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Oligochitosan Mitigates *Vibrio harveyi* Infection in Hybrid Groupers (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀) by Modulating Immune Responses and Disease-Related Pathways

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Abstract: *Vibrio harveyi* is an important pathogen causing enteritis in hybrid groupers. However, its effects on the intestinal microbiota and the regulatory effects of oligochitosan on *Vibrio*-infected groupers are unclear. In this study, we investigated the effects of infection with *V. harveyi* and oligochitosan administration on the growth performance, immune responses, and intestinal transcriptome profiles of hybrid groupers during a 60-day rearing period. Growth parameters were reduced in *Vibrio*-infected groupers, while oligochitosan improved the body weight. Biochemical analysis showed reduced antioxidant enzyme activity and increased lipid peroxidation in *Vibrio*-infected groupers, which were consequently improved by oligochitosan. Transcriptome analysis was used to identify differentially expressed genes in the disease-related and immune system pathways that significantly accumulated in the treatment groups. An evaluation of the immune response showed a significant down-regulation of proinflammatory cytokines, antioxidant genes, and tight-junction proteins in *Vibrio*-infected groupers, which was partially restored by oligochitosan. Additionally, intestinal apoptosis, indicated by TUNEL signaling, was observed to significantly increase in *Vibrio*-infected groupers and was subsequently alleviated by oligochitosan supplementation. These results demonstrate the detrimental effects of *V. harveyi* infection on the growth and immune function of hybrid groupers and the potential of oligochitosan to attenuate these effects.

Keywords: fish diseases; feed additives; gut transcriptome; apoptotic activity

Key Contribution: *Vibrio harveyi* infection impairs hybrid grouper growth; oligochitosan boosts body weight and antioxidants. Transcriptome analysis indicates that *V. harveyi* modulates key genes in disease and immune response pathways. Oligochitosan supplementation reduces intestinal apoptosis, offering protection against *V. harveyi* infection.

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1. Introduction

Aquaculture is a key contributor to global fish production. It has recently exhibited rapid growth, playing a central role in the food industry and contributing to economic stability. However, its expansion has also increased pollution and the risk of disease outbreaks [1]. Various pathogenic diseases have led to significant mortality and economic losses in the aquaculture sector [2]. *Vibrio harveyi*, a Gram-negative marine pathogen, significantly threatens the health of various marine fish species by causing bacterial enteritis and sudden mortality and has become one of the major constraints on the sustainable growth of aquaculture [3,4]. In marine fish aquaculture, developing strategies to prevent and control vibriosis is crucial, and identifying key targets is essential for understanding the underlying mechanism of *Vibrio* infection in groupers.

Oligochitosan, which consists of 2-10 N-acetylglucosamine residues joined through β-1,4-glycosidic linkages, is predominantly derived from the chitin of marine crustaceans via the enzymatic or chemical deacetylation processes applied to chitosan [5]. Oligochitosan is a viable alternative to antibiotics in aquaculture due to its low molecular weight, improved intestinal absorption, non-toxicity, and water solubility. Marine-derived oligochitosan, which exhibits prebiotic effects and a range of bioactivities, such as antioxidant, immunomodulatory, and antimicrobial capabilities, has promising applications in biomedicine and agriculture [6,7]. Previous research revealed that oligochitosan affects growth, immune response, and resistance to bacterial infections in young rainbow trout (*Oncorhynchus mykiss*) [8], blunt snout bream (*Megalobrama amblycephala*) [9], and Nile tilapia (*Oreochromis niloticus*) [10].

The use of bacterial or viral infection models in conjunction with transcriptomic approaches can elucidate the disease resistance and immune-related mechanisms in aquatic organisms [11]. Previous studies have utilized transcriptomics to investigate the immune system gene expression in *Macrobrachium rosenbergii* hepatopancreas, lymphoid tissue, and intestinal tissue following exposure to *Vibrio parahaemolyticus*, white spot syndrome virus, and poly I:C infections [12–14]. Previous research on Chinese tongue sole (*Cynoglossus semilaevis*) revealed intricate gene regulatory networks in both resistant and susceptible families following infection with *V. harveyi* [15]. Consequently, transcriptome sequencing can delineate the immune mechanisms of host resistance to pathogen invasion upon *V. harveyi* infection and oligochitosan intervention and facilitate the identification of genes with significant immunoprotective functions, providing a reference for disease prevention and control.

