Effects of Chinese herbal medicines mixture on antioxidant, immunity and disease resistance against infectious hematopoietic necrosis virus infection in rainbow trout (Oncorhynchus mykiss)

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Abstract

Chinese herbal medicine (CHM) has attracted widespread attention due to its natural, non-toxic, and low side-effect properties. Furthermore, Chinese herbal medicines mixture (CHMM) is often considered to have more beneficial effects than a single CHM. The present study was conducted to evaluate the effects of CHMM on antioxidant, immunity and disease resistance against infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*). The results showed that the total superoxide dismutase (T-SOD), catalase (CAT), acid phosphatase (ACP), alkaline phosphatase (AKP) activities of rainbow trout were significantly enhanced (*P* < 0.05) and the malondialdehyde (MDA) content was significantly reduced (*P* < 0.05) after feeding with CHMM. Meanwhile, the expression of immune and antiviral related genes (NF-KB, TNF-α, IL-1β, IL-8, MDA5, LGP2, IRF-3, IRF-7, IFN1, JAK1, STAT1, TLR3, TLR7, MYD88 and TGFβ) of rainbow trout were down-regulated after feeding with CHMM. After infected IHNV, the results showed that the all CHMM treatment groups increased Antioxidant and immune-related enzyme activities (T-SOD, CAT, ACP, AKP), while significantly reduced (*P* < 0.05) the MDA content. The expression of NF-KB, TNF-α, IL-1β, IL-8, MDA5, LGP2, IRF-3, IRF-7, IFN1, JAK1, STAT1, TLR3, TLR7, MYD88 and TGFβ were up-regulated by CHMM. In summary, based on the current experimental conditions, the CHMM has been discovered to effectively improve the antioxidant, immune, and disease resistance capacities of rainbow trout and the recommended dosage of CHMM supplementation for rainbow trout is approximately 30 g/kg.

Introduction

Rainbow trout (*Oncorhynchus mykiss*), a genus of salmonidae, is one of the economically cold-water fish species recognized worldwide, with global inland aquaculture production exceeding 700 thousand tons (FAO, 2022), and its production is growing rapidly. However, one of the most significant problems arising from the rapid expansion of aquaculture is the outbreak of bacterial and viral diseases. As fish are reared in high densities in aquaculture facilities, the close proximity and limited space provide an ideal environment for the rapid transmission and spread of diseases, making them highly vulnerable to infection, which severely restricts the development of trout farming (Chen et al., 2014; Hoseinifar et al., 2016). Infectious hematopoietic necrosis virus (IHNV) belongs to the genus *Novirhabdovirus* within the family *Rhabdoviridae*, is one of the causes of rainbow trout high mortality. In recent years, IHNV has become a major disease in rainbow trout farming and has caused significant economic losses to the industry. Traditionally, Vaccines are considered to be a primary option for prevent viral diseases, however, their use is still limited by economic and time costs (Adelmann et al., 2008). Antibiotics and synthetic chemical drugs are mainly used to prevent and control disease, but long-term use of these drugs will lead to drug resistance in pathogenic bacteria and excessive drug residues in aquatic animals, which will eventually affect human health (Mohan et al., 2019). The growing emphasis on food safety and quality has led to the popularization of green and pollution-free aquatic products. To meet these needs, environmentally friendly feed additives are needed to ensure the continued health of the aquaculture industry.
Chinese herbal medicines (CHM) have gained significant popularity in many developing countries due to their minimal toxic side effects, low cost, and easy availability (Ahn, 2017; Sasidharan et al., 2017). Meanwhile, the unique natural structure and biological activity of CHM have obvious effects in promoting animal growth, regulating metabolism, improving immunity, and enhancing disease resistance (Zhou et al., 2019). In general, it is believed that Chinese herbal medicines mixture (CHMM) has better therapeutic effects for animal diseases than single ones (Ngo et al., 2015), which are attributed to the synergistic effect created between the CHM (Zhang et al., 2012). The utilization of herbal medicine as a feed additive in aquaculture has gained significant attention in recent years. Studies have shown that CHM such as Astragalus radix, Angelica sinensis, and Hawthorn can improve the immunity and reduce the morbidity and mortality of Nile tilapia (Abarike et al., 2019). By using Massa Medicata Fermentata, Fructus Aurantii, Codonopsis pilosula, and other traditional Chinese medicines, the nonspecific immune and antioxidant capacity of European eel can be significantly improved (Huang et al., 2020). Additionally, the addition of different levels of ginkgo biloba leaf extract to the diet could significantly improve the anti-stress and antioxidant capacity of Nile tilapia (Abdel-Latif et al., 2021). These findings underscore the potential of CHM to improve the overall health and well-being of fish. Furthermore, one of the key reasons behind the effectiveness of CHM in aquaculture lies in its active ingredients. For instance, polyphenols are known for their antioxidant properties. They aid in neutralizing detrimental free radicals in the body, thereby reducing oxidative stress and averting cellular damage. In addition, saponins have been discovered to enhance the immune response in fish. They stimulate immune cell production and enhance the activity of immune-related enzymes, ultimately bolstering the fish's ability to combat pathogens (Kalyanaraman, 2013). Flavonoids, alkaloids, and coumarins are other active ingredients found in herbal medicines that contribute to their antiviral effects. These compounds have been shown to inhibit viral replication and prevent the spread of viruses within the fish population (Francis et al., 2002; Yan et al., 2020; Fichi et al., 2016). By incorporating CHM into the fish's diet, the immune system of the fish can be strengthened and the disease resistance can be improved (Liu et al., 2020). Therefore, the utilization of CHMM as a feed additive plays a crucial role in enhancing fish immunity and disease resistance.

