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Original Research Article

The direct and gut microbiota-mediated effects of dietary bile acids on the improvement of gut barriers in largemouth bass (*Micropterus salmoides*)

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ABSTRACT

Fish gut barrier damage under intensive culture model is a significant concern for aquaculture industry. This study aimed to investigate the effects of bile acids (BAs) on gut barriers in *Micropterus salmoides*. A germ-free (GF) zebrafish model was employed to elucidate the effects of the direct stimulation of BAs and the indirect regulations mediated by the gut microbiota on gut barrier functions. Four diets were formulated with BAs supplemented at 0, 150, 300 and 450 mg/kg, and these 4 diets were defined as control, BA150, BA300 and BA450, respectively. After 5 weeks of feeding experiment, the survival rate of fish fed with BA300 diet was increased ($P < 0.05$). Histological analysis revealed an improvement of gut structural integrity in the BA150 and BA300 groups. Compared with the control group, the expression of genes related to chemical barrier (mucin, lysozyme and complement 1) and physical barrier (occludin and claudin-4) was increased in the BA150 and BA300 groups ($P < 0.05$), and the expression of genes related to immunological barrier (interleukin [*IL*]-6, tumor growth factor β, *IL-10*, macrophage galactose-type lectin and immunoglobulin M [*IgM*]) was significantly increased in the BA300 group ($P < 0.05$), but the expression of genes related to chemical barrier (hepcidin) and immunological barrier (*IL-1β*, tumor necrosis factor-α, *IL-6* and arginase) was significantly decreased in the BA450 group ($P < 0.05$). Gut microbiota composition analysis revealed that the abundance of Firmicutes was augmented prominently in the BA150 and BA300 groups ($P < 0.05$), while that of Actinobacteriota and Proteobacteria showed a downward trend in the BA150 and BA300 groups ($P > 0.05$). The results of the gut microbiota transferring experiment demonstrated an upregulation of gut barrier-related genes, including immunoglobulin Z/T (*IgZ/T*), *IL-6*, *IL-1β* and *IL-10*, by the gut microbiota transferred from the BA300 group compared with the control ($P < 0.05$). Feeding the BA300 diet directly to GF zebrafish resulted in enhanced expression of *IgM*, *IgZ/T*, lysozyme, occludin-2, *IL-6* and *IL-10* ($P < 0.05$). In conclusion, BAs can improve the gut barriers of fish through both direct and indirect effects mediated by the gut microbiota.

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1. Introduction

Aquatic products provide a large amount of high-quality food protein for humans and play an important part in food supply chain. With the gradual decline of capture fishery resources, more than half of the aquatic products come from aquaculture (Dauda et al., 2013; Hu et al., 2021). Aquaculture is the only solution to

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satisfy the world's growing need for aquatic products (Brugère et al., 2019; Food and Nations, 2020; Phillips et al., 2016). Due to the advantages of saving space and water resources, intensive aquaculture system is being developed rapidly. Intensive aquaculture is characterized by high farming density, which requires a high-energy feed to meet fish growth (Ngoc et al., 2021). However, in the intensive aquaculture systems, fish are continuously exposed to stress due to high density and an excessive intake of energy, which affects fish health, especially the health of the gut, the primary organ for nutrition digestion and absorption and the largest immune organ in the body (Dawood et al., 2019; Rohani et al., 2021; Schumann and Brinker, 2020; Sundh et al., 2010; Wang et al., 2020). Therefore, it is urgent to find feed additives that can improve the gut health of fish under the current intensive farming system.

The gut is an important organ for food digestion, nutrients uptake and immunity, while it acts as a barrier between the aquatic animals and the external environment (Gao et al., 2016). Gut barrier functions rely on the chemical, physical, immunological, and microbiological barriers (Anderson et al., 2012; Clayburgh et al., 2004; Cui et al., 2019). The gut chemical barrier comprises various substances that are secreted into the gut, including lysozymes, mucopolysaccharides, gastric acid, mucin, digestive enzymes and glycoproteins (de Aguiar Vallim et al., 2013). The main function of the chemical barrier is to protect gut mucosa from erosion by chemical substances such as digestive enzymes (Cui et al., 2019). The gut physical barrier is a complete and tightly connected gut epithelium structure, mainly comprising the gut mucous layer and epithelial cells. Tight junctions, desmosomes, adherens junctions and gap junctions are intercellular junctional complexes that mainly exist between epithelial cells (Ulluwishewa et al., 2011). Through the action of gut epithelial tight junctions, pathogens can be prevented from invading the body, while a large number of nutrients can enter the body, suggesting that the gut epithelial tight junctions are structural foundation for retaining the selective permeability and barrier function of the gut epithelium (Mu et al., 2017; Suzuki, 2013; Ulluwishewa et al., 2011). The gut immunological barrier is mainly composed of the gut-associated-lymphoid-tissue (GALT) (Martinez-Lopez et al., 2019). In fish, the intestine carries out an immunological barrier function via producing cytokines, acid phosphatase and other humoral immune factors (Pan et al., 2017). The gut immunological barrier can allow the immune system to build a barrier to protect the host by resisting the invasion of pathogenic microorganisms, resisting allergic reactions and inhibiting immune responses (Corthésy, 2010; Kamada et al., 2013; Liu et al., 2019; Russell et al., 2015). The gut microbiological barrier is composed of gut symbiotic microorganisms. The symbiotic microorganisms depend on as well as constrain each other, building a biological defense line for the host through the antagonism between microorganisms. Besides, the gut microbiological barrier can affect the metabolism and proliferation of the gut epithelial cells (Soderholm and Pedicord, 2019). The gut barrier is the host's first important defense line against infection and environmental stress, which is related to the integrity of gut morphology, structure and function, microbial composition and mucus immune compounds (Huang et al., 2018; Jutfelt, 2011). Therefore, the gut barrier plays an extremely important role in preserving the balance of the systemic immune system in fish.

Bile acids (BAs) are amphipathic molecules and have multiple physiological functions in the host (Kortner et al., 2013; Romański, 2007). They can promote the absorption and digestion of dietary lipids in the intestine by emulsifying lipids and improving the activity of lipase of bile salts (Hofmann and Hagey, 2008; Li et al., 2013). Furthermore, BAs participate in shaping of the gut microbial community and inhibiting the proliferation of pathogens in the intestine (Hagey et al., 2010; Ridlon et al., 2014; Romano et al., 2020). Recently, the functions of BAs have been studied in many fish species. Previous studies have reported that BAs can enhance growth performance, improve lipids digestion and absorption, alleviate stress responses and regulate gut microbiota of various fish species including juvenile rainbow trout (*Oncorhynchus mykiss*) (Adhami et al., 2017), Nile tilapia (*Oreochromis niloticus*) (Jiang et al., 2018), grass carp (*Ctenopharyngodon idella*) (Zhou et al., 2018), juvenile black seabream (*Acanthopagrus schlegelii*) (Jin et al., 2019), snakehead (*Channa argus*) (Hou et al., 2019), juvenile large yellow croaker (*Larimichthys crocea*) (Ding et al., 2020), and juvenile largemouth bass (*Micropterus salmoides*) (Yin et al., 2021). However, the function and mechanisms of action of BAs on the gut barrier of fish remain unknown.