The newly developed hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀), known for its rapid growth, nutrient abundance, and stress resistance, is an important species in aquaculture [16]. Studies show that vibriosis is the predominant disease in groupers, with mortality rates of up to 50%, and is particularly common in hybrid groupers, causing significant acute mortality [17,18]. However, the specific mechanisms used by *V. harveyi* to infect hybrid groupers are not yet fully understood. Our previous research has shown that oligochitosan significantly improves growth performance, enhances immune responses, and regulates the gut microbiome of hybrid groupers (*E. lanceolatu* ♂ × *E. fuscoguttatus* ♀) [19]. Nevertheless, the exact mechanisms by which oligochitosan regulates the immune response and intestinal transcriptome in fish with vibriosis infection remain to be thoroughly investigated.

In this study, we investigate the regulatory effects of *V. harveyi* on the comparative transcriptome profile of grouper immune responses and the intervening role of oligochitosan. The study aims to explore the growth parameters, biochemical indexes, and mechanisms regulating the dynamic balance of hybrid grouper immune responses and disease-related signaling pathways following *Vibrio* infection and oligochitosan intervention. The results provide a basis for applying oligochitosan and new insights into the health of marine fish.

2. Materials and Methods

2.1. Experimental Materials

Healthy juvenile hybrid groupers (*E. lanceolatu* ♂ × *E. fuscoguttatus* ♀) were sourced from the Guangdong Marine Fisheries Experimental Center. *V. harveyi* cultures were maintained in our laboratory. High-quality oligochitosan with a deacetylation degree exceeding 90% was purchased from Changlong Biotechnology in Huizhou, Guangdong. The base feed provided by Guangdong Yuequn Marine Biological Research and Development Co, Jieyang, China. consisted of a mixture of imported fish meal, squid meal, flour, lecithin, and enzymatically hydrolyzed proteins, as well as a range of vitamins and minerals.

2.2. V. Harveyi Challenge

Before the experiments, we first acclimatized 600 robust hybrid groupers (average weight 42.7 ± 3.2 g) for one week. The culture conditions for *V. harveyi* followed our

established protocols [20]. The *V. harveyi* X13SZ03 strain was cultured at 28 °C in Luria–Bertani broth, harvested, and re-suspended in sterile PBS (pH 7.2) for infection studies. Following pre-tests, we determined the semi-lethal concentration for hybrid groupers to be 1.01×10^6 CFU/g [19]. After anesthesia with MS-222, half of the groupers received an oral *V. harveyi* suspension at 4.5×10^4 CFU/g; the other half served as controls with PBS.

2.3. Experimental Design

Our previous study utilized an established optimal oligochitosan dosage of 800 mg/kg in hybrid grouper diets [19]. After a low-dose infection with *V. harveyi*, we divided 300 uninfected and 300 infected fish into four experimental groups. The uninfected fish were divided into a control diet group (CON) and an oligochitosan supplementation group (OCS), while the infected fish were divided into an infected control diet group (VH + CON) and an infected oligochitosan supplementation group (VH + OCS). Each group comprised three replicates with 30 fish each, and the experiment was conducted over six weeks.

Following the rearing period, the fish underwent a 24 h fasting regimen. Six groupers were then randomly selected per tank for the experiments. Upon anesthesia induction with 100 mg/L MS-222, the fish were subjected to intestinal tissue extraction; the tissues were subsequently disinfected using a 0.85% saline solution. Subsequently, the harvested tissues were immersed in a 4% paraformaldehyde solution for apoptosis assessment. For biochemical and transcriptomic analyses, the tissues were preserved in RNase-free cryotubes within liquid nitrogen at −80 °C.

2.4. Growth Performance

Throughout the experimental period, weight assessments were conducted at the start and end of each cohort. Detailed analysis was performed on a random sample of two individuals from each replicate with a total of six fish in one group, evaluating key parameters such as body weight, body length, weight gain rate, feed coefficient, relative fatness, and survival rate. These parameters were calculated as follows:

$$\text{Body weight} = \text{final weight} - \text{initial weight} \quad (1)$$

$$\text{Body length} = \text{final length} - \text{initial length} \quad (2)$$

$$\text{Weight gain rate} = (\text{final weight} - \text{initial weight})/\text{initial weight} \times 100 \quad (3)$$

$$\text{Feed coefficient} = \text{total feed intake}/(\text{final weight} - \text{initial weight}) \times 100 \quad (4)$$

$$\text{Relative fatness} = \text{body weight of the fish}/(\text{total length of the fish})^3 \quad (5)$$

$$\text{Survival rate} = \text{number of fish that survived}/\text{initial number of fish} \times 100 \quad (6)$$