Fish gills not only facilitate gas exchange, but also perform functions like ion exchange, acid-base regulation, and excretion of nitrogenous waste (Evans and Nunez, 2015). In addition, the gills also have barrier functions, including microbial barriers, chemical barriers, physical barriers and immune barriers. Among them, the immune barrier in fish is mediated by various cytokines (pro-inflammatory factors, growth transformation factors, anti-inflammatory factors and interleukins, etc.), which can respond critically to various challenges. Therefore, the gills are also one of the important immune organs of fish (Chen et al., 2023). However, the effects of CHMM on immune response and disease resistance in rainbow trout gill have not been known. In this study, we evaluated the effects of adding different proportions of CHMM on antioxidants, immunity, and resistance to IHNV in rainbow trout gills. This study provides scientific basis for the CHMM to improve the immunity and disease resistance of rainbow trout.

**Material and methods**
Fish and rearing condition

Rainbow trout (30 ± 0.5 g) were obtained from Aquatic Science Training Center of Gansu Agricultural University (Gansu, China). Before the experiment, the fish are placed under experimental conditions for 2 weeks and fed basal diet during this phase. Thereafter, a total of 12 identical cylindrical aquariums (four treatments in triplicates) have been used, each randomly stocked with 10 rainbow trout. In order to ensure water quality parameters, 1/3 of the water in the tank is drained every day. Water quality parameters such as temperature, dissolved oxygen and pH were carefully monitored at 12.0 ± 1.0°C, 8.5 ± 0.5 mg/L and 7.3 ± 0.3, respectively. Fish are fed three times a day (at 08:00, 13:00 and 18:00 hours) until they feel satiety. Extra care is taken into account to avoid feed loss. The feeding trial lasted 7 weeks.

Experimental feeds

The basic feed formulation and approximate components of rainbow trout are shown in Table 1. The basic process of the experiment is shown in Fig. 1. As shown in Table 2, a total of ten Chinese medicinal materials are combined into one CHMM, after undergoing an ultra-fine grinding process, the silk is sifted through 100 mesh, evenly mixed based on the same mass ratio. The CHMM were added to the basic feed in the proportions of 0 (control group), 10, 20 and 30 g/kg, mixed evenly, and pelleted feed with a particle size of 1.5 mm was formed by the granulation mechanism and stored in 4°C refrigerator for until use.
Table 1
Composition and proximate analysis of the basal diet (g per 100 g dry matter).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>45</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>20</td>
</tr>
<tr>
<td>Flour</td>
<td>10</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>10</td>
</tr>
<tr>
<td>Fish oil</td>
<td>5</td>
</tr>
<tr>
<td>Lysine</td>
<td>3</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
</tr>
<tr>
<td>Premix</td>
<td>3</td>
</tr>
<tr>
<td>Binder</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>

Composition Proximate analysis (%)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>48</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>10</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>5</td>
</tr>
<tr>
<td>Ash</td>
<td>4</td>
</tr>
<tr>
<td>Moisture</td>
<td>10</td>
</tr>
</tbody>
</table>

Note: premix, amounts per kilogram of diet: vitamin A, 450000 IU; vitamin D3, 200000 IU; vitamin E, 5000 mg; vitamin K3, 550 mg; vitamin B1, 500 mg; vitamin B2, 1000 mg; vitamin B6, 550 mg; vitamin B12, 1.5 mg; vitamin C, 15000 mg; folic acid, 180 mg; niacin amide, 3500 mg; calcium pantothenate, 2000 mg; biotin, 8 mg; manganese, 2500 mg; copper, 6500 mg; ferrous, 4500 mg; zinc, 5500 mg; selenium, 500 mg; iodine, 2500 mg; cobalt, 550 mg; choline chloride, 115000 mg.
### Table 2
Use of Chinese herbal medicine and their main functions in this study.