The largemouth bass (*M. salmoides*) is an economically important freshwater fish species in China. It is characterized by fast growth, strong disease resistance and delicious taste. Its production reached 619,519 tonnes in 2020 (Fisheries Administration Burrau, 2021). The recommended dietary fat levels for largemouth bass range from 80 to 130 g/kg, and the diets containing 15% crude fat are often used to investigate the adverse effects of high-fat diet in the largemouth bass (Bright et al., 2005; Huang et al., 2017a; Liu et al., 2022). Herein, the diet containing 15% crude fat was used to induce gut damage under experimental settings. A 5-week culture trial was carried out to investigate on the importance of dietary BAs on preventing of gut barrier damage, including, chemical, physical, immunological and microbiological barriers in the gut of the largemouth bass. A germ-free (GF) zebrafish model was employed to elucidate the effects of the direct stimulation of BAs and the indirect regulations mediated by the gut microbiota on gut barriers.

2. Materials and methods

2.1. Animal ethics statement

During the whole experimental period, all experiments and animal care procedures were conducted in accordance with the Feed Research Institute of the Chinese Academy of Agricultural Sciences and approved by the China Council for Animal Care (Assurance No. 2020-AFFRI-CAAS-001).

2.2. Experimental diets

Four isonitrogenous and isoenergetic diets were formulated with dietary BAs (provided by Shandong Longchang Animal Health Care Co., Ltd., Dezhou, China, its composition was as follows: 8.00% hyocholic acid, 70.67% hyodeoxycholic acid, and 19.61% chenodeoxycholic acid) of 0 mg/kg (Control), 150 mg/kg (BA150), 300 mg/kg (BA300) and 450 mg/kg (BA450), respectively. Firstly, all ingredients of each diet were accurately calculated and weighed. Secondly, all ingredients were mixed step by step, and an appropriate amount of water was added into the mixed ingredients to make feed. Thirdly, all diets were dried in an oven at 90 °C for 60 min. All diets were sealed and stored at room temperature. Diet formulation and chemical composition are shown in Table 1.

2.3. Experimental fish, feeding, and experimental conditions

A school of Largemouth bass were purchased from Hebei fish farm (China). All fish were acclimated with basic diet for 2 weeks before the start of the experimental feeding. Then, largemouth bass with similar size (5.05 ± 0.02 g, $n = 360$) were randomly allocated to 4 groups. Six replicate tanks were set for each group with 15 fish per 90 L tank. Fish were fed thrice daily (07:00, 12:00 and 18:00) for 5 weeks. The feeding experiment was carried out in an indoor

Table 1
Feed formulation and chemical composition of diets for largemouth bass (g/kg, air dry basis).

Item	Groups			
	Control	BA150	BA300	BA450
Ingredients				
Cassava starch	100.00	100.00	100.00	100.00
Soybean meal	120.00	120.00	120.00	120.00
Groundnut meal	100.00	100.00	100.00	100.00
Fish meal	400.00	400.00	400.00	400.00
Chicken meal	150.00	150.00	150.00	150.00
Bile acid[1]	0.00	0.15	0.30	0.45
Bentonite	6.00	5.85	5.70	5.55
Lys-HCl	8.00	8.00	8.00	8.00
Methionine	2.00	2.00	2.00	2.00
Choline chloride	3.00	3.00	3.00	3.00
$Ca(H_2PO_4)_2$	10.00	10.00	10.00	10.00
Soybean oil	60.00	60.00	60.00	60.00
Phosphatide oil	20.00	20.00	20.00	20.00
Vitamin C	1.00	1.00	1.00	1.00
Vitamin premix[2]	10.00	10.00	10.00	10.00
Mineral premix[3]	10.00	10.00	10.00	10.00
Total	1000	1000	1000	1000
Chemical composition				
Crude protein	516.80	520.00	515.10	517.20
Crude fat	147.10	142.10	144.90	145.80
Gross energy, MJ/kg	18.72	18.72	18.72	18.72

[1] Provided by Shandong Longchang Animal Health Care Co., Ltd., Dezhou, China, its composition was as follows: 8.00% hyocholic acid, 70.67% hyodeoxycholic acid, and 19.61% chenodeoxycholic acid.

[2] Vitamin premix containing the following (g/kg vitamin premix): thiamine, 0.438; riboflavin, 0.632; pyridoxine·HCl, 0.908; D-pantothenic acid, 1.724; nicotinic acid, 4.583; biotin, 0.211; folic acid, 0.549; vitamin B_{12}, 0.001; inositol, 21.053; menadione sodium bisulfite, 0.889; retinyl acetate, 0.677; cholecalciferol, 0.116; DL-α-tocopherol-acetate, 12.632.

[3] Mineral premix containing the following (g/kg mineral premix): $CoCl_2 \cdot 6H_2O$, 0.074; $CuSO_4 \cdot 5H_2O$, 2.5; $FeSO_4 \cdot 7H_2O$, 73.2; NaCl, 40.0; $MgSO_4 \cdot 7H_2O$, 284.0; $MnSO_4 \cdot H_2O$, 6.50; KI, 0.68; Na_2SeO_3, 0.10; $ZnSO_4 \cdot 7H_2O$, 131.93; Cellulose, 501.09.

recirculating water system. Throughout the feeding period, water temperatures were kept between 24.0 and 26.0 °C, the flow rate of the tank water was 60 L/h, the dissolved oxygen was higher than 6 mg/L, the nitrite concentration was lower than 0.01 mg/L, and the content of ammonia nitrogen was kept lower than 0.02 mg/L. The water quality parameters were monitored daily.

2.4. Growth performance

After feeding for 5 weeks, all fish were fasted for 24 h. The number and weight of largemouth bass in each group were counted to calculate parameter of growth performance, including weight gain (WG), feed intake (FI), feed conversion ratio (FCR) and survival rate (SR). The corresponding calculation formulas are as follows: WG (%) = 100 × [(final body weight, g) - (initial body weight, g)]/(initial body weight, g)]; FI = (total feed intake, g)/[(initial number of fish + final number of fish)/2]; FCR = (feed intake, g)/(weight gain of fish, g); SR (%) = (final number of fish/initial number of fish) × 100.

2.5. Liver and intestine histology

At the end of the experimental period, before sampling, fish were anesthetized by the use of 3-aminophenoic acid ester methanesulfonate at a concentration of 100 mg/L. All liver and hindgut samples were collected, and fixed in 4% paraformaldehyde for histology analysis. All fixed liver and hindgut samples were dehydrated and embedded in paraffin. The slides were stained with hematoxylin and eosin (H&E), mounted with neutral resin. Images were examined using a Leica DMIL LED light microscope (Leica, Wetzlar, Germany). The image analysis of slices was performed using the K-Viewer software (version 1.5.3.1, KFBIO Co., Nigbo, China; http://www.kfbio.cn).

