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Original Article

Effects of Mountain Spring Water Purification on Interferon Expression in Yellow Catfish

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Abstract:
The yellow catfish is an important freshwater economic fish in China. Under traditional farming practices, the extensive use of antibiotics has led to frequent diseases and a decline in disease resistance among fish. Mountain spring water purification farming, as a new farming model, offers a better growth environment for fish due to its pure water quality, Rich in minerals and dissolved oxygen, this study compares the interferon expression and histopathological characteristics of yellow catfish in the feeding group (traditional farming model) with those in the purification group (mountain spring water purification farming model). The aim is to investigate the impact of mountain spring water purification farming on the immune function of yellow catfish. The results indicate that the expression levels of IRF3, IRF7, IFNgr, and PfIFN genes in the muscle, intestine, and liver organs of the purification group were significantly higher than those in the feeding group. This suggests that mountain spring water purification can effectively activate the expression of relevant immune genes in yellow catfish, thereby enhancing their disease resistance. Furthermore, histopathological analysis revealed that the lipid metabolism capacity of liver cells and intestinal cells in the purification group was enhanced, with fewer lipid droplets and more regular cell morphology. The findings of this study suggest that mountain spring water purification farming not only provides a high-quality growth environment for yellow catfish but also significantly enhances their immune function by activating immune signaling pathways and regulating lipid metabolism. These results offer new ideas and methods for the healthy farming of yellow catfish, which are of great theoretical and practical significance.
Keywords**: yellow catfish; mountain spring water purification; immune function; gene regulation; lipid metabolism**

1. Introduction

Yellow catfish is one of the important freshwater economic fish in China. It is highly appreciated by consumers for its tender meat and rich nutrition, and it occupies a significant position in aquaculture[1]. In recent years, with the continuous progress of breeding technology and the continuous growth of market demand, the scale of yellow catfish breeding has expanded rapidly, and it has become an important part of Chinas aquaculture industry[2]. However, the traditional farming mode of yellow catfish is faced with many challenges. On the one hand, diseases occur frequently in the farming process of yellow catfish, which are difficult to treat effectively, resulting in a high mortality rate[3]. For example, yellow catfish fry are prone to

Edwardsiella infection, which can cause various diseases such as "red spot" and "big belly disease." Adult fish are also susceptible to viral diseases. On the other hand, the extensive use of antibiotics and other drugs in traditional farming not only disrupts the fishs own immune system but also leads to increased antibiotic resistance in pathogens, further reducing the fishes disease resistance[4].

In contrast, the mountain spring water purification fish farming model offers significant advantages. For instance, the recycling of mountain spring water conserves water resources and reduces pollution in surrounding water bodies. This includes reducing artificial oxygenation and medication use, lowering costs, and minimizing breeding risks, all of which align with green food certification standards. It is also suitable for small-scale production, catering to high-end aquatic markets or export demands. Mountain spring water, rich in minerals and dissolved oxygen, offers pure water quality that provides an excellent environment for fish growth, promoting healthy development and enhancing immune function, thereby reducing disease incidence. The immune system of fish is an important defense line against pathogen invasion[6]. Among them, the immune factor —— interferon (IFN) plays an important role in the immune defense system of fish. It can not only directly inhibit the invasion of pathogens, but also enhance the overall resistance of fish by regulating the immune system[7]. At the same time, the immune organs of fish include the muscle, intestine, and liver. The muscle indirectly supports and regulates the immune system of fish by maintaining tissue integrity, expressing immune-related genes, interacting with immune cells, and regulating metabolic products[8]: The gut of a fish[9]As one of the largest immune organs, it plays an important role in immune defense by its complex barrier structure and the synergistic action of immune cells to maintain the stability and health of the intestinal environment[10];The liver possesses hematopoietic and blood storage functions, is capable of removing large molecules, degrading and processing antigens, and producing antibodies[11].The normal function of these immune organs is crucial for maintaining fish health and enhancing their disease resistance. To understand the impact of mountain spring water purification on the immune function of yellow catfish, this study detected the expression levels of interferon and its regulatory factors in different organs of both the fed group and the purified group. Additionally, tissue pathology was used to analyze lipid content in muscle and intestinal cells of the fish. The results indicated that the mountain spring water purification farming model not only offers a high-quality growth environment for yellow catfish but also significantly enhances their immune function by activating immune signaling pathways and regulating lipid metabolism. The findings provide new insights and methods for the healthy farming of yellow catfish, offering both theoretical and practical significance.