<table>
<thead>
<tr>
<th>Medicinal herbs</th>
<th>Plant part</th>
<th>Family</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Astragalus radix</em></td>
<td>Roots</td>
<td>Leguminosae</td>
<td>Antiviral, antioxidant, immunomodulatory.</td>
<td>Huang et al., 2020</td>
</tr>
<tr>
<td><em>Codonopsis pilosula</em></td>
<td>Roots</td>
<td>Campanulaceae</td>
<td>Immunomodulatory, antioxidant, antiviral, anti-inflammatory.</td>
<td>Pu et al., 2017</td>
</tr>
<tr>
<td><em>Angelica sinensis</em></td>
<td>Roots</td>
<td>Apiaceae</td>
<td>Anti-inflammatory, immune-boosting.</td>
<td>Elumalai et al., 2020</td>
</tr>
<tr>
<td><em>Glycyrrhiza uralensis Fisch</em></td>
<td>Roots</td>
<td>Fabaceae</td>
<td>Immunomodulatory, anti-inflammatory, antioxidant.</td>
<td>Wang et al., 2019</td>
</tr>
<tr>
<td><em>Ophiopogon japonicus</em></td>
<td>Earthnut</td>
<td>Liliaceae</td>
<td>Anti-inflammatory, antioxidant, immunomodulatory.</td>
<td>Huang et al., 2020</td>
</tr>
<tr>
<td><em>Poria cocos</em></td>
<td>Sclerotium</td>
<td>Polyporaceae</td>
<td>Anti-inflammatory, immunomodulatory.</td>
<td>Gong et al., 2014</td>
</tr>
<tr>
<td><em>Lonicera japonica</em></td>
<td>Flower bud</td>
<td>Caprifoliaceae</td>
<td>Anti-inflammatory, antiviral, antioxidant.</td>
<td>Van et al., 2020</td>
</tr>
<tr>
<td><em>Isatidis radix</em></td>
<td>Roots</td>
<td>Brassicaceae</td>
<td>Antiviral, anti-inflammatory.</td>
<td>Zhou et al., 2013</td>
</tr>
<tr>
<td><em>Folium isatidis</em></td>
<td>Leaves</td>
<td>Brassicaceae</td>
<td>Antiviral, anti-inflammatory, immunomodulatory, antioxidant.</td>
<td>Zhu, 2020</td>
</tr>
<tr>
<td><em>Hawthorn</em></td>
<td>Fruits</td>
<td>Rosaceae</td>
<td>antioxidant.</td>
<td>Stratev et al., 2018</td>
</tr>
</tbody>
</table>

## Infection with IHNV

Prior to starting this experiment, the 50% lethality rate (LD50) of rainbow trout was determined based on the same batch of fish. Specifically, five groups of 10 fish each with three replicates per group were injected intraperitoneally with 100 µL of IHNV at a concentration of $1.0 \times 10^2$ to $1.0 \times 10^6$ PFU/ml. Mortality was measured daily for 10 days to determine the appropriate IHNV concentration in the experiment. The LD50 was calculated as $5 \times 10^3$ PFU/ml.

After 5 weeks of the CHMM feeding, all tested rainbow trout were injected intraperitoneally with 100 µl of cell culture medium containing $5 \times 10^3$ PFU/ml of IHNV and other conditions remain unchanged.

## Sampling

Fish fasted for 24 hours prior to sampling. At weeks 1, 3, 5, 7 of feeding, 3 randomly selected rainbow trout per group were completely anesthetized with Methane-Sulfonate-222 (MS-222, Sigma, USA), subsequently, the gill were rapidly excised, quickly transferred to liquid nitrogen and frozen, stored in −80°C freezer until analysis.
Gill biochemical parameters

First, the rainbow trout gill tissue stored in the −80 °C refrigerator were taken out, crush it with a freezer grinder, then add 1:9 saline according to the mass volume ratio, mix well, centrifuged at 4 °C and 4000 r/min for 10 min. The supernatant is then aspirated and the relevant biochemical parameters are detected. The total superoxide dismutase (T-SOD) activity in the gills is determined by the hydroxylamine method. The amount of T-SOD per milligram of tissue protein is calculated when the T-SOD inhibition rate reaches 50% in 1 ml of reaction solution. The malondialdehyde (MDA) content in the gills is assessed through condensation with thiobarbituric acid (TBA), resulting in the formation of red products. At 532 nm, there is a peak absorption rate. The TBA method is named after its substrate, thiobarbituric acid (TBA). The catalase (CAT) content in the gills is determined using the ammonium molybdate method. This method involves adding ammonium molybdate to rapidly stop the reaction of catalase decomposing H₂O₂. The remaining H₂O₂ then reacts with ammonium molybdate to form a yellowish complex, which is measured at 405 nm to determine its content. Both acid phosphatase (ACP) and alkaline phosphatase (AKP) levels in the gills were determined using a colorimetric method. ACP and AKP enzymes break down benzo disodium phosphate, resulting in the production of free phenol and phosphoric acid. In an alkaline solution, phenol reacts with potassium ferricyanide oxidation to generate red quinone derivatives. The depth of the red color indicates the level of enzyme activity. The enzyme activity levels were measured using commercial kits obtained from the Nanjing Jiancheng Institute of Bioengineering in Nanjing, Jiangsu, China.

