High-Starch Diet Consumption Impaired Intestinal Barriers of Juvenile Largemouth Bass (Micropterus Salmoides)

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Research Article

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Abstract

The intestinal barrier is primarily composed of physical, physiological and microbial barriers, and intestine microbe-immune interactions influenced by diet in fish still remain increasingly unexplored. The intestinal barriers of Largemouth bass (*Micropterus salmoides*) (4.1±0.2g) were explored after they were fed three different starch diets for eight weeks (low starch,7%; middle starch, 12%; high starch, 17%). The results showed that high starch diet led slight infiltration of inflammatory cells and moderate loss of mucous membrane layer in midgut. Meanwhile, high starch diet decreased the antioxidant enzymes (T-SOD) activities and genes (*SOD1, SOD2, SOD3a, CAT, GPX*) expression significantly ($P < 0.05$). The MDA content was significantly increased with increasing starch level ($P < 0.05$). High starch diet up-regulated the expression of pro-inflammatory genes (*IL-8, IL-1β* and *TNFα)* and apoptosis genes (*bax*, *caspase3, caspase8* and *caspase9*), whereas down-regulated the expression of anti-apoptosis genes *bcl-2* and tight junction proteins genes (*occludin* and *claudin7*). In addition, the high starch diet increased the relative abundance of *Actinobacteria* and *Firmicutes,* resulted in microbial dysbiosis. In conclusion, high dietary starch impaired intestinal barriers, and increased the risk of disease outbreaks.

1. Introduction

As is known, starch is one of the most important energy sources of carbohydrate and binder in commercial aquafeed. A moderate amount of dietary carbohydrates could reduce production costs in fish, via increase protein deposition, growth, feed efficiency in fish, such as pompano (*Trachinotus ovatus*), snakehead (*Channa argus*) and rainbow trout (*Oncorhynchus mykiss*) (Castro et al., 2015; Ding et al., 2020; KROGDAHL et al., 2005). As a carnivorous fish, the largemouth bass has limited capacity to utilize starch. The growth of juvenile largemouth bass decreased significantly when dietary starch content increased to 15% (Gou et al., 2015). Moreover, long-term feeding of excess carbohydrates in largemouth bass would impair the metabolic homeostasis in liver and dyslipidemia, whereas limited studies focused on intestine (Song et al., 2018).

The intestine is not only the main organ for digestion/absorption, but also executes physical, physiological and microbial barriers function in fish (Arias-Jayo et al., 2018; Wang et al., 2019). After long-time excess carbohydrates intaking, the intestinal mucosal structure of become impaired (Ding et al., 2020). High-starch diet decreased the antioxidant capacity and immunity of the gut, while caused intestinal inflammation in Nile tilapia (*Oreochromis niloticus*) and turbot (*Scophthalmus maximus*)(Bai et al., 2019; Li et al., 2020). The intestinal microbiota constitutes a complex community and interacts with the host to modulate essential biological processes of the health (Makki et al., 2018). Dysbiosis of the microbiota can result in the outgrowth of more pathogenic microbes and the promotion of inflammation (Frank et al., 2007). It is well accepted that diet with high carbohydrate has a considerable effect on the composition of intestinal microbiota in fish (Wang et al., 2018). However, limited reports are available that high dietary carbohydrate affects both intestinal microbial homeostasis, antioxidant and immune function in fish.

To data, there are many reports focused on effects of starch levels on antioxidant and immune function of liver and growth performance in largemouth bass (Li et al., 2020; Lin et al., 2018; Song et al., 2018) . In the present study, intestinal performance and microbiota of largemouth bass caused by different levels of starch were showed, aimed to explore the mechanism of intestinal impairment caused by high starch diet.

2. Materials And Methods

2.1 Experimental diets

In the present study, three isonitrogenous (48% crude protein) and isolipidic (9.5% crude lipid) diets were formulated (Table 1). Three starch levels were 7% (low starch, L), 12% (middle starch, M) and 17% (high starch, H), respectively. All ingredients of each diet were got from Tong Wei Co. Ltd (Chengdu, Sichuan, China) and ground through a particle size 80 mesh. Extruded into pellets with diameter of 3mm, after all ingredients were evenly mixed. After drying in the shade, three diets were stored at −20°C.

2.2 Fish and feeding trial

All of the fish with Same genetic background used in this study were obtained from a commercial farm (Chengdu, Sichuan, China). All fish were acclimated experiment conditions for 2 weeks. 360 healthy fish (average weight: 4.1±0.2g) with similar size fasted for 24 h and were randomly divided into 9 tanks (3 parallels each group). Three starch level diets were prepared to access the effects (L, M and

H, Supplementary Table 1). During 8weeks' feeding trial, fish were fed up to apparent satiation three times a day (9:00am, 2:00pm and 7:00pm). The photoperiod was 12h light:12h dark with the light period from 8:00am to 8:00pm. The water temperature was maintained at 20 ± 1°C. The dissolved oxygen was more than 6 mg/L, and the pH was 6.8–7.5. Remove feces and replace with a quarter of the fresh water daily.

2.3 Sample collection

Fish were fasted for 12h and anesthetized with MS-222 (Sigma, USA) at the end of the trial. Four midguts of each tank were extracted and pooled for microbiota analysis. The remained fish were fasted for 24h and anesthetized with MS-222 (Sigma, USA). The midguts used for biochemical parameter analysis and quantitative real time PCR analysis were frozen in liquid nitrogen immediately and stored at -80 °C until analyzed (30 fish each tank). Midguts used for histological analysis were stored in Bouin's solution (6 fish each tank).

2.4 Histological analysis

In each group, 6 midgut samples were dehydrated, then embedded in paraffin(Zhang, Rahimnejad, Wang, Lu, Song, Wang, Mai, 2018). The embedded intestine segments were sliced into sections (5μm thick), then stained with hematoxylin and eosin (H&E). The midgut morphology was observed Subsequently with a light microscope (OLYMPUS, DP73, Nikon Corporation, Japan).

2.5 Biochemical parameter analysis

The midgut was homogenized on ice in 10vol (w/v) of recommended reagent, then centrifuged at 4000g for 10min at 4°C, and the supernatant was stored for biochemical parameters analysis. Antioxidant enzyme (T-SOD, CAT, GPX and MDA), and immune enzyme （LZM, ACP and AKP）were analysed according to the instruction of the kits(Nanjing Jiancheng Bioengineering Institute, China).

2.6 Quantitative real time PCR analysis

Total RNA samples isolated from the intestine using a BioFast Simply P Total RNA Extraction kit (Invitrogen, Carlsbad, CA, USA), then stored at -80 °C(Lin et al., 2020). The cDNA synthesized by using TianGen® FastKing RT Kit (With gDNase) and stored at -20 °C(Yu et al., 2018). Specific primers of largemouth bass for quantitative real-time PCR were shown in Table 2, which have been published or designed according to the sequences cloned in our lab. All real-time PCR reactions were performed on a CFX96TM Real-Time PCR Detection System(Yang et al., 2018). β-actin and 18S were used as reference genes to evaluate of internal control genes. The target and housekeeping genes were calculated by the $2^{-\Delta\Delta CT}$ method (Livak et al., 2002).

2.7 Intestinal microbiota and metabolites analysis

According to user guide of the Power Soil DNA Isolation Kit (MO BIO Laboratories), total bacterial DNA (midguts) were extracted, then stored at -80°C. The V3-V4 region of the bacterial 16S rRNA gene was amplified with the primer pairs (Forward primer(338F), 5'-ACTCCTACGGGAGGCAGCAG-3'; reverse primer(806R), 5'- GGACTACHVGGGTWTCTAAT-3') as previously described(Sun et al., 2021). PCR products were purified and then quantified(Yu et al., 2020). Bacterial rRNA genes used for High-throughput sequencing analysis were performed by using the Illumina Miseq platform at Shanghai Majorbio Bio-pharm Technology Co Ltd, Shanghai, China.

2.8 statistical analysis

The results were presented as Mean ± SEM, and established database in Excel form. The significance level was determined by one-way analysis of variance (ANOVA) with GraphPad Prism 8.0 (GraphPad Software Inc. USA). Duncan's multi-range tests were used to Multiple comparisons. In present tests, a significance level of 0.05 ($P < 0.05$) and extremely significance level of 0.01 ($P < 0.01$) were used. The GraphPad Prism 8.0 were used for graphics. The correlation coefficient of gut microbe and related genes expression were evaluated by R (pwr package) and the networks were generated using Cytoscape version 3.71.

3. Results

3.1 Intestinal morphology and permeability

As shown in Fig 1, a slight infiltration of inflammatory cells and moderate loss of mucosal architecture were observed in H group. The intestinal sections of M and H groups had no epithelial cell damage and infiltration of inflammatory cells.

The tight junction proteins genes (*occludin, claudin7*) intestinal expression in H group were significantly decreased ($P < 0.05$, Fig 2).