Materials and Methods

Grouping of mountain spring water sources and samples

Samples were collected from natural spring water in the mountainous area of Chongqing, which is unpolluted by industrial activities.The water was pre-filtered through a 0.45 μm microporous membrane to remove large particulate impurities and then divided into two groups:Feeding Group (Control Group): using ordinary adult fish farming water and market-available yellow catfish feed for feeding, with a farming cycle of 20 days. Mountain Spring Water (Experimental Group): using collected mountain spring water, with a farming cycle of 20 days.

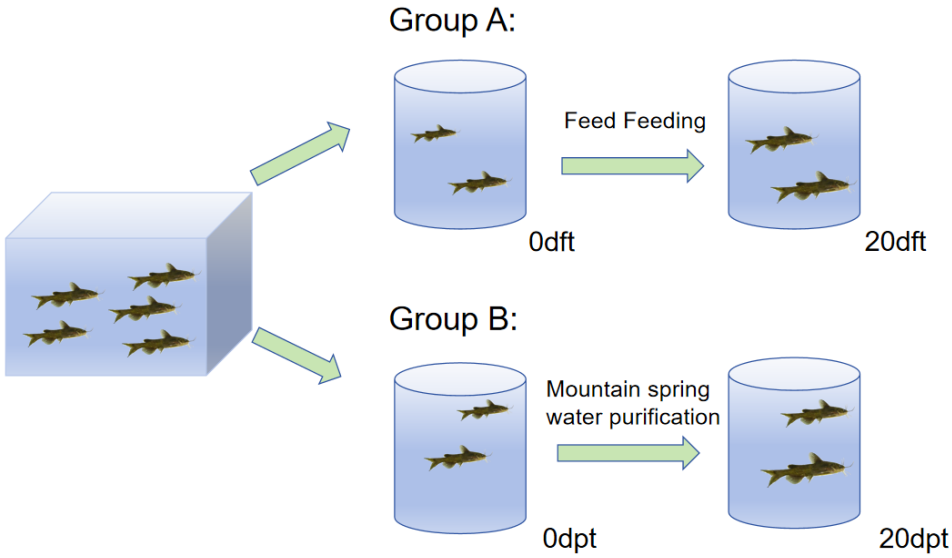

Figure 1 Flow chart of yellow catfish farming

Primer Sequence

Primer name Primer	Primer sequence Sequence5′ -3′
RIG-I-F	TGTTGCTGATGACTCCTC
RIG-I-R	AGTTTGTGCCTCTATGGT
IRF3-F	TGGGTGGGTTGGAGGTGA
IRF3-R	TAGATGGGTTCGGGCTGG
IRF7-F	CAGTAGGGGTCTGCTCCT
IRF7-R	TTCATTCTGGCTTTGGGG
MAVS-F	AACCTTTCGGTCTCAGTC
MAVS-R	CTCTCTTGTCCCTTGCTC
IFNgr1I-F	AACTCCTGGGAATAGTGAAA
IFNgr1I-R	AATAGGGAACTTATGGCTGT
PflFN-a3-F	GATCGATAAGGCCAACACAG
PflFN-a3-R	CAGTGTCCTGCTGTCCCA
β-actin-FW	CCGTGACCTGACTGAATACC
β-actin-BW	GCCCATCTCCTGCTCAAAG

***RNA* Draw**

Dissect the liver, spleen, and head kidney, rinse the surfaces with pre-cooled PBS to remove blood, pat dry with filter paper, and weigh them. Fix some of the tissues in 4% paraformaldehyde for pathological sections, and rapidly freeze the remaining tissue in liquid nitrogen before transferring it to -80°C for storage.This is utilized for the determination of antioxidant enzyme activity (as per the kit instructions, with enzyme activity expressed in units per milligram of protein) and for gene expression analysis.

Add chloroform and shake vigorously for 15-30 seconds, then allow the solution to stand at room temperature for 2-3 minutes to separate the layers. Centrifuge at 4°C, 12,000-15,000 RPM for 15-20 minutes.Transfer the upper aqueous phase, which contains RNA, to a new centrifuge tube. Add an equal volume of isopropanol and gently mix. Allow it to stand at room temperature for 10 minutes to precipitate the RNA.

4°C,12000-15000 rpm centrifuge for 10-15 minutes, discard the supernatant to obtain the RNA pellet. Wash the pellet with 75% ethanol 2-3

times, and after each wash, centrifuge at 4℃,7500-10000 rpm for 5-10 minutes, discard the supernatant. Allow the RNA pellet to dry at room temperature or vacuum-dry it, then add an appropriate amount of water without RNA enzyme to dissolve the RNA.