Relative mRNA expression of Gill immune-related genes

Trizol (Invitrogen, CA, USA) was used to extract total RNA from gills of rainbow trout. The integrity of RNA was determined by 1% agarose gel electrophoresis, while the purity and concentration of RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was removed from RNA using DNase. cDNA was synthesised using the Evo M-MLV Reverse Transcription Kit (Accurate Biology, Changsha, China) following to the manufacturer’s instructions. The expression patterns of 16 immune and antiviral-related genes were analyzed using qRT-PCR. mRNA expression of these genes was quantified by qPCR using SYBR green Master I (Roche, Basel, Switzerland). The qPCR primers are listed in Table 3. Real-time PCR conditions are as follows: 95°C for 30 sec, then 40 cycles at 95°C for 5 sec, and 60°C for 30 sec (LightCycler®480 II, Bioplastics, Roche). Three biological replicates per group were measured and all samples were performed in triplicate. The expression levels of the target genes were normalized to β-actin and expressed as the relative expression ratio. The 2^{-ΔΔCt} method was used to analyze the relative expression levels of immune-related genes.
<table>
<thead>
<tr>
<th>Names</th>
<th>GenBank accession no.</th>
<th>Sequence (5′-3′)</th>
<th>TM (°C)</th>
<th>Amplicon (pb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB</td>
<td>XM_021600117.2</td>
<td>F-CCACAGAACAAGCAGCAT  R-GGTGTTCCACCTGCTCTACT</td>
<td>58.5</td>
<td>139</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_001124357.1</td>
<td>F-ACCCACCATACATTGAAGCAGA  R-GGTGTCAGCAGGAAAGATTAGGA</td>
<td>57</td>
<td>94</td>
</tr>
<tr>
<td>IL-1β</td>
<td>NM_001124347.2</td>
<td>F-CGTCACATTGCCAACCTCATC  R-CAGGTCTTTGTCCTTGAACCTCG</td>
<td>57.5</td>
<td>74</td>
</tr>
<tr>
<td>IL-8</td>
<td>NM_001124362.1</td>
<td>F-ATTGAGACGGAAAGCAGACGA  R-AATGACCCCTCTTGACCACCG</td>
<td>59.8</td>
<td>118</td>
</tr>
<tr>
<td>MDA5</td>
<td>NM_001195179.1</td>
<td>F-TTGATAGATGAGCCAGGTCC  R-TTCCATTGTTGTTCTTGCC</td>
<td>59.5</td>
<td>173</td>
</tr>
<tr>
<td>LGP2</td>
<td>XM_021624830.2</td>
<td>F-GCAGACCTTTGGGCTGTATGAG  R-CCAGGTAGGGATTGAACTCGT</td>
<td>59</td>
<td>181</td>
</tr>
<tr>
<td>IRF3</td>
<td>NM_001257262.1</td>
<td>F-CAAGGGCGTGGGCTGAGGTTA  R-GTGGTGTTGGGGTGCTGC</td>
<td>56.8</td>
<td>90</td>
</tr>
<tr>
<td>IRF7</td>
<td>XM_021600499.2</td>
<td>F-AACGACCCCCCATAAAGTCTACC  R-AGGGGGTTCAAGGTAGGT</td>
<td>57.1</td>
<td>135</td>
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<tr>
<td>IFN1</td>
<td>XM_021624606.2</td>
<td>F-ACAGAATGCCGCCAGTCTTT  R-CTTCTGTCAGGTACGAG</td>
<td>59.8</td>
<td>146</td>
</tr>
<tr>
<td>JAK1</td>
<td>XM_036966729.1</td>
<td>F-GGCTGTGGCACCAGAACTAA  R-TTGGGACCAGCAGATT</td>
<td>58.0</td>
<td>90</td>
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<tr>
<td>STAT1</td>
<td>NM_001124707.1</td>
<td>F-TGAGTAAGGAGGAAGGGAAGGC  R-CAGGCTATTGTATTGGTCG</td>
<td>58.7</td>
<td>88</td>
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<tr>
<td>SOCS2</td>
<td>XM_021616321.2</td>
<td>F-TTCTCATCTTTGGAGGTAAGTGTT  R-AGGAGGTAGCCCTGGTGGAGAT</td>
<td>59.5</td>
<td>93</td>
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<tr>
<td>TLR3</td>
<td>NM_001124578.1</td>
<td>F-CAGACCTTTGTCCTCCTCG  R-CAGCAGAAGGGTGTTGGA</td>
<td>55.9</td>
<td>82</td>
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<tr>
<td>Names</td>
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<td>TM (°C)</td>
<td>Amplicon (pb)</td>
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<td>-------</td>
<td>----------------------</td>
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<tr>
<td>TLR7</td>
<td>XM_021598013.2</td>
<td>F-CTGTGACCTGTGCTGCTCCA R-TGGAGATGGAGAGGGCGAT</td>
<td>59.8</td>
<td>114</td>
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<tr>
<td>MYD88</td>
<td>NM_001124421.1</td>
<td>F-GAGACAGAATAGAGCCCCCA R-ACTTGTGGTCGTCTGCTC</td>
<td>56.4</td>
<td>162</td>
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<tr>
<td>TGF-β</td>
<td>XM_021591332.2</td>
<td>F-AGATGCGGTCAGTATTGGGG R-CAAAATGGGACTTCAGGTAACG</td>
<td>59.5</td>
<td>111</td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_001124235.1</td>
<td>F-ACGCCCTTCCCCCATGCTAT R-CGAACGATTTCCTCGCTCTGC</td>
<td>58.1</td>
<td>122</td>
</tr>
</tbody>
</table>