3.2 antioxidant capacity

As shown in Fig 3, the activity of all antioxidant enzymes (T-SOD, CAT, GPX) decreased with starch level increasing. The activity of T-SOD in H group was significantly decreased compared with L group ($P < 0.05$). There were no significant differences in intestinal CAT and GPX activities in all groups ($P > 0.05$). The MDA in H group was significantly higher than L group ($P < 0.05$).

The intestinal expression levels of antioxidant-related genes (*SOD1, SOD2, SOD3a, CAT, GPX*) in L group were significantly higher than that in H group ($P<0.05$, Fig 4). There were no significant differences between M and H group in mRNA level of *SOD1, SOD2, SOD3a* and *CAT* ($P > 0.05$).

3.3 immune capacity

The LZM in H group was significantly higher than that in L and M group ($P < 0.05$, Fig 5). There were no significant differences in intestinal ACP and AKP ($P > 0.05$, Fig 5).

As shown in Fig 6, mRNA levels of intestinal pro-inflammatory cytokines (*TNF-α, IL-1β*and *IL-8*) and *hepcidin1* in H group were significantly up-regulated than that in L group($P < 0.05$). The mRNA levels of *TNF-α* increased by 46.5% and 47.7% in H group than that in L and M group respectively. The mRNA levels of *IL-1β*increased by 124.0% and 82.5% in H group than that in L and M group respectively. The intestinal anti-inflammatory cytokines (*IL-10*) mRNA levels was increased by53.4% and 60.5% in L and H group respectively than that in M group ($P < 0.05$).

3.4 Apoptosis-related signaling molecules mRNA levels in the intestine

Compared with L and M group, the intestinal *caspase-3, caspase-8, caspase-9* and *bax* mRNA levels in H group were significantly up-regulated, while *bcl-2* was down-regulated significantly ($P < 0.05$, Fig 7). Compared with M group, *bcl-2* in H group was decreased by 42.7%, while *caspase3* was increased by 108.2%.

3.5 Intestinal microbial community

Through high-throughput sequencing, there are 597494 reads were obtained from the bacterial 16S rRNA V3/V4 region. No significant interactions were found in Shannon index and Chao index ($P > 0.05$, Fig 8).

At the phylum level, *Proteobacteria*, *Actinobacteria* and *Firmicutes* were the dominant phylum. The relative abundance of *Proteobacteria* were 94.86% and 92.46% in L and M group respectively . The relative abundance of *Proteobacteria, Actinobacteria* and *Firmicutes* in H group were 36.8%, 30.17% and 32.03% respectively (Fig 9A).

At the genus level, *pseudomonas* was the dominant genus in all groups. The relative abundance of *pseudomonas* were respectively 92.43% ,89.35% and 30.5% in L, M and H group. Together with *pseudomonas, rhodcoccus* and *mycoplasma* dominate the composition of gut microbes in H group. Respectively, the relative abundance of *rhodcoccus* and *mycoplasma* were 29.21% and 31.42% in H group (Fig. 9B).

The picture of the microbial composition of the samples were obtained by PCoA (Fig 10), based on the relative abundance profiles of OTUs. All samples in L and M group clustered in left upper of the coordinate axis, while formed two distinct clusters in H group, two samples clustered in the right of the coordinate axis, one clustered in the bottom left of the coordinate axis.

3.6 The relationship between gut microbes and gene expression

As shown in Fig 11, the expression of antioxidant-related, tight junction proteins genes and *bcl-2* were positively correlated to *pseudomonas*, while negatively correlated with *rhodcoccus, mycoplasma* and *brucella*（$P < 0.05$）.The expression of apoptosis and pro-inflammatory related genes were positively correlated with *rhodcoccus, mycoplasma* and *brucella,* and negatively correlated with *pseudomonas*（$P < 0.05$）.

4. Discussion

Starch is one of the cheapest energy source and a good binder in aquafeed(Song et al., 2018). However, as a typical carnivorous fish, largemouth bass has the limited ability of utilizing starch (Li et al., 2020; Lin et al., 2018). Excess dietary starch could result in negative impact on largemouth bass, such as reduce antioxidant capacity and immune function in liver(Amoah et al., 2008; Lin et al., 2018). The immune and antioxidant were important functions of the intestinal barriers (Yang et al., 2020).However, limited study has been reported about the effects of high starch diet on the combination of intestinal barriers (including physical, immunological and microbial). Thus, this study explored the effects of dietary starch level on the intestinal barriers and the potential regulatory mechanisms, which provides a partial theoretical basis for the mechanism of high starch level of diet effect on the physical health of largemouth bass.

4.1 high-starch diet impaired intestinal physical barrier

It is well known that the intestine is an important physical barrier for fish, and the structural integrity is crucial for resistance to foreign antigens(He et al., 2013; Zhao et al., 2020).This study found that 17% starch diet impaired the intestinal mucosa and increased the number of inflammatory cells. Similar results were found in blunt snout bream (*Megalobrama amblycephala*)(Yu et al., 2020). Moreover, high-fat diet damage the intestinal barrier in zebrafish, carbohydrate and fat are important for energy supply, and when the energy exceeded the body's needs, stress occurred, resulting in intestinal impairment (Arias-Jayo et al., 2018).The physical barrier of animal intestine is not only closely related to the complete cellular structure but also the epithelial permeability (Bergmann et al., 2013; Liu et al., 2014). It is well accepted that decrease in epithelial tight junction protein genes expression (such as *claudin7* and *occludin*) can increase epithelial permeability(Mennigen et al., 2009) . The tight junction was regulated by the MLCK signaling pathway, both the structure and the function (Liu et al., 2014). *Occludin* was a key factor of MLCK signaling pathway(Wang et al., 2019). In this study, 17% dietary starch down-regulated the mRNA of *occludin*, demonstrating that high starch diet increased permeability of midgut.

Apoptosis is mediated by caspases, which is an important process of programmed cell death (Borghetti et al., 2015; Yu et al., 2018). While the death receptor pathway (*caspase8*）and mitochondria pathway (*caspase9*) are differentially triggered and require different initiator caspases, they converge at caspase3 and ultimately leading to apoptosis (Ching et al., 2013). The apoptotic pathway executed is regulated by the *bcl-2* family proteins, *bax* promote apoptosis while *bcl-2* inhibit(Raqib, 2002). In fish, excessive apoptosis led to physical barrier damage (Wu et al., 2018). In the present study, 17% dietary starch up-regulated the genes expression of *caspase3,8,9* and *bax*; and decreased anti-apoptotic *bcl-2*. These results suggest that high starch diet might be via active death receptor pathway and mitochondria pathway to aggravated apoptosis in the fish intestine. Studies indicated that *JNK* was the trigger for death receptor pathway and mitochondria pathway in fish (Cao et al., 2015; Zheng et al., 2014). Thus, high starch diet might stimulate *JNK* genes expression to induced apoptosis in intestine.

4.2 high-starch diet impaired intestinal physiological barrier

The physical health status of animals is closely associated with the antioxidant defense system, which can protect cells and tissues from oxidative stress damage by removing excessive ROS and hydroxyl radicals(Ismail et al., 2010; Liang et al., 2017). Under complex physical and chemical environment, intestine of fish is susceptible to the attack of reactive oxygen species (ROS), due to alters of enteral environment and water environment(Deng et al., 2014). It is well known that ROS could be neutralized by endogenous antioxidant enzymes, such as T-SOD, CAT and GPX(Zhao et al., 2020). As a product of lipid peroxidation, MDA was mainly used to evaluate the damage degree of cell by its content in tissue (Dawood et al., 2017). In present study, the activity of antioxidant enzymes （T-SOD, CAT and GPX） and expression level of antioxidant-related genes (*SOD1, SOD2, SOD3a, CAT* and *GPX*) in H group decreased compared with L and M group, while the concentration of MDA increased significantly, indicating impairment to the intestinal antioxidant activities. The similar results were found in rat, Chinese mitten crab (*Eriocheir inensis*) and snout bream (*Megalobrama amblycephala*) (Miao et al., 2020; Shahid et al., 2018; Xu et al., 2019). whereas, the effects of dietary starch on the antioxidation

activities are controversial. For example, the activity of antioxidant enzymes in golden pompano (*Trachinotus ovatus*) intestine increased with diet starch level increased(Zhao et al., 2020). High carbohydrate diet had no effects on activity of antioxidant enzymes of intestine in European sea bass (*Dicentrarchus labrax*) (Castro et al., 2015). Excessive carbohydrates contribute to persistent hyperglycemia, then cause stress responses in fish(Hemre et al., 2015). In present study, prolonged feeding high-starch diet led to persistent stress, eventually resulted in impairment to the antioxidant system.