Take a small amount of RNA sample and use 1% agarose gel electrophoresis to check the integrity of the RNA, observing the brightness and clarity of the 28S and 18S ribosomal RNA bands, as well as any signs of degradation. Simultaneously, use a NanoDrop 2000 to measure the concentration and purity of the RNA, ensuring that the OD_{260}/OD_{280} ratio is between 1.8 and 2.0, with the RNA concentration unit being ng/μL.

Genetic Reverse Transcription

The reverse transcription reaction system is configured on ice and generally includes RNA template, random primers or oligo dT primers, reverse transcriptase, dNTPs, and buffer. The total volume is 20 μL. The reaction mixture is incubated at 42-50℃ for 30-60 minutes to convert RNA into cDNA, followed by incubation at 70-85℃ for 5-10 minutes to terminate the reverse transcription reaction. After the reaction is complete, the obtained cDNA can be immediately used for real-time PCR.

Quantitative Polymerase Chain Reaction

According to the sequences of target immune genes (such as IFNgr, PfIFN) and reference gene β-actin, design primers using specialized primer design software. The primers should be specific to avoid non-specific amplification. Prepare the fluorescent quantitative PCR reaction system on ice, which generally includes cDNA template, forward and reverse primers, SYBR Green dye, dNTPs, Taq enzyme, and buffer, with a total volume of 20 μL. Place the reaction tubes in the fluorescent quantitative PCR instrument and perform the following steps: 95°C for 30 seconds pre-denaturation, followed by 95°C for 5-10 seconds denaturation, and 60°C for 30-60 seconds annealing, for a total of 40 cycles. Collect the fluorescence signal during the annealing phase of each cycle.

Data Statistics and Analysis

Experimental data were organized using Excel 2021 and subjected to two-factor analysis of variance (Two-way ANOVA) with relative quantification (Relative Quantification Method). When inter-group differences were significant, multiple comparisons were performed using simple effect analysis (Simple Effects Analysis). The significance level was set at $P<0.05$, and results were expressed as "mean ± standard deviation (Mean ± SD)."

Organize Oil Red O Staining

Freeze sections of intestinal and liver tissues, fix with 4% formalin for 10 minutes. Wash three times with PBS, each time for 5 minutes. Dehydrate in 50% ethanol for 2 minutes, then add freshly prepared Oil Red O stain solution and incubate at room temperature for 10 to 15 minutes. Quickly wash with 50% ethanol for 15 to 20 seconds. Wash three times with PBS. Re-stain the cell nuclei with 10% Mayers hematoxylin, rinse three times with tap water. Mount with neutral resin, observe under a microscope, and capture images.

Results

To investigate the expression levels of immune genes under different farming models, fish from various organs were analyzed simultaneously under various farming conditions.The expression of muscle-related genes was examined, and as illustrated in Figure 2, the 20-day feeding group exhibited an approximate two-fold increase in the expression of IRF3 and IRF7 genes in muscle tissues compared to the 0-day feeding group. Conversely, the expression of the RIG-I gene was reduced by roughly fivefold (Figure 2A).As depicted in Figure 2, in comparison to the 0-day purification group, the 20-day purification group demonstrated heightened expression of IFNgr and PfIFN genes within the muscle tissues. Specifically, the expression of the IFNgr gene rose by approximately twofold, while the PfIFN gene expression increased by roughly threefold (refer to Figure 2B).As depicted in Figure 2, in comparison to the 20-day feeding group, the 20-day purification group exhibited heightened expression of IRF3, MAVS, IFNgr, and PfIFN genes within the muscle tissues.In comparison between 20dpt and 20dft, although some impurities were removed during spring water purification, trace amounts of immunogenic substances (such as microbial fragments, viral-like RNA, etc.) may still be retained, which can activate the natural immune pathways of yellow

catfish[12]For example, purifying trace irritants from water may activate the RIG-I receptor (RLR) pathway, which in turn activates the MAVS-TBK1-IRF3/IRF7 pathway, initiating downstream immune signaling. This leads to a approximately 2-fold increase in MAVS and IRF3 gene expression, ultimately promoting the expression of IFNgr and PflFN genes, with IFNgr increasing by about 2 times and PflFN increasing by about 7 times.