**Statistics**

Data analysis was performed using SPSS software version 26.0 (IBM Corp, Armonk, NY, USA). The statistical significance of the differences between treatments was determined using one-way analysis of variance followed by Duncan's multiple-range test. In this experiment, a significance level of \( P < 0.05 \) was considered to indicate statistical significance. The results are presented as mean ± standard deviation (SD).

**Results**

**Gill biochemical parameters**

As shown in Fig. 2, compared with the control group, the gill T-SOD activity significantly increased \( (P < 0.05) \) in the 10, 20, and 30 g/kg CHMM groups at 1, 3, and 5 weeks. There were significant differences \( (P < 0.05) \) observed among all CHMM treatment groups at 3 and 5 weeks, with the highest value found in the 20 g/kg CHMM treatment group. Compared with the control group, the gill MDA content in all CHMM treatment groups showed a tendency to decrease at 1, 3, and 5 weeks. However, the differences among all CHMM treatment groups were not statistically significant \( (P > 0.05) \). Compared with the control group, the gill CAT activity was observed to have increased \( (P < 0.05) \) in all CHMM treatment groups at weeks 1, 3, and 5. The 30 g/kg CHMM treatment group exhibited the highest peak value. The ACP and AKP activities in the gill of the CHMM treatment groups were significantly higher \( (P < 0.05) \) compared to the control group at 1, 3, and 5 weeks. Among the CHMM treatment groups, significant differences \( (P < 0.05) \) were observed, with the 30 g/kg group showing the highest ACP activity and the 20 g/kg group exhibiting the highest AKP activity.

**Gill immune-related genes expression**
As depicted in Fig. 3, the relative expression levels of NF-κB exhibited a significant decrease ($P<0.05$) in all CHMM treatment groups, when compared to the control group. However, there was no significant difference ($P>0.05$) observed among the CHMM treatment groups. Compared with the control group, the relative expression levels of TNF-α, IL-1β, and IL-8 significantly changed ($P<0.05$) in the 10, 20, and 30 g/kg CHMM treatment groups. The 30 g/kg CHMM treatment group exhibited the lowest expression level.

Compared with the control group, the expression of MDA5 and LGP2 showed a significant decrease ($P<0.05$) as the dietary CHMM levels increased. The lowest expression level was observed in the gill of the 30 g/kg group. The relative expression levels of IRF3 and IRF7 were significantly altered ($P<0.05$) in the CHMM treatment groups ($P<0.05$). The gill showed the lowest expression level in the 20 g/kg and 30 g/kg CHMM groups, respectively. Compared with the control group, the relative expression levels of IFN1, JAK1, and STAT1 genes in CHMM groups were significantly altered ($P<0.05$). The 20 g/kg group exhibited the lowest expression level. The relative expression level of the SOCS2 gene in CHMM groups showed no significance ($P>0.05$). Additionally, there were no significant differences ($P>0.05$) among all CHMM treatment groups in the gill. Compared with the control group, the CHMM treatment groups showed a significant decrease ($P<0.05$) in the relative expression of TLR3, TLR7, and MYD88 genes, with the 30 g/kg group exhibiting the lowest expression level. The relative expression levels of TGFβ were not found to be significant ($P>0.05$) in the 10 and 20 g/kg CHMM treatment groups compared with the control group. However, in the 30 g/kg CHMM treatment group, there was a significant decrease ($P<0.05$).