2.6. Observation of the intestine by transmission electron microscopy (TEM)

All hindgut samples were collected, fixed in 2.5% glutaraldehyde overnight, and washed 3 times by PBS (pH = 7). The samples were dehydrated, post-embedded in Araldite, and sectioned (50–70 nm) with an ultramicrotome (Leica EMUC7, Hernalser Hauptstrasse, Germany). The sections were stained with 3% uranyl acetate and lead citrate. Images were examined with a transmission electron microscope (JEM-1230, Japan and JEOL 1010, JEOL Ltd., Tokyo, Japan). The microvillus height was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

2.7. Quantitative real-time PCR analysis

All hindgut samples were collected and put into centrifuge tubes, immediately placed into liquid nitrogen, and then stored at −80 °C for gene expression work. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). The extracted RNA was resuspended in 50 μL RNase-free water then quantified using a spectrophotometer Nano Drop 2000 and assessed by agarose gel electrophoresis. Two μg RNA was reverse transcribed into cDNA according to the instruction of the commercial kit (TianGen, Beijing, China), and then RT-qPCR reaction was performed using the SYBR Green Supermix (TianGen, Beijing, China) on the LightCycler 480 (F. Hoffmann-La Roche AG, Basel, Switzerland). The primer sequences were listed in Table S1.

2.8. Gut microbiota analysis

After feeding for 5 weeks, gut contents of largemouth bass were collected between 4 and 6 h after the last feeding. Gut contents of 3 fish were randomly extracted from each tank and subject to rapid-freeze in liquid nitrogen and were placed in −80 °C before used for bacteria DNA extraction. According to the instructions of manufacturer, Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) was used to extract DNA of each pooled sample. Microbiota analysis was performed by identification of 16S rRNA of V3–V4 regions using high throughput sequencing. The primers 338F_806R-F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 338F_806R-R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify this region. The 16s rRNA gene sequencing was performed on the Illumina platform in Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). By using USEARCH (version 7.0.1090, http://www.drive5.com/uparse/), the qualified reads were clustered to generate operational taxonomic units (OTUs) with 97% similarity level. The representative sequence of each OTU was assigned to the classification level in the Ribosomal Database Project (RDP) database by using the RDP classifier (version 2.11, http://sourceforge.net/projects/rdp-classifier/). Principal component analysis was performed by using R software (Version 3.3.1).

2.9. Preparation of GF zebrafish and transfer of gut microbiota

GF zebrafish were prepared according to a previous study (Guo et al., 2017). To detect the direct influence of BAs on GF zebrafish, diets of zebrafish larvae were sterilized by irradiation (20 kGy gamma ray, Beijing Hongyi Sifang Radiation Technology Co., Ltd, Beijing). The diet formulation and chemical parameters are shown in Table S2.

Zebrafish larvae were verified for sterility at 3 days post fertilization (dpf). Gnotobiotic zebrafish medium (GZM) were renewed at 4 dpf. GF zebrafish were fed the control or BA300 diet at 5 dpf. After 5 days of feeding, GF zebrafish were fasted for 24 h before collection for extracting RNA and analyzing the expression of genes related to gut barrier functions. The primers used for the expression of genes are shown in Table S3.

The gut microbiota of largemouth bass was transferred to GF zebrafish as described previously (Guo et al., 2017). At 4 dpf, the gut microbiota was added to GZM at a concentration of 10^6 CFU/mL suspension. One group of GF zebrafish received no treatment (negative control), and the other 2 groups of GF zebrafish were transplanted with gut microbiota of fish fed the control or BA300 diet ($n = 6$). After 3 days of colonization, zebrafish larvae were collected to extract RNA for gut barrier analysis, and the primers are shown in Table S3.

2.10. Statistical analysis

All data were analyzed using SPSS 24.0 (SPSS Inc., United States). All results were presented as mean ± SEM. Comparisons between multiple groups were analyzed using one-way ANOVA followed by the Duncan's test, while comparisons between 2 groups were analyzed using the Student's *t*-test. $P < 0.05$ was considered statistically significant. The graphics were drawn using Graph Prism 8 software (GraphPad Software Inc. San Diego, USA).

3. Results

3.1. Growth performance and hepatic histological observation of largemouth bass

The results of growth performance are shown in Table 2. There was no significant difference in FI and FCR among the treatment groups ($P > 0.05$). Compared with the control diet, the survival of fish fed the BA150 and BA300 diet was improved ($P < 0.05$). Compared with the BA150 and BA300 diet, WG of fish fed the BA450 diet was increased ($P < 0.05$). As showed in Fig. 1, diffused lipid vacuolization of hepatocyte was reduced by BA supplementation.

3.2. Gut chemical barrier of largemouth bass

After 5 weeks of feeding experiment, compared with the control diet, the expression levels of mucin, lysozyme and complement 1 were clearly increased in fish fed the BA150 and BA300 diet ($P < 0.05$, Fig. 2). Compared with the control diet, the expression levels of hepcidin in fish fed the BA450 diet was decreased ($P < 0.05$, Fig. 2).

3.3. Gut physical barrier of largemouth bass

H&E staining was used to evaluate the effect of BAs on gut histomorphology in largemouth bass (Fig. 3A). Loosening of the basement membrane and the infiltration of inflammatory cell were observed in the intestine of fish fed the control diet. Compared with the control group, the structure of the intestine became orderly, disorders of gut villus structure were ameliorated and infiltrations of inflammatory cells were decreased in largemouth bass fed the BA150 and BA300 diets (Fig. 3A). TEM analysis showed that compared with the control group, the structural integrity of gut microvilli was improved in the BA150 and BA300 groups (Fig. 3B). The microvilli were higher in the BA300 group than in the control group ($P < 0.05$, Fig. 3C).

The effects of dietary BAs on expressions of the gut tight junction-related genes of largemouth bass are shown in Fig. 4. The results indicated that BA150 group had no marked influence on the expressions of zona occludens-1 (*ZO-1*) and claudin-1 ($P > 0.05$, Fig. 4), but increased the level of occludin and claudin-4 compared with the control diet ($P < 0.05$, Fig. 4). Moreover, the relative mRNA levels of *ZO-1*, occludin, claudin-1 and claudin-4 in largemouth bass fed the BA300 diet were clearly increased compared with the control diet ($P < 0.05$, Fig. 4). Those in the BA450 group were not changed compared with the control diet.

3.4. Gut immunological barrier of largemouth bass

The expression of inflammation-related genes of largemouth bass fed the experimental diets is presented in Fig. 5. The relative expression of pro-inflammatory gene interleukin-6 (*IL-6*) was significantly increased in the BA150 and BA300 groups, while the relative mRNA levels of the pro-inflammatory genes (interleukin-1β [*IL-1*β], tumor necrosis factor-α [*TNF-*α], and *IL-6*) were down-regulated in largemouth bass fed the BA450 diet compared to those fed the control diet ($P < 0.05$, Fig. 5A).

Furthermore, compared with the control diet, the relative mRNA levels of anti-inflammatory genes (arginase, macrophage galactose-type lectin [*MGL*]) of intestine were increased in fish fed the BA150 diet ($P < 0.05$, Fig. 5B) and the relative mRNA levels of anti-inflammatory genes (interleukin-10 [*IL-10*], tumor growth factor β2 [*TGF-β2*], *TGF-β3*, *MGL*) of the intestine were increased in fish fed the BA300 diet ($P < 0.05$, Fig. 5B). However, when the dose level of BAs was increased up to 450 mg/kg, the relative mRNA levels of anti-inflammatory gene (arginase) were decreased compared with the control diet ($P < 0.05$, Fig. 5B). In addition, compared with the control diet, the relative mRNA level of immunoglobulin M (*IgM*) was increased significantly in fish fed the BA300 diet ($P < 0.05$, Fig. 5C).

