nano-Se, followed by a significant decrease in the expression of *p38 MAPK* and *NF-κB p65* was observed. As a result, the expression of proinflammatory factors (*IL-8*, *IL-1β*, *IFN-γ*, *TNF-α* and *IL-6*) in intestine was also significantly reduced (Fig. 6), indicating that nano-Se can alleviate intestinal inflammation induced by HFD.

Research has shown that IFN-γ up-regulated the expression of hypoxia-inducible factor-1 alpha (Hif-1α) through the NF-κB pathway, thereby inducing the loss of epithelial barrier function and the disruption of tight junction proteins (Yang et al., 2014b). Meanwhile, the role of Hif-1α in protecting mucosa in vivo has been confirmed by a growing volume of literature (Hindryckx et al., 2010; Tambuwala et al., 2010). As such, the mRNA expression of *Hif-1α* was further tested in this research, and the result revealed that nano-Se supplementation markedly reduced the mRNA level of *Hif-1α* (Fig. 8) which was induced by HFD, indicating that nano-Se supplementation might prevent the loss of epithelial barrier function and disruption of tight junction proteins induced by HFD by regulating the NF-κB-Hif-1α pathway. However, this needs further verification.

Glutathione peroxidase 4, as a member of the GPx family, catalyzes the reduction of hydrogen peroxide and lipid

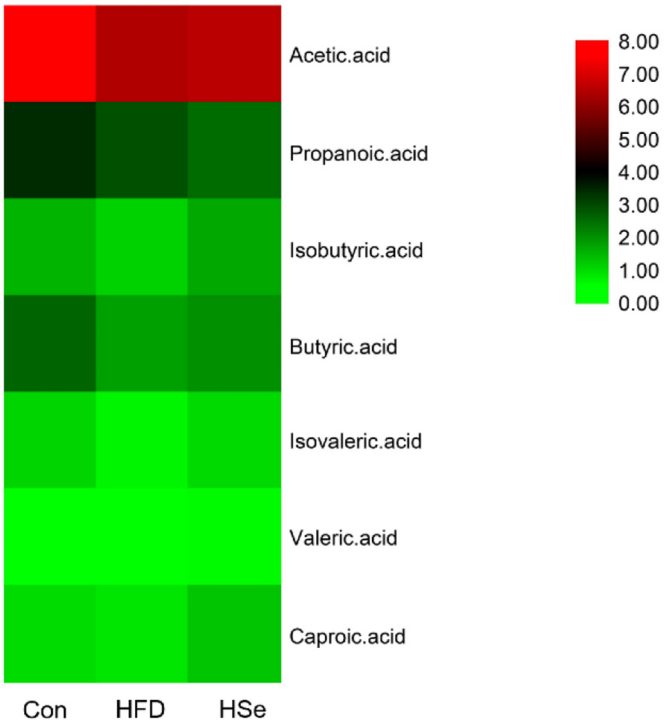


Fig. 12. Effect of dietary nano-Se on intestinal SCFA concentration in juvenile grass carp fed with high-fat diet (HFD) (red, high concentration; green, low concentration) ($n = 3$). HSe = nano-Se group.