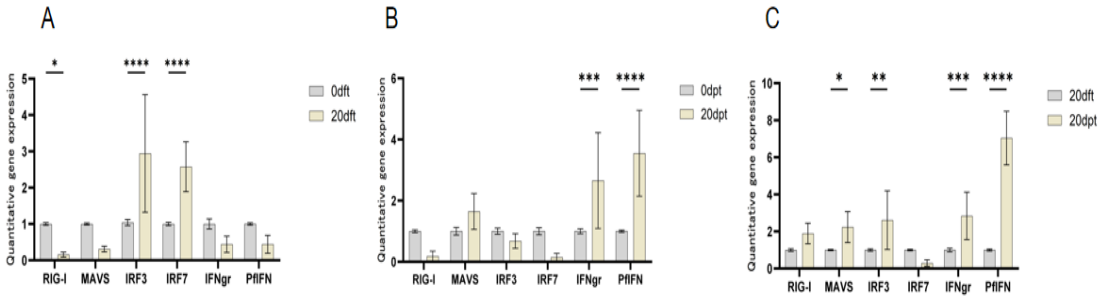

Figure 2A Comparison of 20-day feeding group with 0-day feeding group; Figure 2B Comparison of 20-day purification group with 0-day purification group; Figure 2C Comparison of 20-day purification group with 20-day feeding group, expression of RIG-I, MAVS, IRF3, IRF7, IFNgr and PfIFN in muscle organs.

The expression of immune-related genes in the fish's gut was also detected. As illustrated in Figure 3, compared to the 0-day feeding group, the expression of the RIG-I gene in the intestinal organ increased by approximately 2 times, and the expression of the IRF7 gene increased by about 1.5 times (Figure 3A). Compared to the 0-day purification group, the expression of the RIG-I gene in the intestinal organ was reduced by fourfold, whereas the expressions of the IRF7 and IFNgr genes increased by approximately 1.5 times, and the expression of the PfIFN gene increased by about 2 times (Figure 3B).Additionally, compared to the group fed for 20 days, the expression of IRF3, IRF7, IFNgr, and PfIFN genes in the intestinal organ increased by approximately 2-fold (Figure 3C).

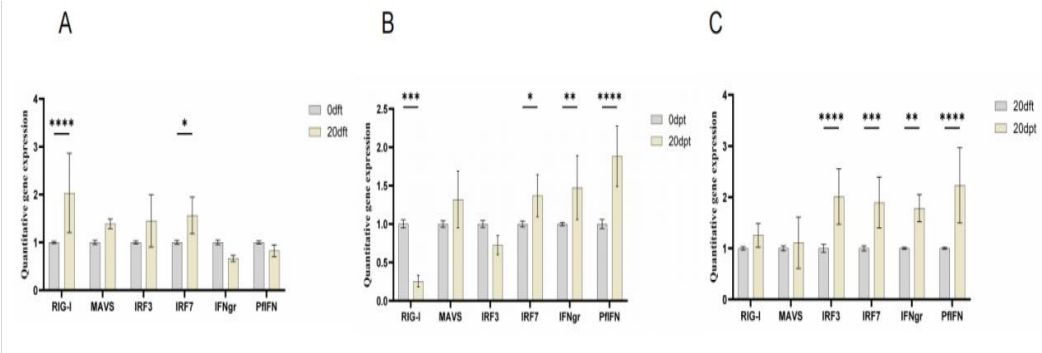

Figure 3A Comparison of the 20-day feeding group with the 0-day feeding group; Figure 3B Comparison of the 20-day purification group with the 0-day purification group; Figure 3C Comparison of the 20-day purification group with the 20-day feeding group. Expression levels of RIG-I, MAVS, IRF3, IRF7, IFNgr, and PfIFN in intestinal organs.

The expression levels of genes associated with the liver of fish were examined. As depicted in Figure 4, in comparison to the 0-day feeding group, the expression levels of MAVS, IRF7, and PfIFN in the liver tissues of the 20-day feeding group were found to be approximately 1.5 times greater, while the expression level of IRF3 was approximately 2 times higher (Figure 4A).Compared to the 0-day purification group, the expression levels of IRF3 and PfIFN in the liver organs of the 20-day purification group were approximately 1.5 times higher, the expression level of MAVS was approximately 2 times higher, and the expression level of IRF7 was approximately 2.5 times higher

(Figure 4B). Additionally, compared to the 20-day feeding group, the expression levels of IRF7, IFNgr, and PfIFN in the liver organs of the 20-day purification group were approximately 1.5 times higher, the expression levels of MAVS and IRF3 were approximately 2 times higher, and the expression level of RIG-I was approximately 3 times higher (Figure 4C).