3.5. Gut microbiota (microbiological barrier) of largemouth bass

Using high-throughput sequencing to detect the gut microbiota of largemouth bass, about 2,172,859 sequences were acquired, with an average sequence length of 418 bp. Due to supplementation of BAs, there was no obvious difference in Shannon index and Simpson index between the treatment groups. Compared with the control diet, Ace and Chao indexes were decreased significantly in BA-supplemented groups ($P < 0.05$, Table 3), indicating that the addition of BAs reduced the richness of the gut microbiota.

Table 2
Growth performance of largemouth bass fed with control, BA150, BA300 and BA450 diet for 5 weeks.

Growth parameters	Control	BA150	BA300	BA450
IBW, g/fish	5.06 ± 0.01	5.06 ± 0.01	5.06 ± 0.01	5.04 ± 0.02
WG, %	108.77 ± 5.84^{ab}	103.99 ± 5.79^{a}	103.42 ± 2.96^{a}	115.57 ± 5.84^{b}
FI, g/fish	6.37 ± 0.02	6.37 ± 0.01	6.37 ± 0.01	6.35 ± 0.02
FCR	1.28 ± 0.06	1.25 ± 0.04	1.21 ± 0.05	1.21 ± 0.03
SR, %	80.00 ± 11.55^{a}	96.67 ± 3.33^{b}	98.33 ± 2.89^{b}	85.00 ± 7.26^{ab}

IBW = initial body weight; WG = weight gain; FI = feed intake; FCR = feed conversion ratio; SR = survival rate.
$^{a, b}$ Different superscripts in the same row indicate significant differences (Duncan's test; $P < 0.05$).

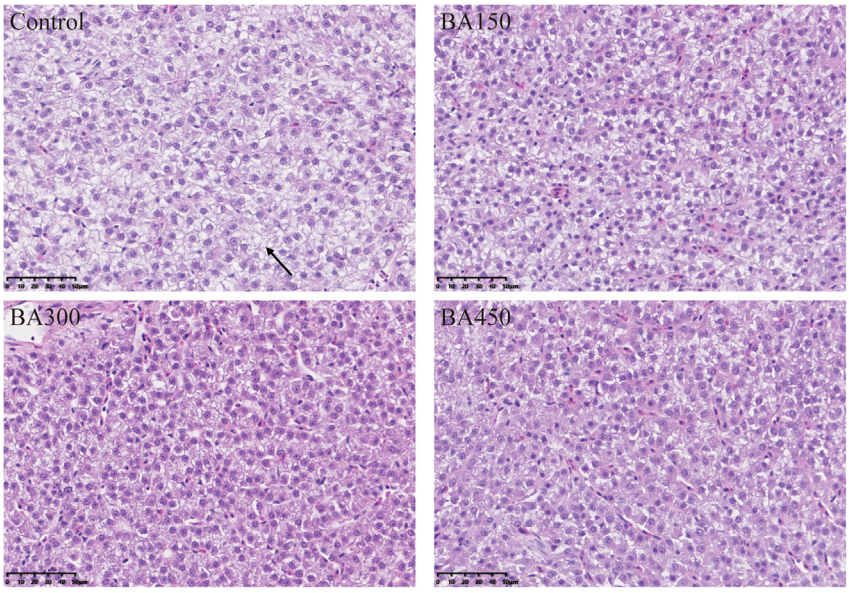

Fig. 1. Histology of liver in largemouth bass fed on one of the experimental diets for 5 weeks (scale bar = 50 μm). Black arrows indicate the presence of liver cell swelling and vacuolar degeneration.

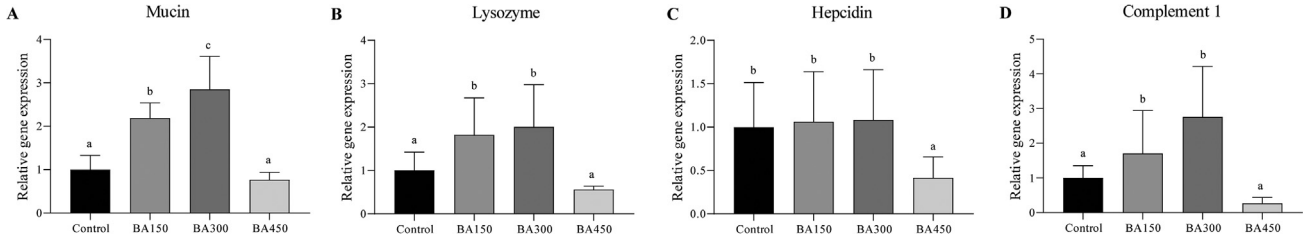

Fig. 2. The expression of genes related to gut secretions in the intestine of largemouth bass fed with one of the experimental diets for 5 weeks. Results are presented as the means (±SEM) ($n = 6$). Bars with different letters are significantly different (Duncan's test; $P < 0.05$).

At the phylum level (Fig. 6A; Table 4), Firmicutes, Proteobacteria and Actinobacteriota were the dominant phyla in the gut of largemouth bass. Compared with the control diet, the abundance of Firmicutes increased apparently in the BA150 and BA300 groups ($P < 0.05$), and the abundance of Proteobacteria and Actinobacteriota were in a decreasing trend in the gut of fish fed the BA150 and BA300 diets ($P > 0.05$). The abundance of Firmicutes showed an increasing tendency, whereas the abundance of Actinobacteriota exhibited a decreasing trend in the BA450 group compared with the control group ($P > 0.05$). Additionally, the ratio of (Firmicutes + Fusobacteria + Bacteroidetes) to Proteobacteria was significantly higher in the BA150 and BA300 groups compared to the control group ($P < 0.001$, Fig. 6C). At the genus level (Fig. 6B; Table 5), the dominant genera were *unclassified_f__Peptostreptococcaceae*, *Staphylococcus* and *Acinetobacter*. In fish fed on the BA150 and BA300 diets, the abundance of *unclassified_f__Peptostreptococcaceae* was significantly increased ($P < 0.05$) while that of *Acinetobacter* was considerably decreased compared with the control diet ($P > 0.05$). Principal coordinate analysis (PCoA) indicated that the gut microbiota of largemouth bass fed BA-supplemented diets significantly changed at the genus level (Fig. 6D).

3.6. Effects of direct feeding of BAs on the gut chemical, physical, immunological barriers in GF zebrafish model

A GF zebrafish model was used to investigate how BAs improve the gut barriers of largemouth bass. In our experiment, we observed that 300 mg/kg of BAs was optimal dosage for largemouth bass normal growth. Hence, to investigate the influence of BAs on the gut barriers and its potential regulation mechanisms of action, we compared the control with the BA300 group.

Based on this, a diet containing 300 mg BAs was fed to sterile zebrafish for 5 days and the transcription level of gut barriers was analyzed. The results showed that the levels of gut secretion gene (lysozyme) ($P < 0.01$, Fig. 7A) and tight junction gene (occludin-2) ($P < 0.01$, Fig. 7B) in zebrafish larvae were increased. Moreover, the levels of pro-inflammatory gene (*IL-6*), anti-inflammatory gene (*IL-10*), *IgM* and immunoglobulin Z/T (*IgZ/T*) were increased ($P < 0.05$, Fig. 7C). The results indicated that direct feeding of BAs apparently improved the function of gut barrier of GF zebrafish.