2.5. Biochemical Analysis

Midgut samples were obtained from six fish per tank and processed by homogenization at a 10% dilution ratio using a 0.8% saline solution. The homogenates were then centrifuged at 3500 rpm for 10 min at a temperature of 4 °C. Total protein content in the intestinal homogenates was determined using the Bradford protein assay kit provided by Beyotime (Shanghai, China). The supernatant was promptly analyzed for biochemical markers with a microplate reader from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Enzymatic activities, including catalase (CAT, A007-1-1), total superoxide dismutase (T-SOD, A001-1-1), glutathione peroxidase (GSH-Px, A005-1-2), malondialdehyde (MDA, A003-1-1), lipase (LPS, A054-1-1), and amylase (AMS, C016-1-1), were quantified using specific commercial assay kits sourced from Jiancheng Bioengineering Research Institute Co Ltd, Nanjing, China, adhering to the manufacturer's recommended protocols.

2.6. RNA Extraction, cDNA Synthesis, and Transcriptomic Sequencing Analyses

Total RNA was extracted from hybrid grouper samples following the manufacturer's instructions with TRIzol reagent (Life Technologies, Carlsbad, CA, USA). RNA concentra-

tion and integrity were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). cDNA libraries were constructed following the NEBNext Ultra RNA Library Prep Kit manual. The libraries were sequenced on an Illumina Hi-Seq X Ten platform (OE Biotech Co., Ltd., Shanghai, China).

Low-quality sequencing reads, characterized by the presence of only adaptor sequences, more than 5% unknown nucleotides, or a Q20 score below 20%, were eliminated using a Perl script. This filtration process ensured the retention of high-quality reads, which were then mapped to the reference genome using TopHat2 software. Gene expression levels were quantified by calculating fragments per kilobase of exon per million fragments mapped (FPKM) values with the Cufflinks 2.2.0 software (https://cole-trapnell-lab.github.io/cufflinks/manual/, accessed on 19 October 2024). To identify differentially expressed genes (DEGs), q-values were utilized, and a stringent threshold was applied: genes with false discovery rate (FDR) significance score < 0.05 and an absolute value of log2 ratio ≥ 1 were selected for further analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses and heatmap visualization of the DEGs were performed using Perl scripts in the TopGo R package.

2.7. Expression of Immune-Related Genes Using qRT-PCR

Total RNA was extracted from the intestinal tissues of fish in various experimental groups using the TRIzol reagent (Sangon, Shanghai, China). The PrimeScript™ RT reagent kit (Takara Bio, Tokyo, Japan), supplemented with gDNA Eraser (Takara Bio, Tokyo, Japan), was employed for cDNA synthesis under the protocols provided by Takara (Dalian, China). The quality assessment and quantification of cDNA samples were conducted using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expression levels of interleukin-1β (*IL-1β*), interleukin-8 (*IL-8*), interleukin-10 (*IL-10*), catalase (*CAT*), glutathione peroxidase (*GPx*), claudin-3α (*CLDN-3α*), occludin (*OCLN*), Zonula occludens 2 (*ZO-2*), and zonula occludens 3 (*ZO-3*) were monitored by quantitative real-time PCR (qRT-PCR). As internal controls, *β-actin* and *18S rRNA* genes were used. All primers for the targeted genes are itemized in Table 1. qRT-PCR was conducted following the methodologies detailed in our previous study [21]. The experiments were conducted using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the specified conditions: 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 58 °C for 10 s, 72 °C for 10 s, and 4 °C for 5 min. The relative expression levels of the target genes were determined by normalizing against the housekeeping genes *β-actin* and *18S rRNA*. After amplifying the target gene and two internal reference genes to obtain Ct values, we calculated the ΔCt for each reference gene and then determined the average ΔCt. The relative expression of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method [22].

Table 1. Primer sequences for qRT-PCR analysis.