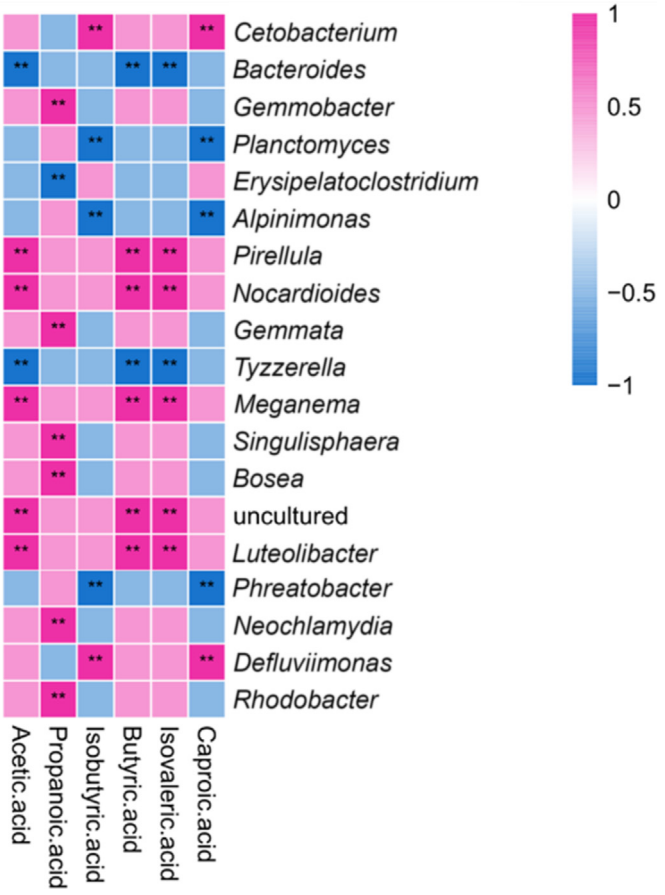


Fig. 13. Heatmap of Spearman's correlation between the abundance of gut microbiota (at the genus level) and short chain fatty acid levels. The intensity of the colors represented the degree of association (red, positive correlation; blue, negative correlation). Significant correlations are marked by $*P < 0.05$; $**P < 0.01$.

hydroperoxides by GSH (Ezea, 2013). Moreover, GPx4 plays essential roles in the prevention of selenium-dependent gastrointestinal oxidative injury in mammals (Speckmann et al., 2011). In the current study, the HFD suppressed the expression of *GPx4a* and *GPx4b* in the intestine, which might lead to the decline of antioxidant capacity and the aggravation of oxidative stress. However, expressions of *GPx4a* and *GPx4b* increased significantly in the intestine of the juvenile grass carp in group H, indicating that nano-Se supplementation might reduce the intestinal oxidative stress caused by HFD. Similar results were also found in a previous study in which the optimal level of dietary selenium up-regulated the expression of *GPx4* in the liver of chicken (Zoidis et al., 2010). Moreover, many studies have revealed that selenium supplementation in the diet significantly enhanced the activity of GPx (Boostani et al., 2015; Cai et al., 2012; Chen et al., 2013; Marković et al., 2018). The improved antioxidant capacity by dietary nano-Se might also contribute to the decrease in intestinal inflammation and injury, and consequently an improvement of intestinal barrier function.

These results indicated that the alleviating the effects of dietary supplementation of nano-Se on intestinal pathological injury of juvenile grass carp caused by HFD (revealed by pathologic section) might be achieved by upregulating the expression of genes involved in tight junction and anti-oxidization, and reducing the expression of inflammation-related genes and signal molecules. However, the specific regulatory mechanism needs further exploration.

4.4. Dietary nano-Se modulated composition and function of gut microbiota

Intestinal flora colonize the intestinal tract, forming a biological barrier in the intestinal tract, promoting the expression and secretion of mucin by intestinal cells, and maintaining the integrity of the intestinal barrier function (Chen et al., 2017). It has been reported that HFD could cause intestinal flora imbalance in mice (Liu et al., 2011; Zhou et al., 2020) and humans (Li and Cui, 2013). It has also been reported that HFD reduced species richness, accompanied by a significant reduction in the diversity of the gut microbiota in eels (Peng et al., 2019). Similarly, in the present study, results of 16 S rDNA sequencing revealed that HFD reduced the alpha diversity of gut microbiota, including the observed species, and reflected species richness and diversity index (Chao-1 index and Shannon index). Moreover, the beta diversity of gut microbiota, which is often used to measure the similarity of community composition between different treatments, was also shifted by HFD in juvenile grass carp (analyzed by NMDS). When the intestinal microecology is disordered, the increase in abundance of harmful microorganism will reduce the expression level of intestinal mucin, increase intestinal permeability, and promote the occurrence and development of host system inflammation and inflammatory intestinal diseases (Etienne-Mesmin et al., 2017; Thevaranjan et al., 2017). As expected, an increase in intestinal inflammation and the dysfunction of the intestinal barrier in juvenile grass carp induced by HFD were observed in this study.