Lysozyme (LZM) is discharged from lysosomes of neutrophils and aggravates the responses to inflammation(Wu et al., 2006). As an effector molecule of the non-specific immune system, Hepcidin with antibacterial effects and is regarded as a marker of inflammation(Park et al., 2001). Hepcidin expression is strongly induced by inflammation cytokines such as IL6 and IL1β in mice(Wang et al., 2016). Pro-inflammatory cytokines (*TNF-α, IL-8* and *IL-1β*) performed important roles in fish intestinal immune response, and were used to assess the intestinal inflammation(Zhang et al., 2018). However, excessive occurrence of intestinal inflammation can lead to tissue damage and dysfunction(Pan et al., 2017). In present study, the activity of LZM conspicuously increased in H group, 17% starch diet up-regulated the mRNA levels of *hepcidin1*、*TNF-α*、*IL-8* and *IL-1β* in the intestine, which indicated high starch diet could induce intestinal inflammation. Similarly, high starch diet up-regulated intestinal inflammation-related mRNA levels in Nile tilapia, turbot and blunt snout bream(Bai et al., 2019; Li et al., 2020; Yu et al., 2020). It was observed that 17% starch diet impaired the intestinal epithelium and increased intestinal permeability, making it easier for harmful substances during food digestion and pathogens produced to enter the intestinal tissues, and ultimately resulted intestinal inflammation.

4.3 high-starch diet impaired intestinal microbial barrier

As is known, the gut microbiota has crucial effects on immune and inflammatory responses in host(Maslowski et al., 2011). Gut microbiota is variable and shifty with nutrient supplies and water environment (such as temperature, pH and so on) (Giatsis et al., 2015; Maslowski et al., 2011). *Fusobacteria, Proteobacteria* and *Firmicutes* are the dominant bacterial phylum of largemouth bass intestine at 27.6 to 34℃ (Lin et al., 2020; Zhou et al., 2018). The present study showed that *Proteobacteria, Actinob*acteria and *Firmicutes* were the dominant bacterial phylum in largemouth bass at 20 ± 1°C. The differences in gut microbial composition may be due to the alter in water temperature. In this study, the relative abundance of *Proteobacteria, Actinobacteria* and *Firmicutes* in H group obviously changed, compared with L and M group. Studies reported that dietary energy source (corn starch and lipid) and additives (brewer's yeast and sanguinarine) levels had considerable effects on intestinal microbial relative abundance in phylum level. (Arias-Jayo et al., 2018; Ding et al., 2020; Zhang et al., 2019; Zhou et al., 2018). So, we speculated that the relative abundance of intestinal microbe was considerably affected by the dietary starch level in phylum level.

Pseudomonas is a common dominant bacterial in water environment and intestine of aquatic animals(Zhou et al., 2018). Although a few *Pseudomonas* species (such as *Pseudomonas aeruginosa and Pseudomonas anguilliseptica*) have been isolated and identified as a pathogenic for humans, animals and fish (Berthe et al., 1995; Peix et al., 2018). Most species of *Pseudomonas* could produce a diverse set of antibacterial peptides, which could activate the immune response without triggering the activation of a large number of harmful inflammatory factors(Auvynet et al., 2010; Ghequire et al., 2018). In present study, the negative correlation between relative abundance of *pseudomonas* and the expression of apoptosis (*caspase-3,8,9* and *bax*) and inflammatory related genes(*hepcidin1,2, IL-1β, IL-8* and *TNF-α*) was observed. The antimicrobial peptides produced by *pseudomonas* could relieve the inflammation and apoptosis caused by pathogenic bacteria or diets (Ghequire et al., 2018) . Intestinal inflammation and apoptosis caused by high starch diet might be related with decreasing of *Pseudomonas*. *Pseudomonas* is the most important microbial component in this study, and may represent a normal inhabitant of the intestinal tract of largemouth bass playing a key role in intestinal microbial barrier. High starch diet decreased intestinal *Pseudomonas*, resulting in a reduced in immunity.

Mycoplasmas are the smallest bacteria, depending on glycolysis as an ATP-generating system(Arraes et al., 2007). As controversial bacteria, *Mycoplasmas* have various relationships with fish which extends from innocuous commensals to etiologies of disease(Brown, 2002). Some studies reported that *Mycoplasmas* were the dominant bacteria in Long-Jawed Mudsucker (*Gillichthys mirabilis*) and salmon, as harmless symbiotic microbes rather than pathogens in intestine(Bano et al., 2010; Holben et al., 2002). Whereas, most literature reported that *Mycoplasmas* act as a mucosal pathogen and exert its pathogenic capabilities through cytoadherence (Brown et al., 1994; Jakeen et al., 2020). In present study, high starch diet feeding resulted in increasing relative abundance of *Mycoplasmas,* and there was a positively correlation was observed between relative abundance of *mycoplasma* with inflammatory(*TNF-α, IL-1β*, *IL-8* and *hepcidin1,2*) and apoptotic(*caspase-3,8,9* and *bax*) genes. Carbohydrates are the main source of energy for *mycoplasma* and converted to lactic acid and acetic acid, which are essential for immune and inflammation regulation

(Brown, 2002; Kashinskaya et al., 2019). Furthermore, host immune system also plays a key role in shaping gut microbial communities(Kokou et al., 2019). The increased relative abundance of *mycoplasma* in H group may be a strategy for largemouth bass to adapt to the high-starch diet. At the same time, *mycoplasma* may act as the pathogenic bacteria leading to the outbreak of disease. *Mycoplasma* is beneficial for starch metabolism in intestine of largemouth bass and increases the risk of disease.

As emerging opportunistic pathogens*, rhodcoccus* have been shown to cause infections in human, animals and fish(Majidzadeh et al., 2018; Olsen et al., 2006). *rhodcoccus* is the most important causes of diseases such as: fulminating bacteremia in American alligator (*Alligator missippiensis*), pneumonia in pig and purulent meningitis in human(Cui et al., 2018; Jasmin et al., 1969; Woodroofe et al., 1950). Similar to *mycoplasma*, the relative abundance of *rhodcoccus* was positively correlated with apoptosis and inflammatory related genes, indicating *rhodcoccus* may act antigenic stimulus stimulate intestinal cells and cause intestinal inflammation and apoptosis. Our results suggest that high dietary starch consumption induced increased in the relative abundance of *rhodcoccus,* which might activate inflammatory response (mediated by *IL1β* and *TNF-α)* and apoptosis (mediated by *bax/bcl-2*). The similar results were found in zebrafish, high-fat diet enriched the relative abundance of bacteria (*Flectobacillus, Flavobacterium* and *Runella*) which may activate inflammatory response (Arias-Jayo et al., 2018) . Therefore, high-energy diet (starch or fat) could alter the composition of intestinal microbes and induce inflammation. There is a complex and tight relationship between intestinal microbial composition with host physiological system (antioxidant and immune), whereas the mechanism of their interaction remains to be explored.

5. Conclusion

In summary (Fig12), high starch diet impaired intestinal barriers of fish as displayed in the following aspects. 1) high starch diet led a slight infiltration of inflammatory cells and moderate loss of mucosal architecture, 2) impaired antioxidant capacity, 3) Induced inflammation and apoptosis. 4) remodeled the composition of intestinal microbes and increased the relative abundance of opportunistic pathogens.

In conclusion, high starch diet impaired physical, physiological and microbial barriers of intestine, and increased the risk of disease outbreaks.

Declarations

author declarations

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All authors declare no conflict interest.

The University of Sichuan Agricultural Animal Care Advisory Committee approved the procedures used in this study.

Consent to participate is not applicable for this study.

The authors declare Consent for publication.

The datasets used or analysed during the current study are available from the corresponding author on reasonable request. Sequence data has been deposited in the SRA under the study accession code PRJNA623189.

Code availability is not applicable for this study.

Authors' contributions: Song Yang supported the fund, conceived and designed the experiments; Liulan Zhao conceived and designed the experiments, assisted with critical review, commentary and revision; Ji Liang conceived and designed the experiments, written the Original Draft; Lei Liao, Xiaohong Tang and Qiao Liu performed the experiments; Kuo He and Jie Luo analyzed data; Wei Luo and Zongjun Du collected samples.

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Tables

Table1 Composition and nutrient levels of the experimental diets (%)

Ingredients	L	M	H
Fish meal	52	52	52
Vital gluten	6.5	6.5	7.5
Beer yeast powder	1	1	1
Soy protein concentrate	6.5	6.5	7
Corn Gluten Meal	6.5	6.5	4.5
Shrimp paste	2	2	2
Cassava starch	7	12	17
soybean oil	4	4	4
Lecithin oil	1.5	1.5	1.5
Microcrystalline Cellulose	9.5	4.5	0
$Ca(H_2PO_4)_2$	1.5	1.5	1.5
Choline	0.5	0.5	0.5
Vitamin premix [a]	0.5	0.5	0.5
Mineral premix [b]	1	1	1
Total	100	100	100
Nutrient levels			
Crude lipid	9.31	9.44	9.99
Crude protein	48.04	48.19	48.17
Ash	10.62	10.18	10.02
H_2O	10.12	10.62	11.34

[a] Vitamin Premix (mg. kg $^{-1}$ diet): vitamin A, 16000 IU; vitamin D3, 8000 IU; vitamin K3, 14.72; vitamin B1, 17.80; vitamin B2, 48; vitamin B6, 29.52; vitaminB12, 0.24; vitamin E, 160; vitamin C, 800; niacinamide, 79.20; calcium-pantothenate, 73.60; folic acid, 6.40; biotin, 0.64; inositol, 320; choline chloride,1500; L -carnitine, 100(Li et al., 2019).