Gene	Primer	Sequence (5′-3′)
IL-1β	Forward	TACGATGCCTATGTGGTC
	Reverse	CTCTGCTTTATGCTGTCC
IL-8	Forward	GCCGTCAGTGAAGGGAGTCTAG
	Reverse	ATCGCAGTGGGAGTTTGCA
IL-10	Forward	TTCGACGAGCTCAAGAGTGAG
	Reverse	TGCCGTTTAGAAGCCAGATACA
CAT	Forward	GCGTTTGGTTACTTTGAGGTGA
	Reverse	GAGAAGCGGACAGCAATAGGT
GPx	Forward	TACCCTACCAAGTCCTCCAACC
	Reverse	AACAAACACCCGACACCCA
CLDN-3α	Forward	ACTCTATGCTCGCCCTCTCT
	Reverse	TGGATGCCTCGTCGTCA
OCLN	Forward	TCAGAACATCCAGGGCAATC
	Reverse	CCACCATCAGACCCAAAACT

Table 1. *Cont.*

Gene	Primer	Sequence (5′-3′)
ZO-2	Forward	CAAGATTCTCCTCCGACCA
	Reverse	AACATCATTACCTCCTGCCA
ZO-3	Forward	GAGCCAATCTACTCCCTTCC
	Reverse	CTGGTCTCCCTCTTTCATCC
β-actin	Forward	TACGAGCTGCCTGACGGACA
	Reverse	GGCTGTGATCTCCTTCTGC
18S rRNA	Forward	GCAATTATTCCCCATGAACG
	Reverse	GGTCGATCGAGGTCCTCAAA

2.8. Apoptosis Analysis

For the evaluation of apoptotic activity, we utilized the TdT-mediated dUTP nick-end labeling (TUNEL) assay. This technique was applied to paraformaldehyde-fixed sections of midgut tissue using a one-step TUNEL apoptosis detection kit (Guge Biology, Wuhan, China). Nuclear counterstaining was executed with a 4′,6-diamidino-2-phenylindole (DAPI) solution (Servicebio, Wuhan, China) at 37 °C for 10 min. Following this, fluorescent images of the intestinal tissues were acquired using a Leica DMI8 fluorescence microscope (Wetzlar, Germany).

2.9. Statistical Analysis

Data are expressed as mean ± standard deviation. Statistical significance was ascertained utilizing one-way analysis of variance (ANOVA), complemented by Dunnett's post hoc test to adjust for multiple comparisons. The statistical analyses were also performed utilizing R 2.6-8 software in conjunction with the 'vegan' package. Histograms were constructed with GraphPad Prism 9.0 (San Diego, CA, USA).

3. Results

3.1. Changes in Growth Performance

The growth performance data obtained after a 60-day culture of the hybrid groupers are presented in Figure 1. Compared with the CON group, several growth parameters, including body weight, body length, and weight gain rate, significantly decreased in the VH + CON group. In addition, body weight was higher in the OCS group compared to the CON group. No significant differences were observed in the feed coefficient and relative fat content between the different treatment groups. Furthermore, the survival rate decreased significantly in OCS compared to CON, while no significant differences were determined in VH + OCS compared to VH + CON.

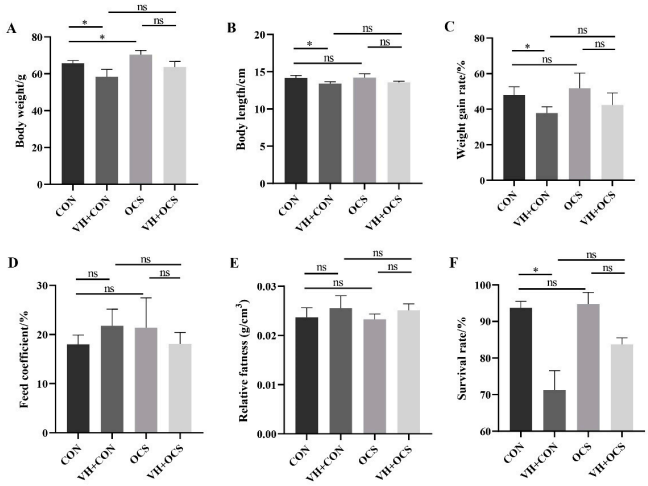

Figure 1. Growth performance of hybrid groupers in different treatments (a control group (CON), a group treated with *V. harveyi* (VH + CON), a group treated with oligochitosan (OCS), and a group

treated with a combination of *V. harveyi* and oligochitosan (VH + OCS)). (**A**) Body weight; (**B**) Body length; (**C**) Weight gain rate; (**D**) Feed coefficient; (**E**) Relative fatness; (**F**) Survival rate. The asterisks indicate significant differences (*, $p < 0.05$ and ns, not significant) among different groups.