Diet can rapidly and reproducibly alter the structure of the gut microbial community, regardless of its genetic influence (Zhang et al., 2020). Some studies in mice have shown that dietary selenium supplementation can affect the intestinal barrier and immune responses by regulating the gut microbiota (Kasaikina et al., 2011; Zhai et al., 2018). In the current study, dietary nano-Se supplementation increased the alpha diversity of gut microbiota, including the observed species, Chao-1 and Shannon index, and mitigated the loss

of species richness and diversity of gut microbiota induced by HFD. Further, dietary nano-Se partially recovered the beta diversity of gut microbiota that was shifted by the HFD. Moreover, nano-Se affected the composition of intestinal flora. At the phylum level, HFD increased the abundance of Bacteroides and (https://fanyi.so.com) Firmicutes and decreased the abundance of Fusobacteria, but nano-Se decreased the abundance of Bacteroides, Firmicutes (https://fanyi.so.com), Proteobacteria and Chloroflexi, and increased the abundance of Fusobacteria which had a positive effect on fish health by participating in the synthesis of vitamins and butyric acid (Huda et al., 2020). At the genus level, HFD increased the abundance of *Bacteroides* and decreased the abundance of *Cetobacterium*, which is considered a beneficial bacterium in fish (Noor-Ul et al., 2020), but nano-Se decreased the abundance of *Bacteroides*, *Gemmobacter* and increased the abundance of *Cetobacterium*.

Intestinal microorganisms catabolized carbohydrates that cannot be used by the host to produce SCFA, mainly acetic acid, propionic acid and butyric acid. Short chain fatty acids not only supply energy to a host, but also influence the intestinal immune cells through a variety of protein inflammatory complexes to regulate the immune response (Laszczyńska et al., 2019). Moreover, SCFA are closely related to the health of the intestinal barrier, especially butyric acid (Ohata et al., 2005). Studies have shown that the increase of intestinal SCFA levels induced by the increase of *Bifidobacterium* abundance inhibited the growth of pathogenic bacteria, reduced intestinal permeability, maintained the integrity of intestinal barrier and reduced inflammatory reaction (Cani et al., 2009; Moreira et al., 2012). Further, another study has documented that selenium in the diet significantly promoted gastrointestinal fermentation and resulted in increased levels of SCFA (Benazir et al., 2016). The current data also showed that nano-Se supplementation increased the concentrations of intestinal butyric acid and isobutyric acid that were reduced by the HFD (Fig. 12). As such, the decrease in intestinal inflammation and the improvement of intestinal barrier function in grass carp supplied with HFD containing nano-Se were observed.

Short chain fatty acid levels in the intestinal tract are influenced by the composition of the intestinal microbiota (Rooks and Garrett, 2016). In order to further confirm the relationship between the change of intestinal flora structure (at genus level) and the change of intestinal SCFA levels, the spearman's correlation analysis was carried out (Fig. 13). The result revealed that *Cetobacterium* was significantly positively correlated with isobutyric acid and caproic acid, but *Planctomyces* and *Alpinimonas* were opposite ($P < 0.01$). The abundance of *Pirellula*, *Nocardioides*, *Meganema*, *Luteolibacter* was significantly positively correlated with the butyric acid ($P < 0.01$). Hence, dietary nano-Se could promote the colonization of beneficial bacteria and consequently increase the production of SCFA, so as to alleviate intestinal damage caused by HFD. Furthermore, it provided a potential target to regulate the fish health, and it also provided a reference for the development of probiotics.

5. Conclusion

This study explored the alleviative effects of dietary nano-Se on the intestinal damage induced by HFD in grass carp. The above results demonstrated that supply of nano-Se (0.6 mg/kg) in HFD effectively protected the intestine of juvenile grass carp by improving intestinal barrier function and reducing intestinal inflammation and oxidative stress, and consequently improved both the survival rate of juvenile grass carp and feed utilization efficiency. These positive effects may be due to the regulation of nano-Se on intestinal microbiota and the subsequently increased beneficial SCFA levels.

Author contributions

Sha Liu: Methodology, Investigation, Data curation, Writing original draft, Formal analysis; **Haibo Yu:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing - review & editing; **Pengju Li:** Methodology, Investigation, Data curation; **Chi Wang:** Methodology, Investigation, Data curation; **Guohao Liu:** Investigation; **Xiaotian Zhang:** Investigation; **Cheng Zhang:** Investigation; **Meng Qi:** Funding acquisition; **Hong Ji:** Resources.

Declaration of competing interest

We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Paper is containing original research and has not been submitted/published earlier in any journal and is not being considered for publication elsewhere. All authors have seen and approved the manuscript and have contributed significantly for the paper.

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