[b] Mineral Premix (mg.kg $^{-1}$ diet): Cu ($CuSO_4$), 2.00; Zn ($ZnSO_4$), 34.4; Mn ($MnSO_4$), 6.20; Fe ($FeSO_4$), 21.10; I ($Ca(IO_3)_2$), 1.63; Se (Na_2SeO_3), 0.18; Co ($COCl_2$), 0.24; Mg ($MgSO_4 \cdot H_2O$), 52.70(Li et al., 2019).

Table 2 Primer sequences for real-time PCR

Genes	F/R	Primer sequence (5'-3')	Target size(bp)	TM (℃)
CAT	F*	GTTCCCGTCCTTCATCCACT	85	60.4
	R*	CAGGCTCCAGAAGTCCCACA		
SOD1	F	CCCCACAACAAGAATCATGC	178	58.0
	R	TCTCAGCCTTCTCGTGGA		
SOD2	F	CTGACCTACGACTATGGTGC	147	58.0
	R	CGTCACATCTCCCTTCGCTA		
SOD3a	F	ATCCATCAGTACGGAGACCT	181	58.8
	R	TTCCAATCGCAGACATCCCT		
SOD3b	F	CTCACAGCACAATGGCACTC	101	61.0
	R	CTGCTTAAACAGCGCCTGACC		
GPX	F	CCCTGCAATCAGTTTGGACA	94	58.0
	R	TTGGTTCAAAGCCATTCCCT		
IL-1β	F	CGTGACTGACAGCAAAAAGAGG	166	59.4
	R	GATGCCCAGAGCCACAGTTC		
IL-10	F	CGGCACAGAAATCCCAGAGC	113	62.1
	R	CAGCAGGCTCACAAAATAAACATCT		
TNF-α	F	CTTCGTCTACAGCCAGGCATCG	161	63.0
	R	TTTGGCACACCGACCTCACC		
	R	TCCTCTACCATTCGCAATCC		
hepcidin-1	F	CATTCACCGGGGTGCAA	107	58.0
	R	CCTGATGTGATTTGGCATCATC		
hepcidin-2	F	TCAGTGGAATCCTGGAAGATGC	69	58.0
	R	ACAGCAAAAGCGACATTTAATGC		
Caspase-3	F	GCTTCATTCGTCTGTGTTC	98	54.0
	R	CGAAAAAGTGATGTGAGGTA		
Caspase-8	F	GAGACAGACAGCAGACAACCA	195	56.0
	R	TTCCATTTCAGCAAACACATC		
Caspase-9	F	CTGGAATGCCTTCAGGAGACGGG	125	66.0
	R	GGGAGGGGCAAGACAACAGGGTG		
bax	F	ACTTTGGATTACCTGCGGGA	133	61.0
	R	TGCCAGAAATCAGGAGCAGA		
bcl2	F	CGCCATCCACAGAGTCCT	145	59.4
	R	CCGGAACAGTTCGTCTATCACC		
occludin	F	ACTTTGGATTACCTGCG	118	60.3
	R	GGGAGGGGCAAGACAACAGT		
claudin7	F	CATTTCAGCAAACACATC	135	58.4

	R	CGGCACAGAAATCCCAGAG

*F: forward primer; R: reverse primer

Figures

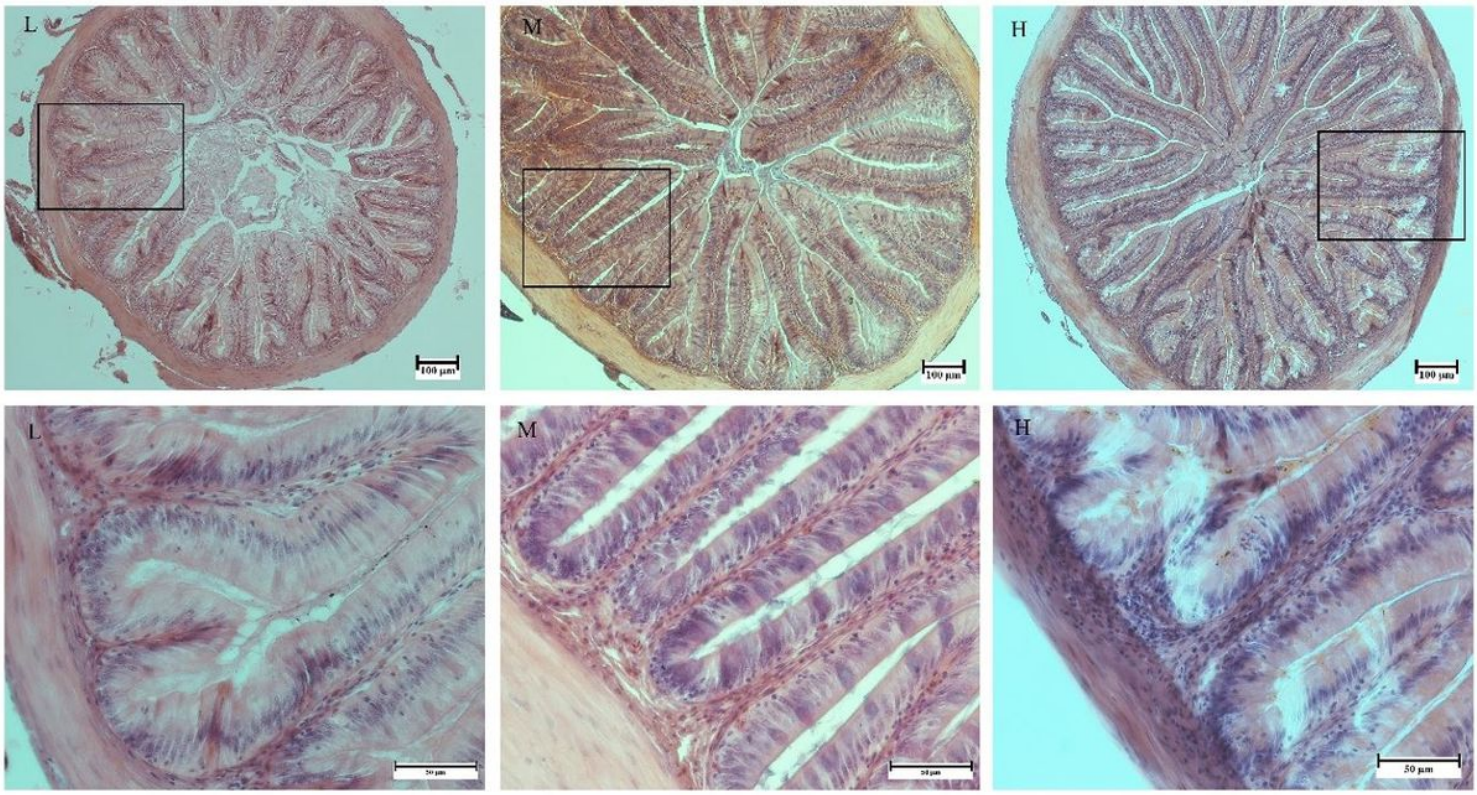

Figure 1

The histology of the midgut (upper: H&E×100, below: H&E××400) of largemouth bass fed diets containing different levels of starch. L: 7% starch, M: 12% starch, H: 17% starch. Note: The following figure corresponds to the squared part of upper figure.