3.7. Effects of gut microbiota from largemouth bass on the gut chemical, physical, immunological barriers of GF zebrafish

To evaluate influence of the transferred gut microbiota on gut barriers of GF zebrafish, we compared the effects of gut microbiota of largemouth bass by transferring to the GF zebrafish. The results are shown in Fig. 8A–C. The treatment group colonized with the gut microbiota of largemouth bass fed the BA300 diet showed increased the levels of pro-inflammatory genes (*IL-6, IL-1β*), anti-inflammatory gene (*IL-10*) and *IgZ/T* ($P < 0.05$, Fig. 8C). However, there were no significant differences in the expression of genes related to chemical and physical barriers ($P > 0.05$; Fig. 8A and B).

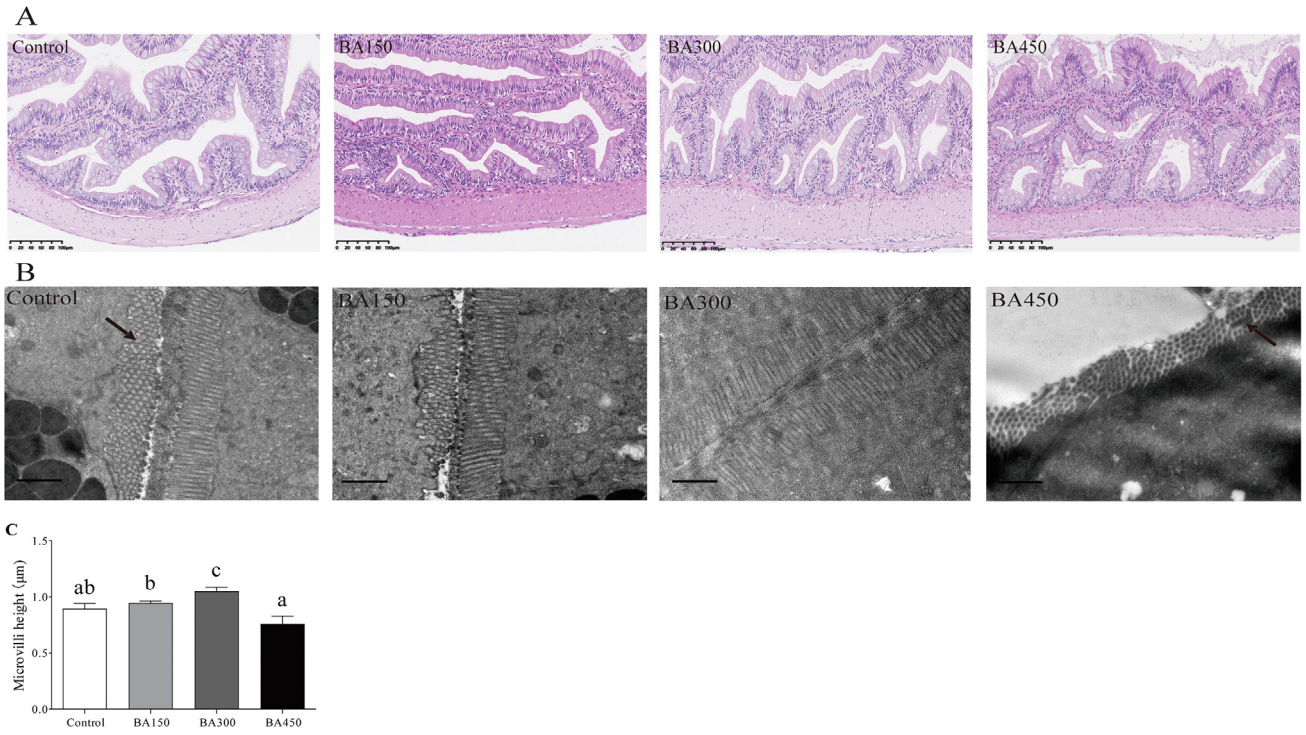

Fig. 3. Effects of bile acids on the intestine of largemouth bass fed with one of the experimental diets for 5 weeks. (A) Hematoxylin and eosin (H&E) staining for histology examination (scale bar = 100 μm). (B) Transmission electron microscopy (TEM) micrographs (Black arrow: imperfect microvilli texture or the microvilli of the intestine were peeled) (scale bar = 1 μm). (C) Microvilli height of largemouth bass. Results are presented as the means (±SEM) ($n = 6$). Bars with different letters are significantly different (Duncan's test; $P < 0.05$).

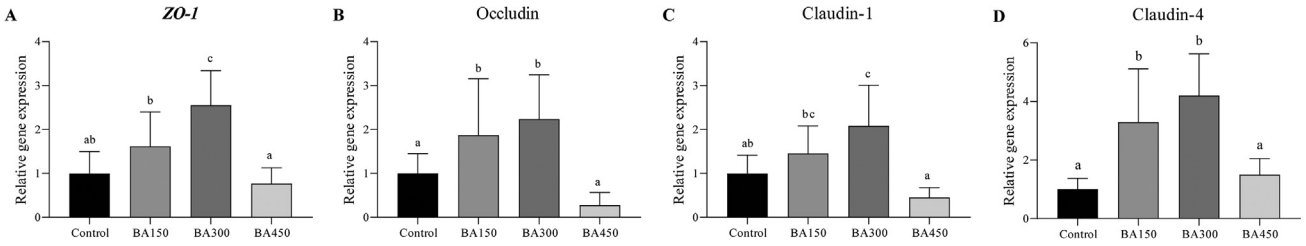

Fig. 4. Effects of the gene levels of tight junction genes in the intestine of largemouth bass fed with one of the experimental diets for 5 weeks. Results are given as the means (±SEM) ($n = 6$). Bars with different letters are significantly different (Duncan's test; $P < 0.05$).

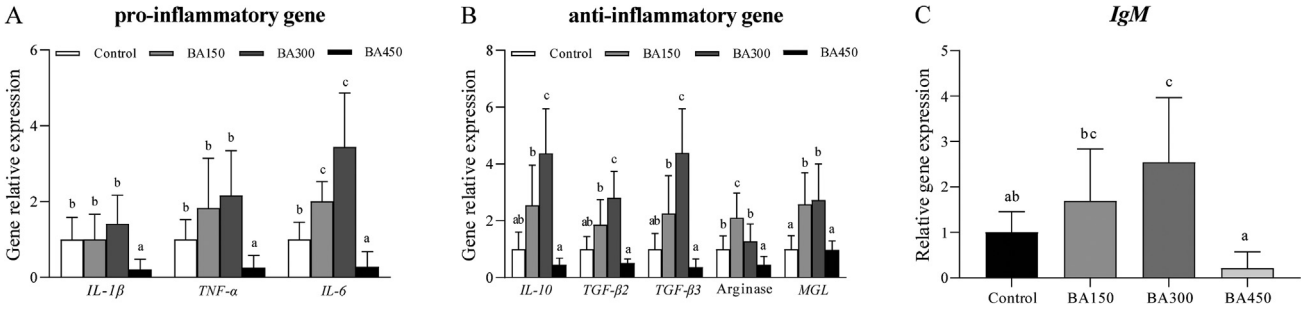

Fig. 5. Gut expression levels of inflammation-related genes in largemouth bass fed with one of the experimental diets for 5 weeks. Results are given as the means (±SEM) ($n = 6$). Bars with different letters are significantly different (Duncan's test; $P < 0.05$).