**Gill Biochemical parameters after infected IHNV**

As shown in Fig. 4, the gill T-SOD activity was found to be higher in all CHMM groups when compared to the control group. It reached its peak in the 30 g/kg CHMM group. Additionally, the MDA content showed a significant decrease ($P<0.05$), reaching its lowest value in the 30 g/kg CHMM group. The gill CAT and AKP activities were noticeably increased ($P<0.05$) in comparison with the control group. On the other hand, the gill ACP activities showed an increase, but the difference was not statistically significant ($P>0.05$) when compared with the control group.

**Gill immune-related genes expression after infected IHNV**

As shown in Fig. 5, the expression level of NF-κB, TNF-α, IL-1β, and IL-8 in rainbow trout was significantly up-regulated ($P<0.05$) after IHNV infection when supplemented with CHMM diets. In rainbow trout infected with IHNV, the CHMM group exhibited up-regulated ($P<0.05$) expression levels of MDA5, LGP2, IRF3, and IRF7 compared to the control group. Compared with other CHMM treatment groups, the expression of MDA5, LGP2, IRF3 and IRF7 related expressions reached the maximum in the 10g/kg CHMM treatment group. The CHMM treatment groups significantly up-regulated ($P<0.05$) the relative expression lever of IFN1, JAK1, STAT1 and SOCS2 in rainbow trout after infected IHNV. Compared with the control group, the expression levels of IFN1, JAK1, and STAT1 were highest in the 10 g/kg CHMM group. Additionally, the expression levels of SOCS2 were highest in the 30 g/kg CHMM group. In addition, IFN1, JAK1 and SOCS2 also had significant differences among the treatment groups of CHMM. The CHMM group significantly up-regulated ($P<0.05$) the relative expression lever of TLR3, TLR7, MYD88 and TGFβ.
in rainbow trout gill after infected IHNV. Compared with the control group, the expression levels of \textit{TLR3}, \textit{TLR7}, and \textit{MYD88} peaked in the 30 g/kg CHMM group. In addition, the expression level of \textit{TGF\beta} decreased with the increase of CHMM does.

**Discussion**

The body's non-specific immune and antioxidant capacity not only reflects the health status of the animal, but also has a close relationship with the survival rate of the animal. Many studies have reported that CHM are not only rich in antioxidants, such as polyphenolic compounds, flavonoids, vitamins C and E, etc. These substances have an antioxidant effect, which can neutralize free radicals and reduce oxidative damage. Moreover, some CHM can enhance the vitality of immune cells, promote the proliferation and differentiation of immune cells, thereby enhancing the body's immune function. At the same time, Chinese herbal medicines can also regulate the secretion function of immune cells, increase the antioxidant substances produced by immune cells, and improve the antioxidant capacity of the body. This regulatory effect helps balance the immune system and enhance the body's ability to adapt to the external environment (Huang et al., 2020; Van Hai, 2015). In this experiment, we observed that CHMM significantly increased the antioxidant and non-specific immune-related enzyme activity of rainbow trout, and also significantly changed the expression of immune and antiviral related genes.