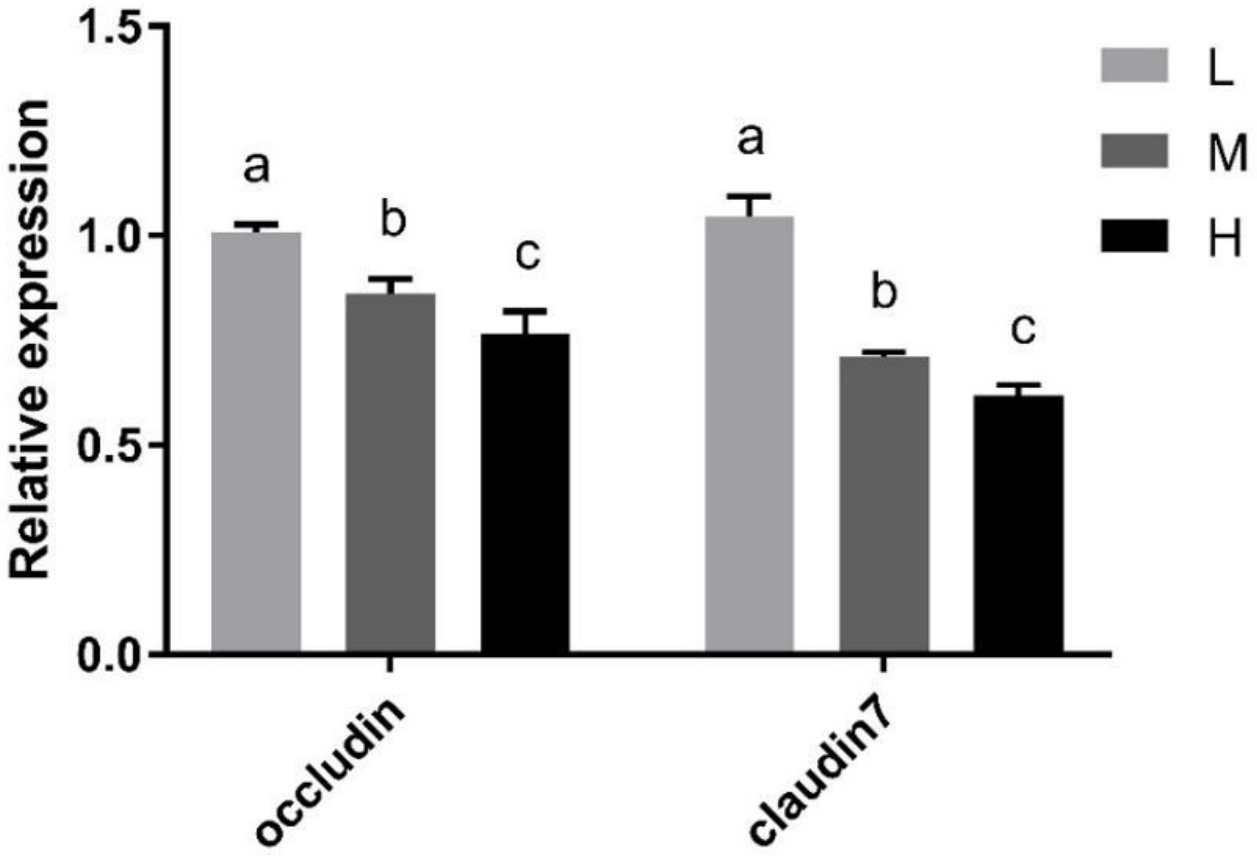

Figure 2

Intestinal expression levels of tight junction proteins genes (occluding, claudin7). Data are expressed as mean (SEM). The superscript small letters in the same column means the significant difference at $P < 0.05$. L: 7% starch, M: 12% starch, H: 17% starch.

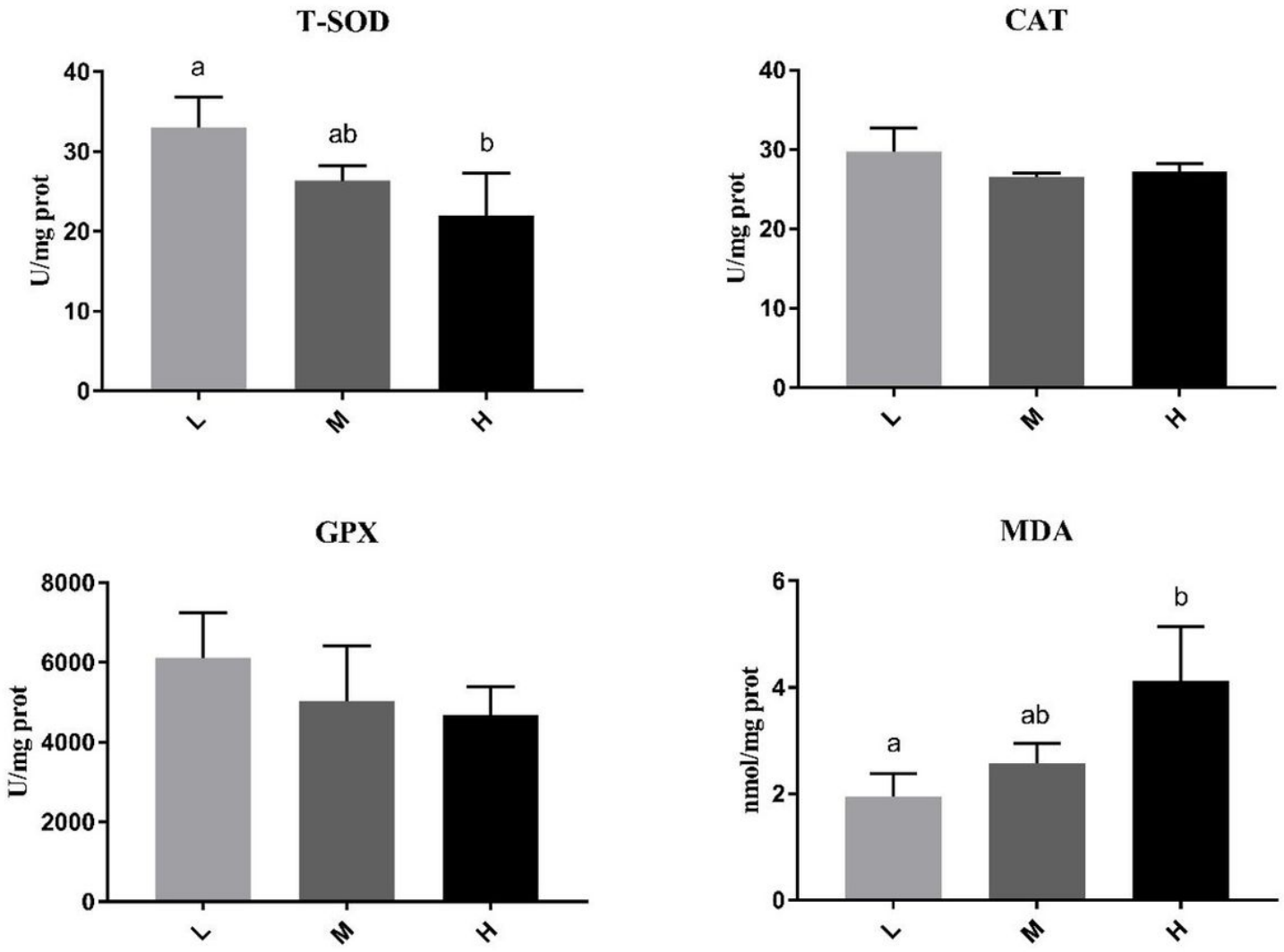

Figure 3

Effect of starch levels on Intestinal antioxidant parameters (CAT, T-SOD, GPX, MDA). The superscript small letters in the same column means the significant difference at $P < 0.05$. L: 7% starch, M: 12% starch, H: 17% starch.