4. Discussion

BAs are regarded as safe and efficient feed additives. In fact, numerous studies show that BAs play an important role in promoting fish growth (Yu et al., 2019b; Zhai et al., 2020). However, dietary BA supplementation in different fish species may yield completely different effects. Our study indicates that there was no significant difference in the growth characteristics of the

Table 3
Effects on α diversity of gut microbiota of largemouth bass fed with control, BA150, BA300 and BA450 diet for 5 weeks.

Estimators	Control	BA150	BA300	BA450
Shannon	3.54 ± 0.63	3.05 ± 1.89	2.96 ± 1.10	3.17 ± 1.15
Simpson	0.12 ± 0.08	0.29 ± 0.41	0.24 ± 0.24	0.18 ± 0.14
Ace	788.28 ± 54.14^{c}	463.85 ± 206.75^{b}	436.05 ± 98.39^{a}	485.36 ± 81.75^{a}
Chao	789.12 ± 53.04^{c}	452.79 ± 244.71^{b}	443.19 ± 97.71^{a}	480.32 ± 92.64^{a}

$^{a, b, c}$ Different superscripts in the same row indicate significant differences (Duncan's test; $P < 0.05$).

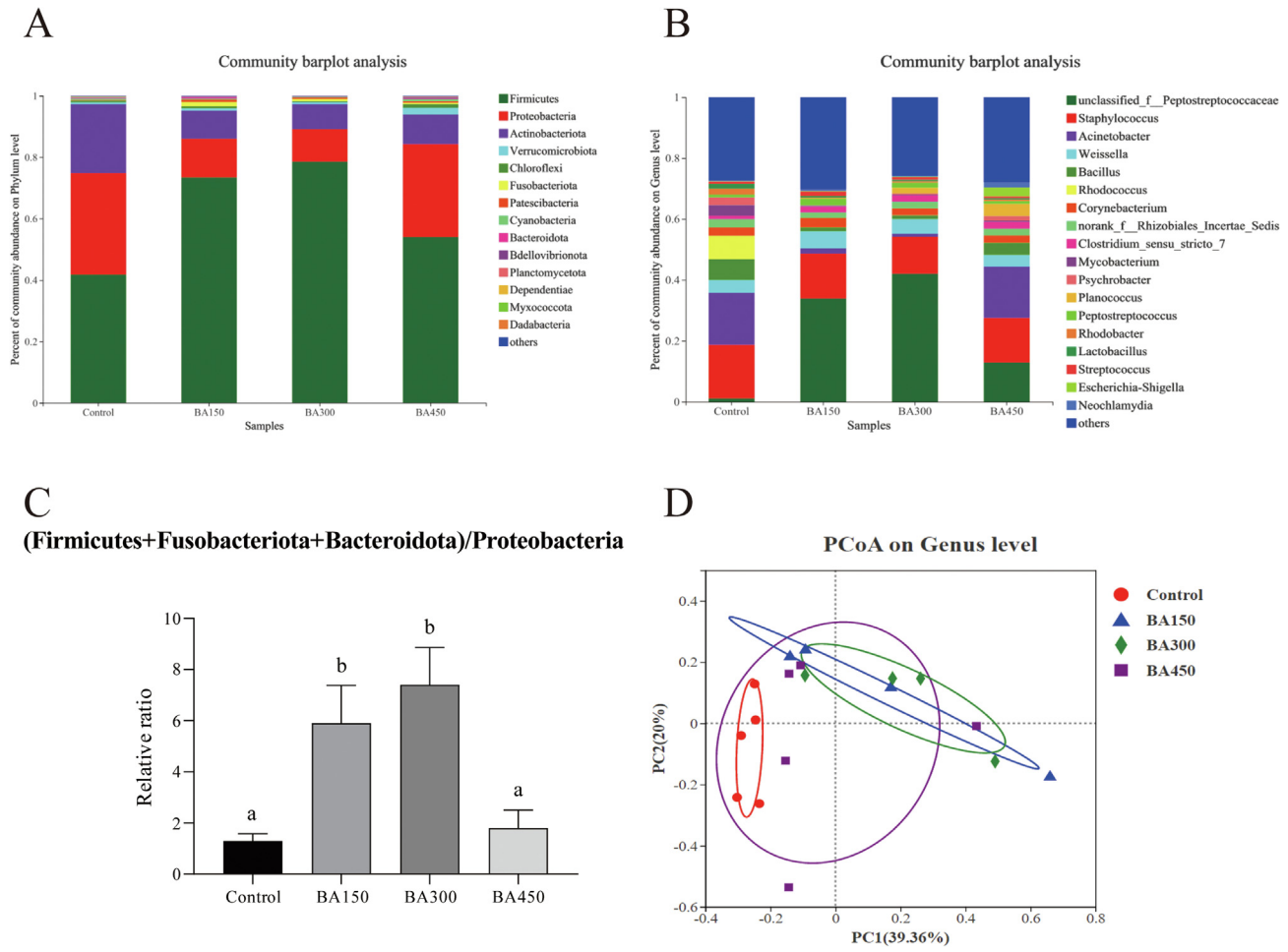

Fig. 6. Effects of supplementation of BAs on gut microbiota of largemouth bass, at (A) phylum level, (B) genus level, (C) relative ratio of (Firmicutes + Fusobacteria + Bacteroidetes) to Proteobacteria and (D) principal coordinate analysis (PCoA) analysis, fed with 4 different diets for 5 weeks ($n = 4$ in BA150 and BA300 groups, $n = 5$ in Control and BA450 groups). Bars with different letters are significantly different (Duncan's test; $P < 0.05$).

Table 4
The predominant gut bacterial phyla in largemouth bass fed with control, BA150, BA300 and BA450 diet for 5 weeks (%).

Bacteria phylum	Control	BA150	BA300	BA450
Firmicutes	41.95 ± 9.36^{a}	73.13 ± 18.55^{b}	77.91 ± 15.80^{b}	54.03 ± 20.66^{a}
Proteobacteria	32.61 ± 14.80	12.68 ± 10.52	10.62 ± 9.66	30.37 ± 23.06
Actinobacteriota	22.71 ± 8.61	9.46 ± 7.15	8.87 ± 7.55	9.57 ± 6.37
Verrucomicrobiota	0.56 ± 0.31	0.78 ± 1.20	0.58 ± 0.78	2.17 ± 4.72
Chloroflexi	0.58 ± 0.31	0.64 ± 0.83	0.33 ± 0.37	1.17 ± 2.35
Fusobacteriota	0.06 ± 0.06	1.31 ± 1.48	0.66 ± 0.82	0.51 ± 1.05
Patescibacteria	0.17 ± 0.07	0.79 ± 0.92	0.51 ± 0.52	0.49 ± 0.50
Cyanobacteria	0.42 ± 0.44	0.28 ± 0.18	0.20 ± 0.13	0.59 ± 1.00
Bacteroidota	0.14 ± 0.11	0.42 ± 0.64	0.08 ± 0.05	0.22 ± 0.17
Bdellovibrionota	0.14 ± 0.07	0.21 ± 0.21	0.10 ± 0.16	0.35 ± 0.60

$^{a, b}$ Different superscripts in the same row indicate significant differences (Duncan's test; $P < 0.05$).