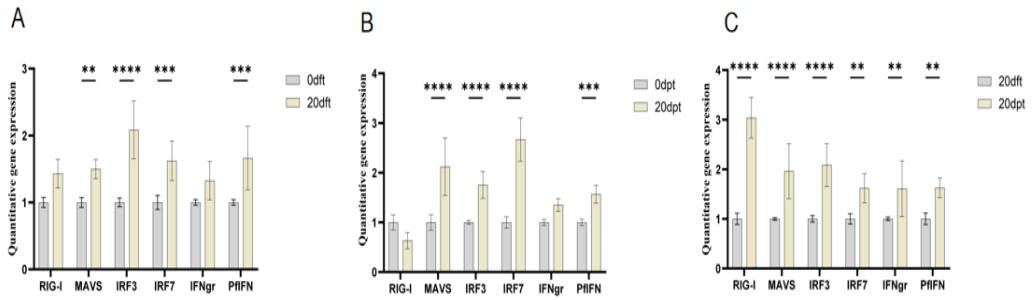

Figure 4A Comparison of feeding group at 20 days with feeding group at 0 days; Figure 4B Comparison of purification group at 0 days with purification group at 0 days; Figure 4C Comparison of purification group at 20 days with feeding group at 20 days, expression of RIG-I, MAVS, IRF3, IRF7, IFNgr and PfIFN in liver organ.

The Oil Red O staining of liver tissues reveals (as depicted in Figure 5) that, in comparison to the 20-day feeding group, the 0-day feeding group exhibits: In Figure A, hepatocytes contain a moderate number of lipid droplets that are evenly distributed; conversely, in Figure C, lipid droplets are extensively fused and enlarged, the cytoplasm appears turbid, the nucleus is notably diminished, and the intercellular spaces are filled with lipids, suggesting that prolonged feeding has resulted in a significant impairment of lipid metabolism in hepatocytes and a substantial accumulation of lipids. Comparing the 0-day purification group with the 20-day purification group: In Figure B, some hepatocyte nuclei are condensed, resulting in the aggregation of lipid droplets and a slight turbidity of the cytoplasm; in Figure D, the number of lipid droplets in hepatocytes decreases, and the cytoplasm is clear, indicating that purification treatment has markedly enhanced the lipid metabolism status of hepatocytes. When comparing the 20-day feeding group with the 20-day purification group: In Figure C, hepatocytes display severe lipid accumulation and reduced metabolic function, with a significant decrease in the nucleus; in Figure D, hepatocytes have very low lipid content and exhibit active metabolism.

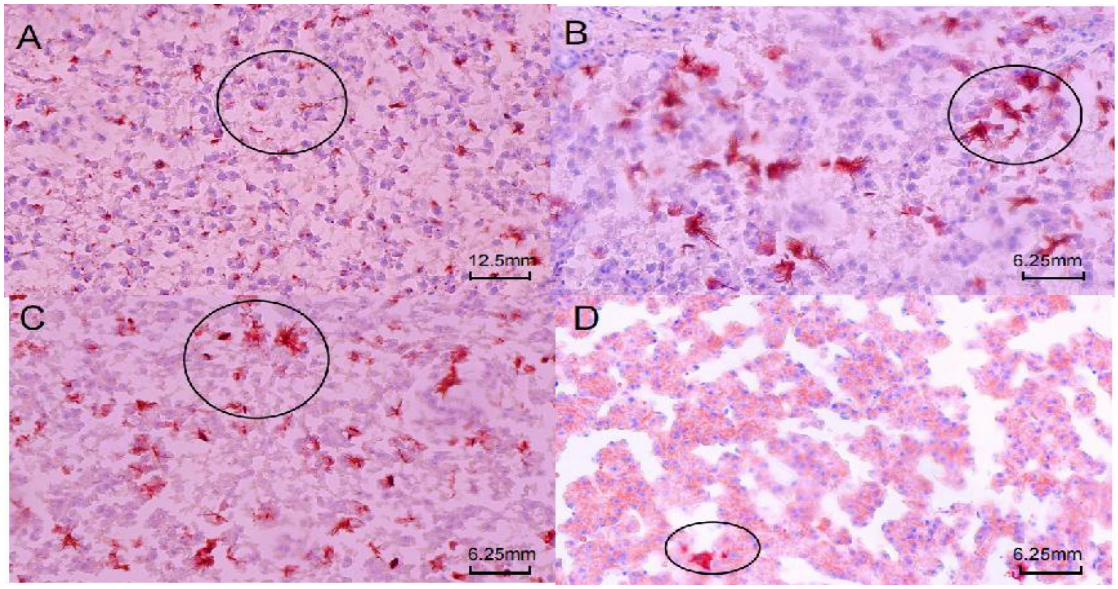

Figure 5 Oil red stained sections of liver organs at different days A x 80, B, C and D x 160
A was the feeding group at 0 days, B was the purification group at 0 days, C was the feeding group at 20 days, and D was the purification group at 20 days