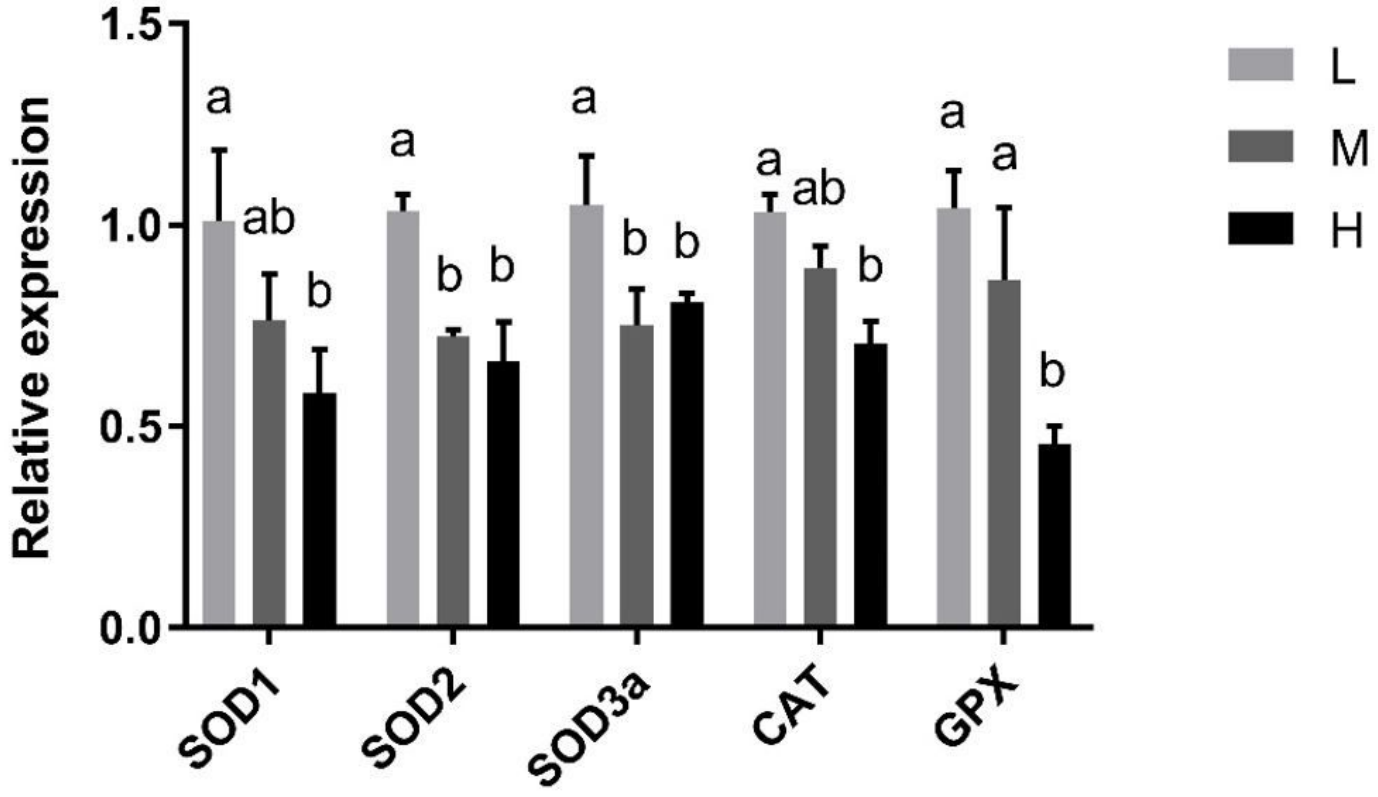

Figure 4

Intestinal expression levels of antioxidant-related genes (SOD1, SOD2, SOD3a, CAT and GPX) in largemouth bass fed the experiment diet. Data are expressed as mean (SEM). The superscript small letters in the same column means the significant difference at $P < 0.05$. L: 7% starch, M: 12% starch, H: 17% starch.

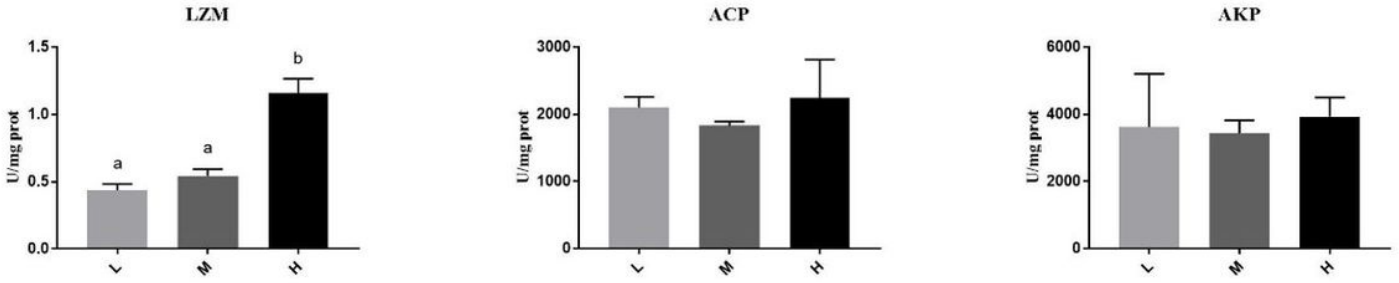

Figure 5

Effect of starch levels on Intestinal immune parameters (LZM, ACP, AKP). Data are expressed as mean (SEM). The superscript small letters in the same column means the significant difference at $P < 0.05$. L: 7% starch, M: 12% starch, H: 17% starch.

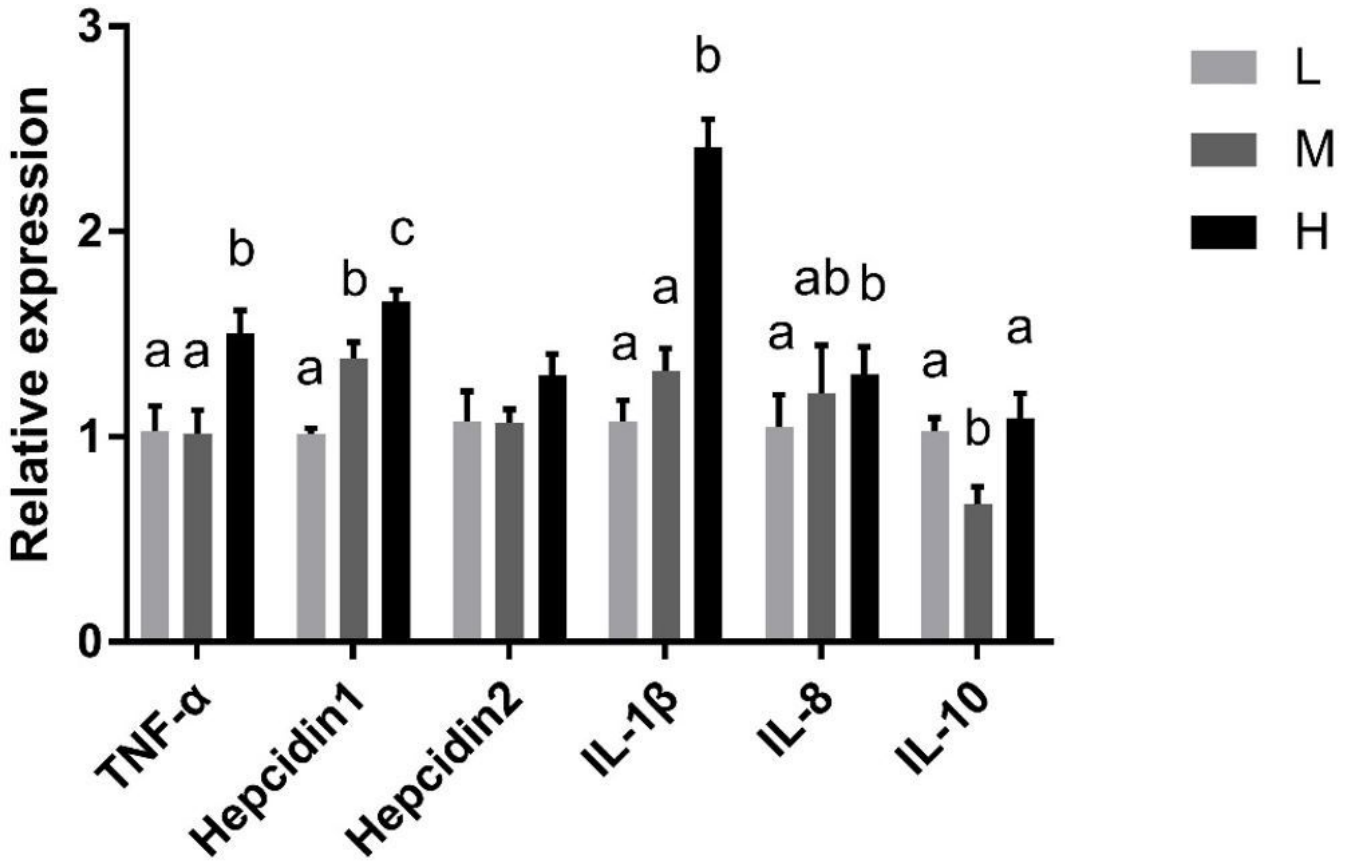

Figure 6

Intestinal expression levels of immune-related genes （TNF-α, Hepcidin1, Hepcidin2, IL-1β, IL-8 and IL-10）. Data are expressed as mean (SEM). The superscript small letters in the same column means the significant difference at $P < 0.05$. L: 7% starch, M: 12% starch, H: 17% starch.