Table 5
The predominant gut bacterial genus in largemouth bass fed with control, BA150, BA300 and BA450 diet for 5 weeks (%).

Bacteria genus	Control	BA150	BA300	BA450
Peptostreptococcaceae	1.05 ± 0.93[a]	32.71 ± 43.22[b]	38.96 ± 29.11[b]	12.28 ± 20.53[a]
Staphylococcus	18.19 ± 10.07	15.16 ± 11.93	13.00 ± 7.20	15.10 ± 15.25
Acinetobacter	16.54 ± 18.57	1.65 ± 2.19	0.98 ± 0.76	17.29 ± 25.71
Weissella	4.23 ± 2.65	5.73 ± 4.21	5.14 ± 2.37	3.81 ± 2.23
Bacillus	6.57 ± 10.30	1.28 ± 0.88	1.19 ± 0.15	3.90 ± 5.19
Corynebacterium	2.69 ± 1.52	3.10 ± 2.05	2.49 ± 1.42	2.45 ± 2.81
Rhizobiales_Incertae_Sedis	2.72 ± 2.13	1.82 ± 2.69	2.03 ± 1.87	2.23 ± 2.02
Rhodococcus	7.84 ± 6.07[a]	0.06 ± 0.04[b]	0.05 ± 0.04[b]	0.05 ± 0.05[b]
Clostridium_sensu_stricto_7	1.08 ± 0.60	1.96 ± 1.33	2.42 ± 1.12	2.25 ± 1.20
Planococcus	0.02 ± 0.03	0.04 ± 0.07	2.03 ± 3.75	4.19 ± 8.34

[a, b] Different superscripts in the same row indicate significant differences (Duncan's test; $P < 0.05$).

largemouth bass fed BA-supplemented diets. Similar results were also observed in the same fish species fed a high-fat diet supplemented with 300 mg/kg BAs (Yin et al., 2021). However, the opposite results have been reported that a diet supplemented with 300 mg/kg of BAs could significantly improve WGR and SGR of largemouth bass (Yu et al., 2019b). We speculate that the causes for this different growth performance results might mainly be related to the levels and types of BAs, as well as diet composition.

BAs have a key effect on reducing hepatic triglyceride deposition and protecting liver health (Cipriani et al., 2010; Jain et al., 2012; Watanabe and Fujita, 2014). Accordingly, BA-enriched diets reduced vacuolization of hepatocyte of the fish. Similar findings have been observed in *C. idella* (Zeng et al., 2017), *A. schlegelii* (Jin et al., 2019), *Scophthalmus maximus* (Sun et al., 2014) and *Schizothorax prenanti* (Zheng et al., 2016), because diets supplemented with BAs have been proved to decrease lipid accumulation in the fish body (Sun et al., 2014) or increase lipid catabolism in the fish body (Hu et al., 2015).

The gut mucosa and the mucus layer of fish play a key role in the host defense against threats present in the immediate environment (Jin et al., 2018; Wei et al., 2019; Yang et al., 2017). Furthermore, this barrier function is closely dependent on mucins, complement proteins, lysozymes and antimicrobial peptides (Rauta et al., 2012; Salinas, 2015). The present study demonstrated that diet supplemented with BAs increased the expression of genes mucin, lysozyme and complement 1, indicating that diets supplemented with BAs could impair the gut chemical barrier function of fish. Similar results were also detected in other experiments that BAs could enhance lysozyme activity and alternative complement activity in rainbow trout (Deng et al., 2013) and lysozyme activity of largemouth bass (Guo et al., 2020).

The intestine is the main place for the absorption and digestion of nutrients in fish. The structural integrity of the intestine has an important impact on the digestive capacity of animals. Studies have reported that feeding fish high-fat diets for an extended period of time could compromise the integrity of intestine structure, however addition of BAs could attenuate their negative effects (Ma et al., 2018; Peng et al., 2019). In our study, dietary BAs were shown to significantly improve gut integrity compared with the control diet, which agreed with previous findings (Jiang et al., 2018). Tight junction proteins, such as the cytoplasmic protein ZO-1 and the transmembrane proteins including claudins and occludin are also associated with the functions of the gut physical barrier (Chen et al., 2018; Yu et al., 2019a). Moreover, a plethora of studies show that tight junction proteins are also important to control the changes of gut permeability in fish (Ding et al., 2019; Oshima et al., 2008; Suzuki, 2013). However, there are few studies examining the importance of BAs on the functions of tight junction proteins of intestine of largemouth bass. Our study indicated that the expression of genes including, *ZO-1*, occludin, claudin-1 and claudin-4 was increased in largemouth bass fed a diet containing 300 mg/kg BAs. These results indicated that supplementation of BAs in the diet of largemouth bass can improve the gut physical barrier, leading to a decrease in gut permeability and improve gut health of the fish. Our research provided a reference for the involvement of BAs in regulating the integrity of the gut physical barrier in fish.

The gut immunological barrier plays an important role in preventing from entering of pathogens from the intestine to the body of the fish. Inflammation is a protective response that eliminates the initial cause of cellular damage and initiates tissue repair. Inflammatory cytokines produce a marked effect in this process (Karin and Clevers, 2016). To a certain extent, cytokines are a good indicator of the immune status of the host in aquatic animals. Cytokines are a class of important small-molecule proteins secreted by lymphoid tissues, which exist in the epithelial layer and lamina propria of gut mucosa and are involved in the protection of fish (Dalmo et al., 1997; McMillan and Secombes, 1997). Cytokines have

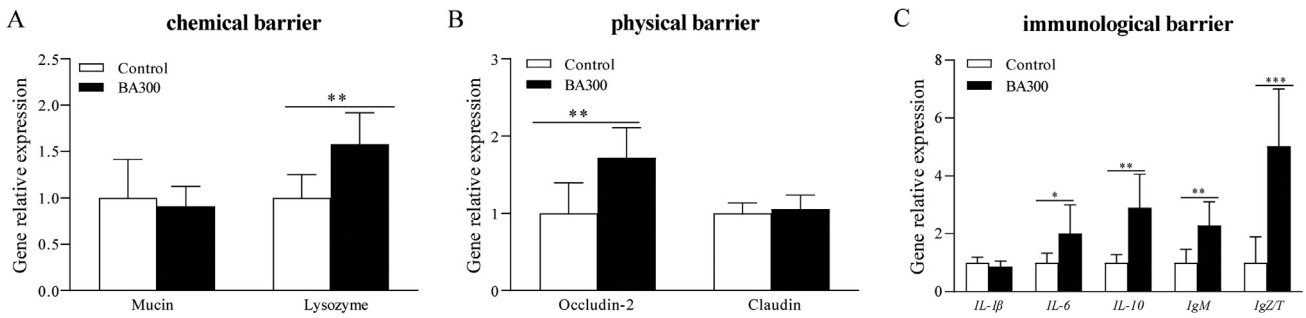

Fig. 7. Effect of direct feeding of bile acids on the gut barriers: (A) chemical barrier, (B) physical barrier, (C) immunological barrier, in GF zebrafish. Results are given as the means (±SEM) ($n = 6$). Values marked with asterisks are significantly different (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