The Oil Red O staining of intestinal tissues revealed that in the 20-day purification group, as opposed to the 20-day feeding group, there was a notable difference. In Figure C, numerous lipid droplets were visible within the tissue sections of the intestinal organs, and the cytoplasm appeared relatively turbid. The lipid droplets were widely distributed, and in some cells, lipid droplet fusion was observed (Figure 6). The cell morphology was slightly irregular, with parts of the intercellular spaces filled with lipids, making the intercellular structures less distinct. This indicates that under pure water farming conditions, the lipid metabolism of yellow catfish intestinal cells is relatively weak, resulting in a large accumulation of lipids within the cells that are not effectively consumed or transported. In contrast, in the 20-day purification group, the number of lipid droplets inside the cells decreased, and the number of lipid droplets in the intestinal epithelial and glandular cells was significantly reduced. The cytoplasm became clearer, suggesting a decrease in intracellular lipid content. The overall cell shape was more regular, with clear intercellular spaces, and no obvious lipid accumulation was observed. This suggests that under mountain spring water farming conditions, the lipid metabolism of yellow catfish intestinal cells is more active, with lipids being extensively consumed or transported, leading to a reduction in intracellular lipid accumulation.

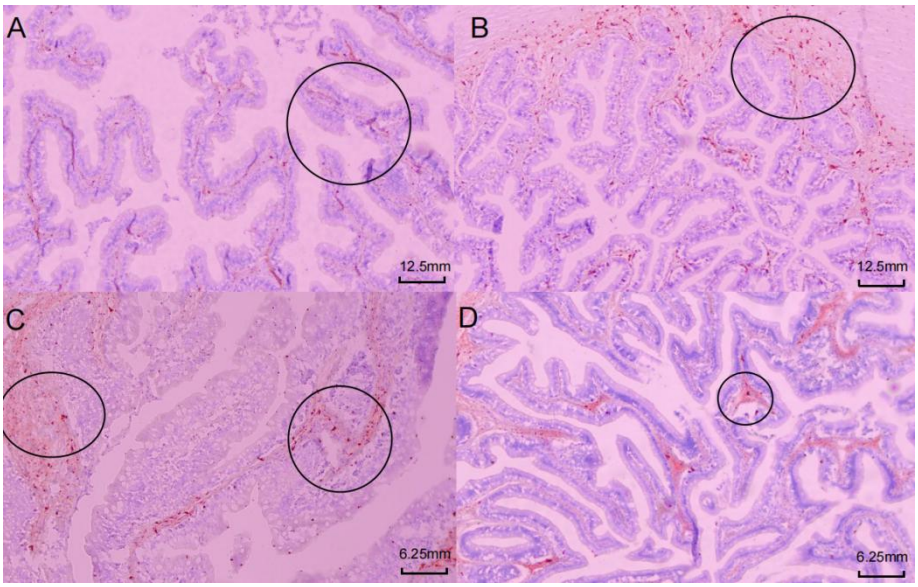

Figure 6 Oil red stained sections of intestinal organs at different days A and B x80, C and D x160 A was the feeding group at 0 days, B was the purification group at 0 days, C was the feeding group at 20 days, and D was the purification group at 20 days

Discussion

Mountain spring water is naturally filtered, typically free of impurities and pollutants, and boasts clear, transparent water quality. It is a natural high-quality water source that can provide a suitable environment for fish growth and reduce the occurrence of diseases[13].At the same time, the dissolved oxygen levels in mountain spring water are sufficient to meet the high demand for dissolved oxygen required by fish, thereby promoting their healthy growth[14].This study investigates the effects of mountain spring water purification on the immune function of yellow catfish. The experimental design targets the liver, muscle, and intestinal organs for research. To uncover potential mechanisms at the molecular level, RNA extraction and reverse transcription were conducted to obtain precise data on gene expression[15].Simultaneously, we conducted an in-depth analysis using quantitative methods and experimental techniques. The findings indicated that the purification of mountain spring water had a moderate enhancing effect on the immune function of yellow catfish.