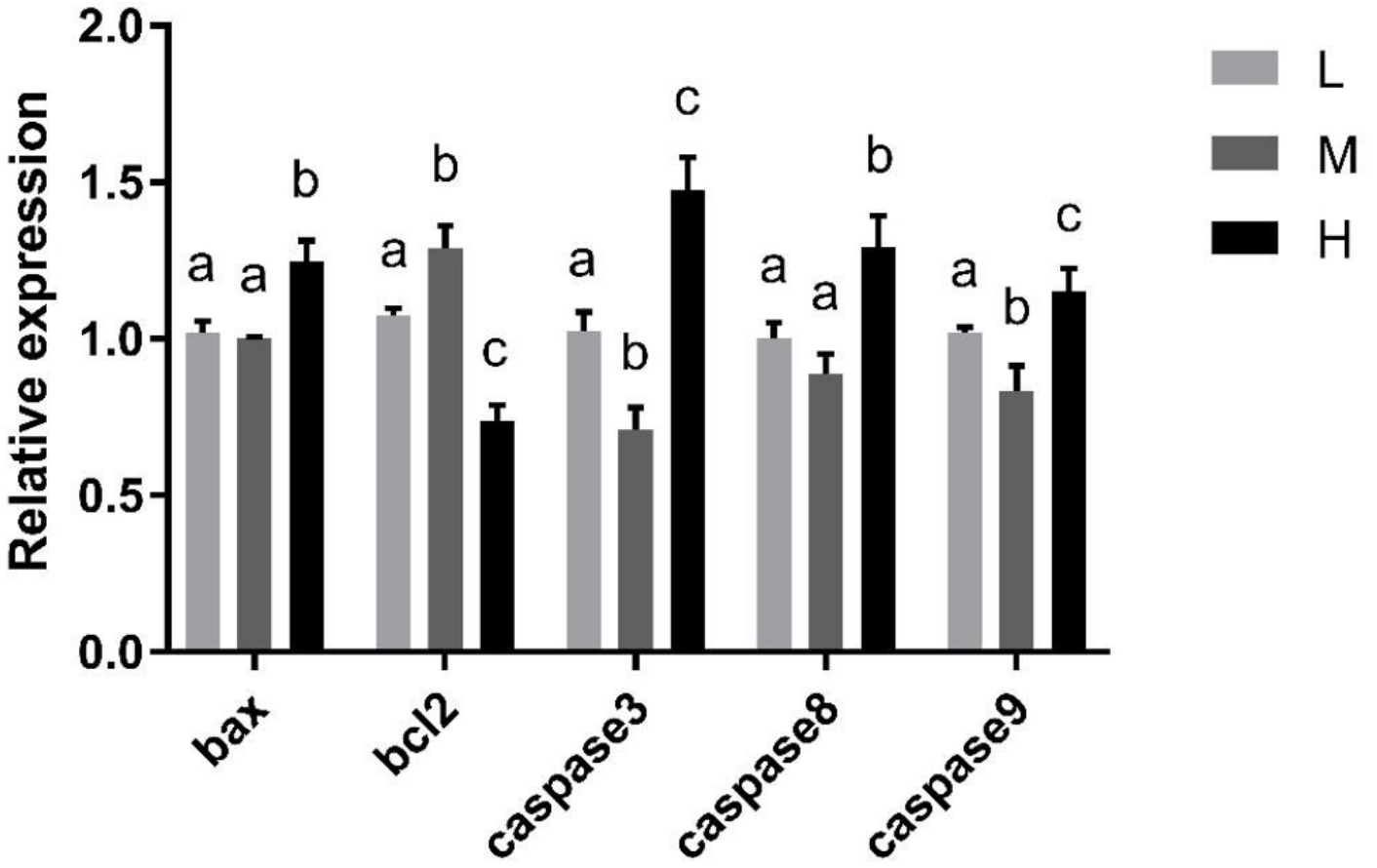

Figure 7

Intestinal expression levels of apoptosis genes (bax, bcl2, casepase3, casepase8 and casepase9). Data are expressed as mean (SEM). The superscript small letters in the same column means the significant difference at P < 0.05. L: 7% starch, M: 12% starch, H: 17% starch.

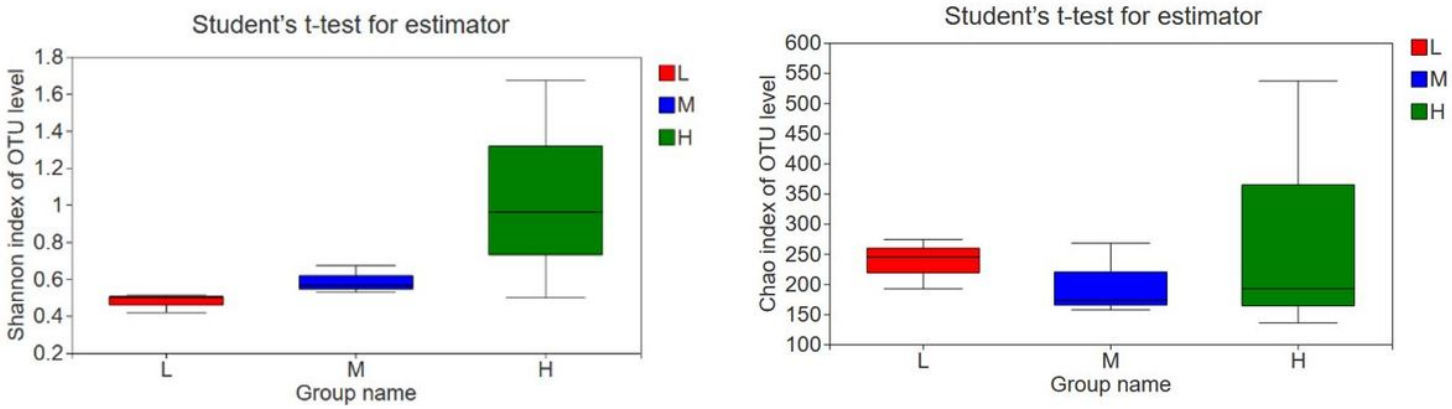

Figure 8

the shannon index and chao index of the Intestinal bacterial on OTU level

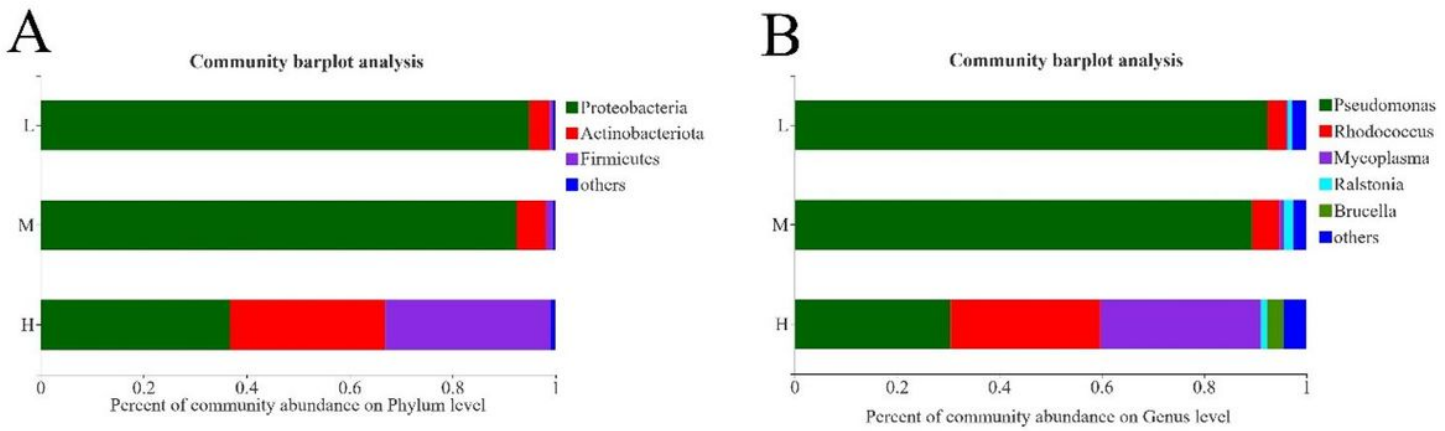

Figure 9

Intestinal bacterial composition in three groups at the phylum (A) and genus(B) levels.