Oxidative stress in the organism is caused by the excessive production of oxidation products, which leads to a disruption of the intracellular redox balance and thus causes a series of harmful reactions (Abdel-Daim et al., 2019; Martínez-Álvarez et al., 2005). Fish, on the other hand, scavenge these oxidative products mainly by enhancing the activity of antioxidant enzymes. As an important antioxidant enzyme in the body, SOD has the effect of scavenging harmful superoxide anion free radicals in the body. Generally, its activity reflects the body's antioxidant capacity (Winston, 1991). Previous studies have shown that adding CHMM to feed significantly increases the SOD activity and enhances the antioxidant capacity of European eel (Huang et al., 2020). As the end result of lipid peroxidation, MDA induces cross-linking and polymerization of vital macromolecules like proteins and nucleic acids, resulting in cytotoxicity. Therefore, its content is generally related to the body's oxidative stress response. CAT is widely distributed in organisms, which can remove free radicals from the body, and maintain normal body function. In this study, the dietary supplement with CHMM significantly increased SOD and CAT activities, while decreasing MDA content in the gill of rainbow trout. Similarly, the addition of CHM to the feed significantly increased SOD and CAT activities of yellow catfish (Wang et al., 2020); The addition of curcumin to the diet significantly increased SOD activity and reduced MDA content in the liver of grass carp after injection with \textit{A. hydrophila} (Ming et al., 2020). After infected IHNV, the activity of antioxidant enzymes T-SOD and CAT increased in rainbow trout fed with all CHMM treatment groups, while the content of MDA significantly decreased. The results demonstrated that the CHMM decreased oxidative damage and improved the antioxidant capacity of rainbow trout by scavenging free radicals generated within the body.
ACP and AKP play crucial roles in the immune response and are often used as important indicators for evaluating the immune status of organisms. They also participate in the animal immune response as important metabolic regulating enzymes, promoting the absorption and utilization of nutrients in fish, and improving the immunity and disease resistance of fish (Liu et al., 2022; Liu et al., 2021; Liu et al., 2020). Previous study has shown that the addition of astragalus extract to feed significantly improved ACP and AKP activity in large yellow croaker (Wu et al., 2019). Similarly, feeding Allium mongolicum Regel flavonoids significantly increased AKP activity in juvenile northern snakehead fish (Li et al., 2019); The addition of Radix Rehmanniae Preparata polysaccharides to the feed significantly increased serum ACP and AKP activity in Luciobarbus capito (Wu et al., 2019). In the current study, the dietary supplement with CHMM significantly increased ACP and AKP activities in rainbow trout. The results of this study were similar to previous. After infected IHNV, the activities of ACP and AKP in the CHMM group were improved. The results suggest that the CHMM have the ability to enhance both the immune and the metabolic functions of infected rainbow trout.

The inflammatory response is the process by which the immune system recognizes and eliminates external stimuli (e.g., immunostimulants, pathogens, damaged cells, toxic compounds, or radiation) and is primarily mediated by cytokines (Han and Ulevitch., 2005). As the main transcription factor that coordinates the expression of inflammation-related genes, NF-κB activation can promote the release of pro-inflammatory factors, which in turn promotes inflammatory response. IL-1β, IL-8 and TNF-α are pro-inflammatory cytokines, and their expression levels are important indicators to measure whether inflammatory response occurs. Previous study showed that Allium mongolicum Regel can downregulate the expression of NF-κB p65, IL-1β, IL-8 and TNF-α (Li et al., 2018). Similarly, Astragalus Propinquus Schischkin polysaccharides significantly reduced IL-1, TNF-α, IL-8, NF-κB p65 expression levels in the liver, spleen, kidney, and intestines of Channa argus (Zhu et al., 2021). The study's results revealed that the expression levels of NF-κB were notably reduced in all CHMM groups, as compared with the control group. Additionally, the expression of IL-1β, IL-8, and TNF-α showed significant decrease in the 20 g/kg CHMM group when compared with the control group. The results indicate that CHMM may hinder the TLRs-Myd88-NF-κB signaling pathway and RLR signaling pathway in rainbow trout, thereby decreasing the inflammatory response. After infected IHNV, the CHMM groups could significantly increase the expression of NF-κB, TNF-α, IL-1β and IL-8 genes. This suggests that CHMM have a dual effect on the immune response in rainbow trout. On one hand, they can inhibit the pro-inflammatory response, which is beneficial in preventing excessive inflammation and tissue damage. On the other hand, when the fish are infected with IHNV, the CHMM seem to stimulate the expression of pro-inflammatory factors, potentially enhancing the immune response against the virus.

RLRs are cytoplasmic RNA helicases, which include MDA5 and LGP2, and play a vital role in the host's antiviral response (Hiscott et al., 2006). MDA5 recognizes pathogen-associated molecular patterns (PAMPs), which results in the activation of interferon regulatory factors (IRF-3, IRF-7). This process eventually triggers the activation of different antiviral genes, including interferon and pro-inflammatory factors. These genes work to hinder the virus's replication and transmission (Chen and Jiang, 2012; Saito et al., 2007). The study results demonstrated a significant decrease in the expression levels of MDA5,
LG2, IRF3, and IRF7 in the CHMM treatment groups, as compared to the control group. The findings indicated that the CHMM may impede the signaling pathway of the RIG-I-like receptors and the expression levels of genes related to antiviral responses. After being infected with IHNV, the CHMM effectively enhances the expression of antiviral-related genes MDA5 and LG2, and also activates the expression of interferon-releasing factors IRF3 and IRF7. These results demonstrate that CHMM effectively boosts the immunity of rainbow trout infected with IHNV and improves their ability to fight against viruses.