In the intestinal organs, compared to 0dft, some fish may become infected with viruses or carry them during the breeding process. For instance, in the wild, the yellow catfish is particularly susceptible to infection by the yellow catfish cup

virus[16].During infection, RIG-I activates the MAVS-TBKI-IRF3/IRF7 pathway by recognizing viral RN A, resulting in a 1.5-fold increase in IRF7 expression at 20dft[17].Compared with 0dpt, 20dpt also increased the expression of IRF7 by about 1.5 times through the MAVS-TBKI-IRF3/IRF7 pathway. The increase of IRF7 further enhanced the production of type I interferon (IFNgr, PfIFN), forming a positive feedback loop[18].However, due to the high expression of PfIFN and IFNgr genes, they will induce the expression of a variety of interferon-stimulating genes (ISGs), such as LGP2. LGP2 can competitively bind viral RNA with RIG-I, thus reducing the binding of RIG-I and viral RNA[19], Which leads to a decrease in RIG-I gene expression, which helps maintain the balance of immune response and avoid immune pathological damage caused by overactivation. In the muscle organs of yellow catfish, compared to 0 dft, the expression of RIG-I gene is inhibited at 20 dft, while the expression of IRF3 and IRF7 genes increases by about two times. Under normal conditions, RIG-I recognizes viral RNA, activates IRF3 and IRF7, thereby inducing the production of type I interferon. If RIG-I is inhibited, melanoma differentiation-related gene 5 (MDA5) compensates for this by activating to maintain the cells antiviral response[20].Therefore, MDA5 activates the MAVS-TBKI-IRF3/IRF7 pathway, which increases the expression of IRF3 and IRF7 genes.In the liver organ, compared with 0dft, in the traditional farming mode, the water of ordinary adult fish pond may contain residual metabolic waste, drugs or chemicals, such as sulfonamides (such as sulfamethizole) and tetracyclines (such as oxytetracycline), which can cause irritation or damage to the skin of fish, making it easier for pathogens such as yellow catfish cup-shaped virus to invade the fish body[21], Triggering the activation of the RIG-I signaling pathway, which in turn upregulates the expression levels of MAVS, IRF3, IRF7, and PfIFN genes at 20 dft. Compared to 20 dft, as shown in Figure 4C, the measured gene expression levels all increased. This is due to the improvement in water quality and reduction in environmental stress from mountain spring water, indirectly enhancing the immune system activity of yellow catfish[22].In addition, mineral elements (such as selenium) in natural spring water can further enhance the immune ability of yellow catfish by enhancing antioxidant capacity[23].This result indicates that mountain spring water purification not only maintains the efficiency of immune response, but also further enhances the expression of immune-related genes.

The oil red staining of liver cells indicated that the lipid content in the purified group was significantly lower than that in the fed group. This suggests that the use of mountain spring water for purification in farming can effectively enhance the lipid metabolic capacity of yellow catfish liver cells, compared to conventional feeding methods. Consequently, when compared to 20 days of conventional feeding, 20 days of mountain spring water purification significantly enhanced the lipid metabolic capacity of yellow catfish liver cells. After feeding, a large amount of lipid accumulation was observed in the liver cells, primarily due to the long-term continuous feeding causing the yellow catfish to consume far more nutrients than their body can metabolize and utilize[24].These excess nutrients, especially fat, are difficult to be transported or consumed in time, and can only continue to accumulate in hepatocytes, which leads to a series of changes such as lipid droplet fusion and cytoplasm turbidity, and finally leads to a significant decrease in the efficiency of lipid metabolism in liver organs[25].After purification, the lipid metabolism of liver cells was significantly improved, which is due to the ability of type I interferon to regulate the expression of genes related to fatty acid oxidation and lipid synthesis. For example, IFN-β can promote the expression of genes associated with mitochondrial fatty acid oxidation (such as CPT1A), thereby increasing fatty acid oxidation and reducing lipid accumulation[26].On the other hand, the JAK-STAT signaling pathway regulated by IFN gene can also enhance the immune defense ability of the body, provide a strong guarantee for the maintenance of normal function and metabolic state of hepatocytes, so that the morphology of hepatocytes gradually returns to regular, the cytoplasm becomes clear, and the cell gap tends to be clear[27].The oil red staining of intestinal cells showed that the lipid content of intestinal cells in the purification group was significantly lower than that in the feeding group. The internal activation of MAVS-TBKI-IRF3/IRF7 pathway regulated the synthesis of type I interferon, which in turn could regulate the expression of lipid metabolism

genes in adipocytes[28].For example, IFN-β can inhibit the expression of genes related to fat synthesis in adipocytes (such as FASN, ACC), reducing fat synthesis[29]Moreover, as a key signaling protein, MAVS not only regulates the production of IFN, but also indirectly regulates lipid metabolism by affecting mitochondrial function and membrane potential[30].This enhanced lipid metabolism, which is activated by the IFN signaling pathway, requires cells to produce more energy and biosynthetic substances to support the immune response, that is, the enhanced lipid metabolism is the result of enhanced cellular immunity[31].