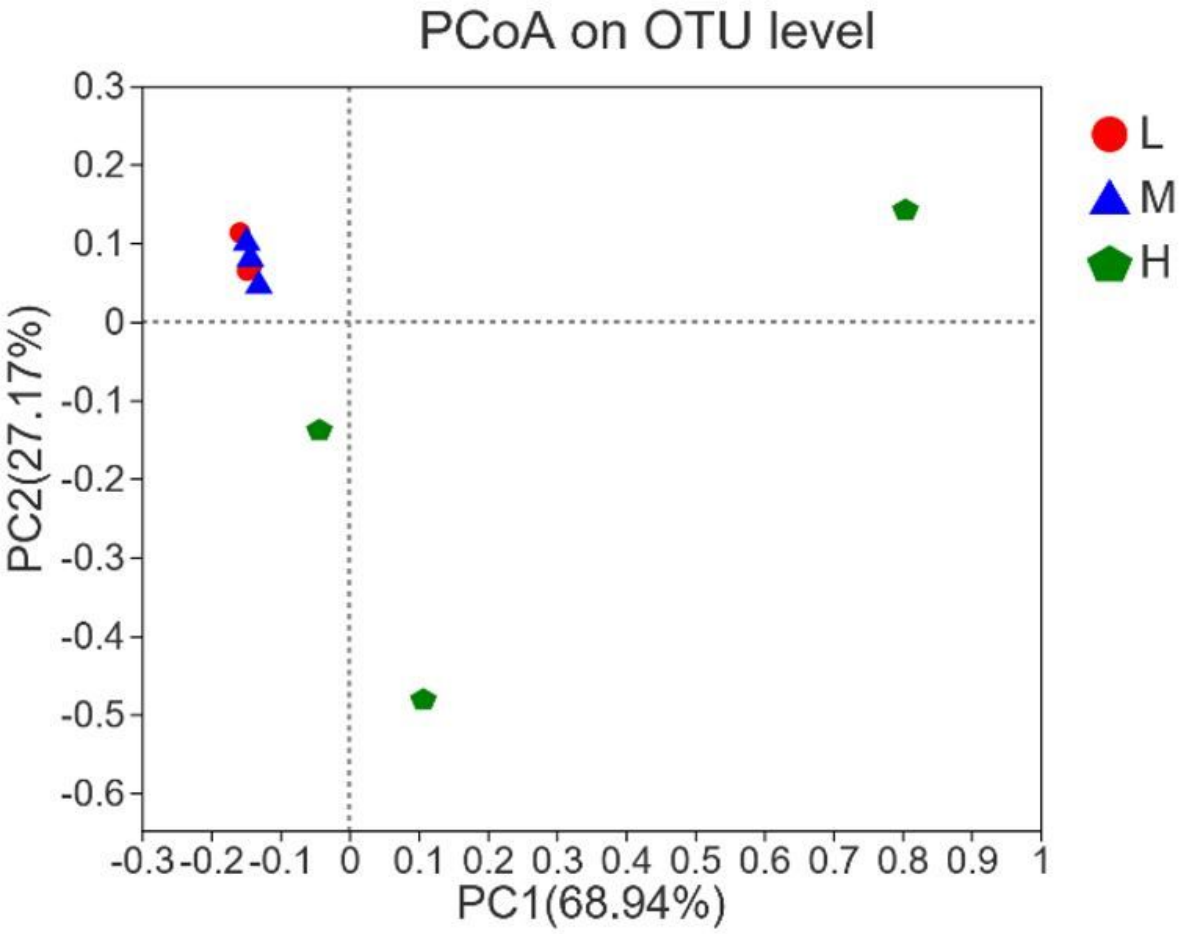

Figure 10

Principal Component Analysis (PCoA) of intestinal microbiology on OTU level

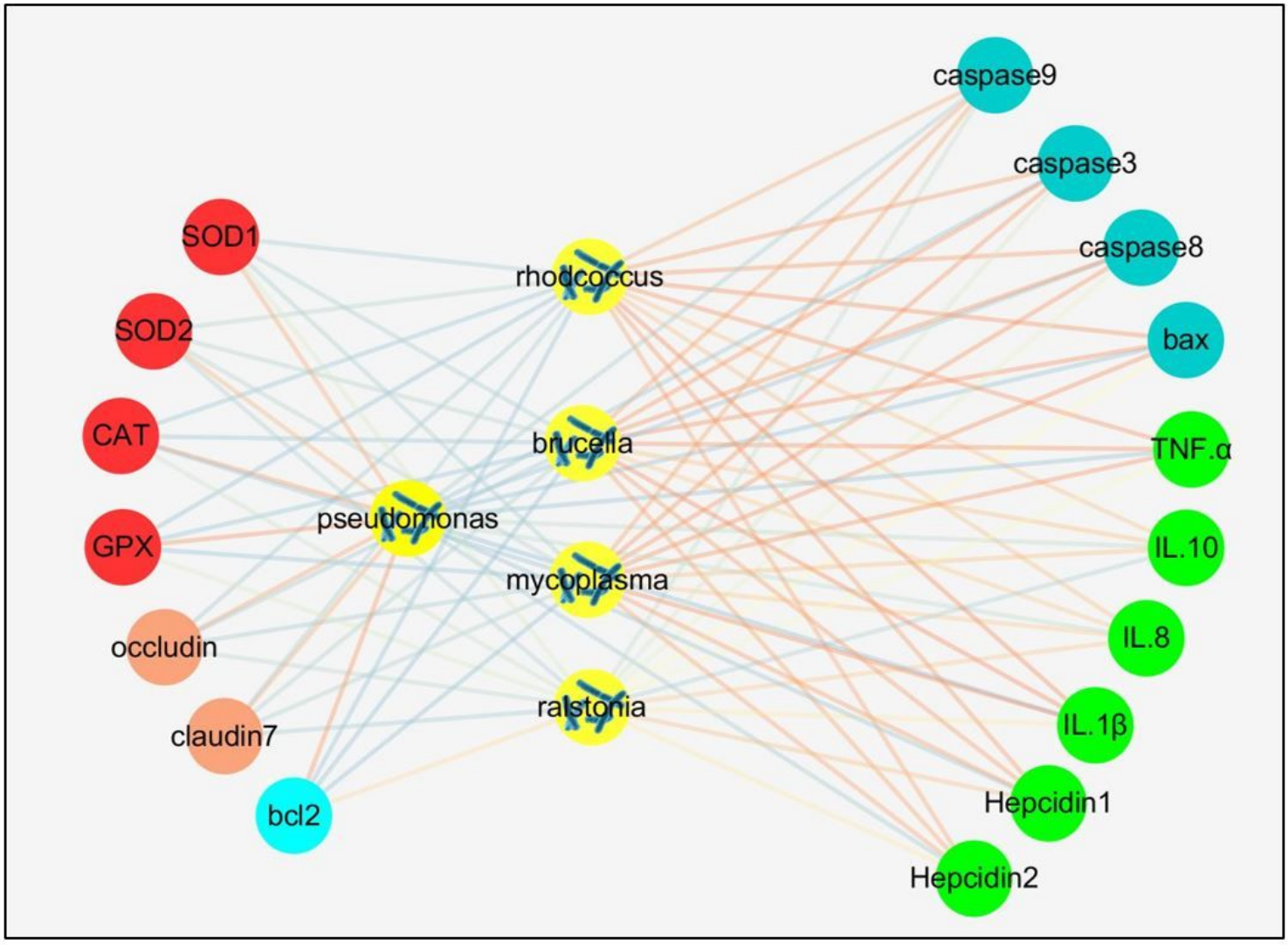

Figure 11

Correlation analysis network diagram of gut microbe and related genes expression of antioxidant, tight junction, apoptosis and inflammation. The red and blue lines represent positive and negative correlations respectively, and the depth of the color represents the size of the correlation.

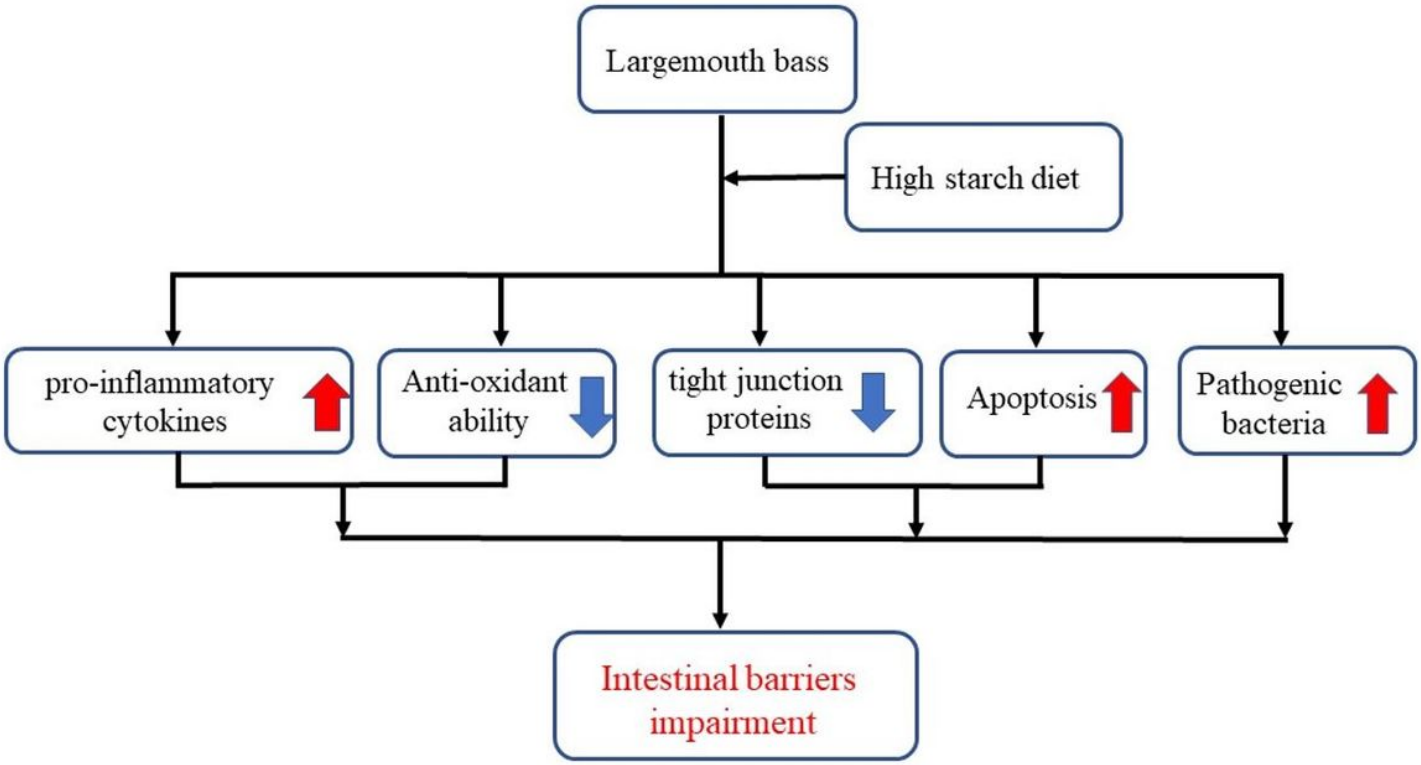

Figure 12

The potential pathways of high starch diet impair intestinal barriers of largemouth bass.