IFN1 belongs to the cytokine family and possesses potent antiviral and immunomodulatory properties. It exerts its antiviral function by inducing activation through IRF3 and IRF7, and by inducing the expression of downstream genes via the protein tyrosine kinase-transcription activator (JAK-STAT) pathway (Sato et al., 2000; Honda et al., 2005; Xu et al., 2010). SOCS negatively regulates the JAK-STAT pathway, specifically inhibiting the kinase activity of JAKs, thereby inhibiting the phosphorylation of STAT (Shan et al., 2023). In this study, the CHMM effectively inhibited the expression of interferon regulatory factors (IRF-3, IRF-7), resulting in a significant decrease in IFN1 and inhibited the activation of the JAK-STAT pathway. However, after infected IHNV, compared with the control group, the expression of IFN1, JAK1 and STAT1 was significantly increased. SOCS2 showed a downward trend, but not significant. This result indicated that the CHMM also promoted the improvement of rainbow trout’s immunity. The above results demonstrate that CHMM has an immunoprotective effect and play a crucial role.

Toll-like receptors (TLRs) play an important role in the innate immune response, TLR3 and TLR7 are important pattern recognition receptors, which plays important role in recognizing pathogens in the receptor defense system. TLR can activate downstream signaling cascades upon specific ligand recognition, such as the MyD88-dependent pathway. This ultimately results in the release of inflammatory mediators and cytokines, playing a crucial role in anti-inflammatory and immunomodulatory processes. (Zhang et al., 2017; Thompson and Locarnini, 2007). In the present study, the CHMM could significantly decrease the expression of TLR3, TLR7 and MYD88 in gill of rainbow trout. These results were similar to previous studies (Bu et al., 2018; Sun et al., 2020). TGF-β is a crucial immunomodulatory cytokine that regulates the inflammatory response in fish by controlling the production of pro-inflammatory cytokines. (Hoseinifar et al., 2020). Previous studies have shown that different plant species, doses, and treatment times can lead to different expression patterns of TGF-β (Bilen et al., 2016; Reverter et al., 2016; Tan et al., 2017). The study results indicated that the expression of TGF-β remained unchanged in the 10 and 20 g/kg CHMM group. However, there was a significant decrease in the expression of TGF-β in the 30 g/kg CHMM group. These results indicated that the CHMM probably suppressed the expression of the anti-inflammatory factor TGF-β with the increase of dose. The findings above demonstrate that including CHMM in the diet can decrease the expression of pro-inflammatory factors and enhance the anti-inflammatory capacity of rainbow trout.

Conclusion
In summary, the findings of this study suggest that the CHMM can stimulate the immune response, boost antioxidant activity, and regulate the expression of genes related to immunity and antiviral defense in rainbow trout. Furthermore, the recommended dosage of the CHMM is 30 g/kg. These findings demonstrate the significant potential of the CHMM in enhancing fish immunity and provide a scientific basis for improving the resistance of rainbow trout to IHNV infection.

Declarations

Author contributions QW data curation, writing-original draft. JH conceptualization, funding acquisition, project administration, resources, supervision, writing-review and editing. YL conceptualization, project administration. SW writing-review and editing. LZ writing-review and editing. YP methodology, validation, formal analysis, investigation. YK writing-review and editing. ZL conceptualization, project administration.

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Data availability Data will be made available on request.

Ethics approval The animal study was reviewed and approved by the Animal Ethics Committee of Gansu Agricultural University, China.

Conflict of Interest The authors declare no conflict of interest.

References


Figure 1

Experimental design and timeline: The control group was designated as NC, while the compound Chinese herbal medicine groups were designated as NC+CHMM.
Figure 2

Effects of dietary CHMM on T-SOD (A), MDA (B), CAT (C), ACP (D), AKP (E) in the gill of rainbow trout, data are represented as mean ± S.D. with distinct superscript values significant ($P< 0.05$), determined by the Duncan's test.
Figure 3

Relative expression of NF-κB, TNF-α, IL-1β and IL-8 (A); MDA5, LGP2, IRF3 and IRF7 (B); IFN1, JAK1, STAT1 and SOCS2 (C); TLR3, TLR7, MYD88 and TGFβ (D) in the gill of rainbow trout fed diet supplemented with CHMM, data are represented as mean ± S.D. with distinct superscript values significant ($P < 0.05$), determined by the Duncan's test.
Figure 4

Effects of dietary CHMM on T-SOD (A), MDA (B), CAT (C), ACP (D), AKP (E) in rainbow trout gills after IHNV infection, data are represented as mean ± S.D. with distinct superscript values significant ($P < 0.05$), determined by the Duncan's test.
Figure 5

Relative expression of NF-κB, TNF-α, IL-1β and IL-8 (A); MDA5, LGP2, IRF3 and IRF7 (B); IFN1, JAK1, STAT1 and SOCS2 (C); TLR3, TLR7, MYD88 and TGFβ (D) in rainbow trout gills after IHNV infection, data are represented as mean ± S.D. with distinct superscript values significant ($P < 0.05$), determined by the Duncan’s test.