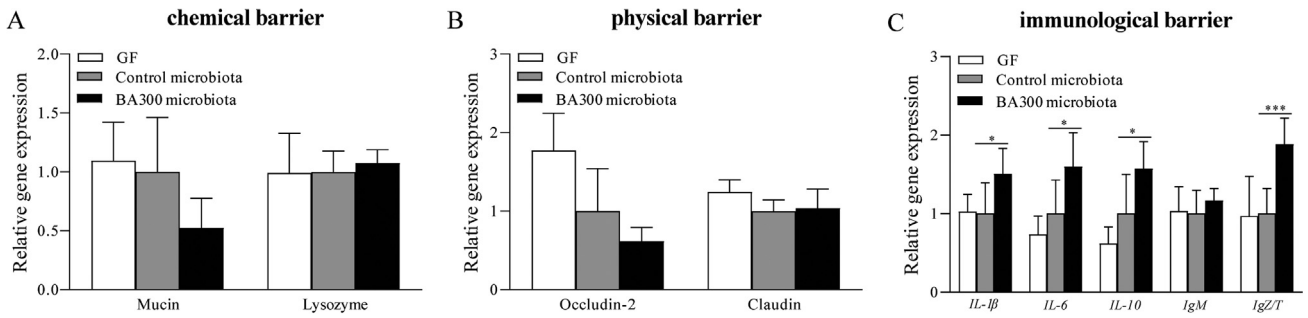

Fig. 8. The expression of genes related to gut barriers: (A) chemical barrier, (B) physical barrier, (C) immunological barrier, in GF zebrafish colonized with gut microbiota from the control or from the BA300 group. Results are given as the means (±SEM) ($n = 6$). Values marked with asterisks are significantly different (*$P < 0.05$, and ***$P < 0.001$).

a vital effect on regulating inflammatory response and immune function of aquatic animals, including IL-1β, TNF-α, IL-6, IL-10 and TGF-β (Standen et al., 2016). Our results indicated that the levels of both pro-inflammatory gene (*IL-6*) and anti-inflammatory genes (*IL-10*, *TGF-β* and *MGL*) were increased in the BA300 groups, while the levels of both pro-inflammatory gene (*IL-6*) and anti-inflammatory gene (arginase) were decreased in the BA450 group, which indicated that adding a certain amount of BAs could decrease gut inflammatory responses. Similarly in a previous study, the levels of both pro-inflammatory genes (*TNF-α* and *IL-8*) and anti-inflammatory genes (*IL-10*, *IL-1β*, and *TGF-β1*) were increased in fish fed a diet containing 300 mg/kg BAs (Yu et al., 2019b). However, Jiang et al. (2018) reported fish fed high levels of BAs may contribute to chronic inflammation. Our findings also found that addition of high levels of BAs did not inhibit the gut inflammatory responses. The possible reason for the differences may be related to the accumulation of cholesterol in the fish body. Studies have suggested that high levels of BAs lead to the accumulation of serum cholesterol. High concentrations of cholesterol may impair immunity of fish and disrupt the inflammatory response of fish (Jiang et al., 2018; Wang et al., 2018). Similar results were observed in grass carp, where supplementation with higher levels of BAs impaired the gut immunity and increased serum cholesterol level compared to lower levels of BAs (Peng et al., 2019).

BAs can affect the intestinal microbiota composition via various ways. The gut microbiota is considered as an indispensable metabolic "organ" in fish, which could be involved in the biotransformation of BAs (Molinero et al., 2019). Our results illustrated that the gut microbiota of largemouth bass fed the control diet consists mainly of Firmicutes, Proteobacteria and Actinobacteria at the phylum level, which is consistent with the findings reported in other freshwater fish species (Merrifield et al., 2009; Navarrete et al., 2008; Roeselers et al., 2011). At the phylum level, the present results showed that BA supplementation significantly altered the structure of gut microbiota. The abundance of Firmicutes in the BA150 and BA300 groups was increased significantly, while that of Proteobacteria and Actinobacteria was decreased. This agrees with the findings of Islam et al. (2011) which showed that increased BA levels promoted the abundance of Firmicutes and decreased that of Actinobacteria in rats. In addition, study using grass carp also showed that the addition of BAs inhibited the abundance of Proteobacteria (Zhou et al., 2018). Butyrate is a key signaling molecule between the gut microbiota and the immune system production and its production is associated with Firmicutes (Huang et al., 2017b). Therefore, it is possible that an increased abundance of Firmicutes and hence a higher butyrate level in the intestine might help attenuate gut inflammation induced by high-fat diets. At the genus level, diets supplemented with BAs significantly increased the abundance of *unclassified_f__Peptostreptococcaceae*. As described earlier, some members of the *Peptostreptococcus* may be important for the digestion and absorption of proteins and the production of ammonia and other compounds (Attwood et al., 1998; Whitehead and Cotta, 2004).

These results clearly demonstrate the effect of BAs on the structure of the gut microbiota. To further study the influence of BAs on gut barrier, particularly on the question whether the gut microbiota mediates regulation of BAs, the expression of gut barrier-related genes was analyzed using a GF zebrafish model. Both the direct action of BAs and the change mediated by the gut microbiota were investigated. Thus, the current study showed that transferring the gut microbiota of largemouth bass fed the BA300 diet to the GF zebrafish fed the BA300 diet induced a significant increase in the gut barrier-related genes, including, *IL-6*, *IL-1β*, *IL-10* and *IgZ/T* by the BA300 microbiota, indicating that the gut microbiota acted primarily through the immunological barrier. The direct administration of BAs to GF zebrafish resulted in an increasing of the expression of genes such as lysozyme, occludin-2, *IL-6*, *IL-10*, *IgM* and *IgZ/T*, indicating that BAs could directly stimulate the gut barrier. Therefore, BAs appear produce a significant effect on the gut barrier functions through both their direct action as well as via altering of the gut microbial composition. To the best of our knowledge, this was the first study to verify the effects of BA-mediated gut microbiota on the gut barriers in largemouth bass.

5. Conclusion

BA supplementation at levels between 150 and 300 mg/kg in the diet of largemouth bass can improve the gut non-specific immunity, enhance gut tight junction functions, and improve the gut microbiota composition, thereby significantly improving fish gut health. However, BA supplementation at 450 mg/kg, a level perhaps outside the optimum supplementation range, significantly disrupted the function of gut chemical, physical, immunological barriers. Supplementation of 300 mg/kg of BAs to the feed affected the gut chemical, physical, immunological barriers both directly and indirectly, mediated by the gut microbiota.

Author contributions

Zhigang Zhou and **Zhen Zhang**: designed study and provided the laboratory resources. **Rui Xia** and **Qingshuang Zhang**: conducted the experiments and biochemical analyses. **Dongmei Xia**: participated in revision. **Qiang Hao**, **Qianwen Ding, Chao Ran**, **Yalin Yang** and **Aizhi Cao**: developed the methodology. **Rui Xia**, **Qingshuang Zhang** and **Zhen Zhang**: analyzed the data. All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.03.008.

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