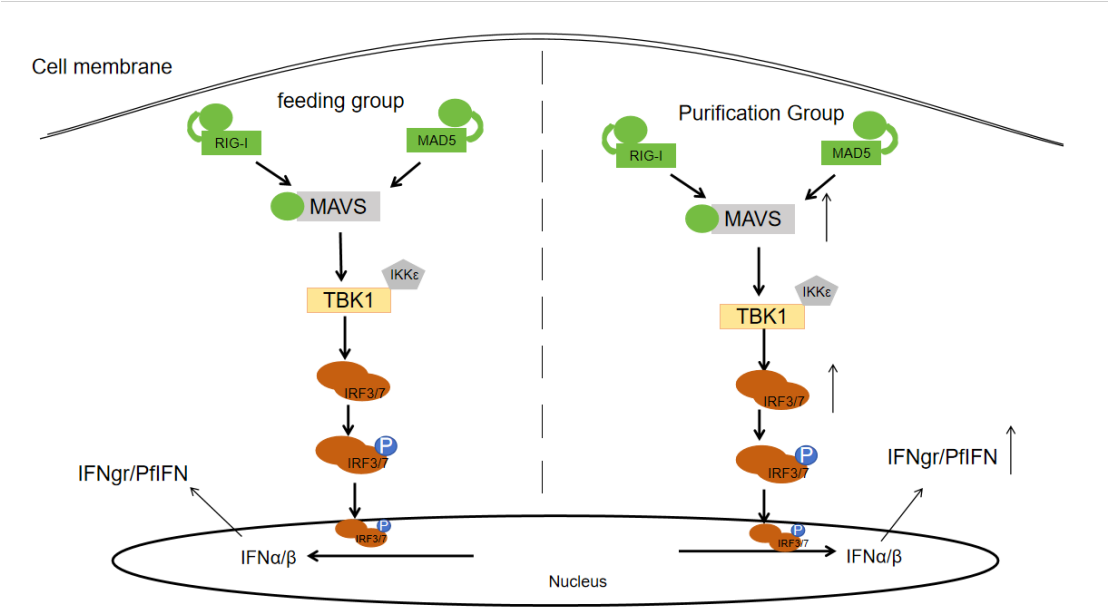

Figure 6. Signal transduction pathway diagram

Conclusions

This study investigates the impact of mountain spring water purification on the immune function of yellow catfish. By comparing the immune gene expression and histopathological characteristics of the group fed with purified water to those of the group fed with untreated water, it was determined that the purification process significantly enhanced the immune capacity of the yellow catfish.In both muscle and intestinal tissues, the expression levels of immune genes such as IRF3, IRF7, IFNγ, and PfIFN were significantly increased in the purified group, indicating activation of their immune systems.Additionally, the lipid metabolism capacity of muscle and intestinal cells in the purified group was enhanced, as evidenced by a reduction in lipid droplet numbers and more regular cell morphology.This is associated with the expression of genes that regulate lipid metabolism, following the activation of interferon signaling pathways. The study suggests that purifying mountain spring water by enhancing its quality and altering the structure of aquatic microbial communities can activate immune signaling pathways, which in turn promotes interferon production and thereby improves the immune function and lipid metabolism of yellow catfish.This model provides new insights into the healthy farming of yellow catfish and holds significant application value.

Author contributions

Data Collation: Qibiao Xiao, Juzhe Wu. Official Analysis: Qibiao Xiao, Juzhe Wu, Xingchen Li,Shengao Xiao,Junhong Liu. Resources: Xingchen Li,Shengao Xiao, Zhuoqi Ying,Zhang Chi. Software: Qibiao Xiao, Junzhe Wu. Supervision: Zhuoqi Ying. Verification: Zhang Chi, exceptional. Draft Writing: Qibiao Xiao, Junzhe Wu, Xingchen Li, Shengao Xiao, Junhong Liu. Review and Editing: Zhang Chi, Zhuoqi Ying.

Declaration of conflict of interest

The authors declare that they have no conflict of interest in this work.

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