3.2. Changes in Biochemical Activities

To assess the effects of *Vibrio* infection and the mitigating effect of oligochitosan on the enzyme activities in the midgut of hybrid groupers, we quantified the biochemical activities of CAT, T-SOD, GSH-Px, MDA, LPS, and AMS in different experimental groups (Figure 2). The activity of the antioxidant enzyme CAT significantly decreased in VH + CON compared to CON and significantly increased in VH + OCS compared to VH + CON. In addition, the activity of T-SOD significantly decreased in VH + CON compared to CON, while no significant changes were observed in the other groups. In contrast, the MDA levels significantly increased in VH + CON compared to CON and decreased in VH + OC compared to VH + CON. In terms of digestive enzyme activity, the LPS activity increased in VH + CON compared to CON and also showed an increase in VH + OCS compared to OCS. Remarkably, no significant differences were observed in T-SOD and AMS between the treatment groups.

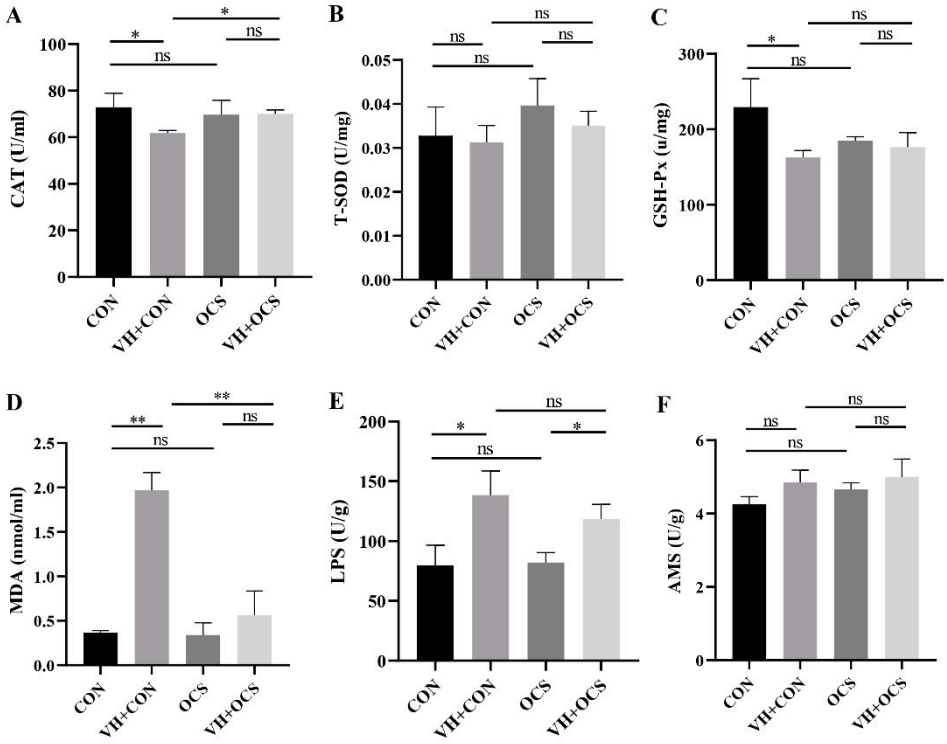

Figure 2. Antioxidant and digestive enzyme indices in the midgut of hybrid groupers among all treatments. (**A**) Catalase (CAT); (**B**) Total superoxide dismutase (T-SOD); (**C**) Glutathione peroxidase (GSH-Px); (**D**) Malondialdehyde (MDA); (**E**) Lipase (LPS); (**F**) Amylase (AMS). The different symbols indicate significant differences (**, $p < 0.01$; *, $p < 0.05$; or ns, not significant) among the groups.

3.3. Changes in Gut Transcriptome Profiles

Transcriptomic analysis was performed to decipher the molecular mechanisms of *V. harveyi* infection and the modulating effect of oligochitosan on gene expression in the midgut of hybrid groupers (Figure 3). The mRNA sequencing revealed 70.43 Gb of clean data in all samples, with each sample contributing 3.96 to 5.04 Gb and 22,384 annotated genes. The Venn diagram depicts 531 DEGs between the treatment groups (Figure 3A). A detailed examination of the DEG expression levels in the midgut of hybrid groupers

is shown in Figure 3B–D. Compared to CON, VH + CON exhibited 35 upregulated and 99 downregulated DEGs, while OCS showed 21 upregulated and 91 downregulated DEGs. Moreover, VH + OCS presented 148 upregulated and 185 downregulated DEGs compared to VH + CON and 15 upregulated and 40 downregulated DEGs compared to OCS.

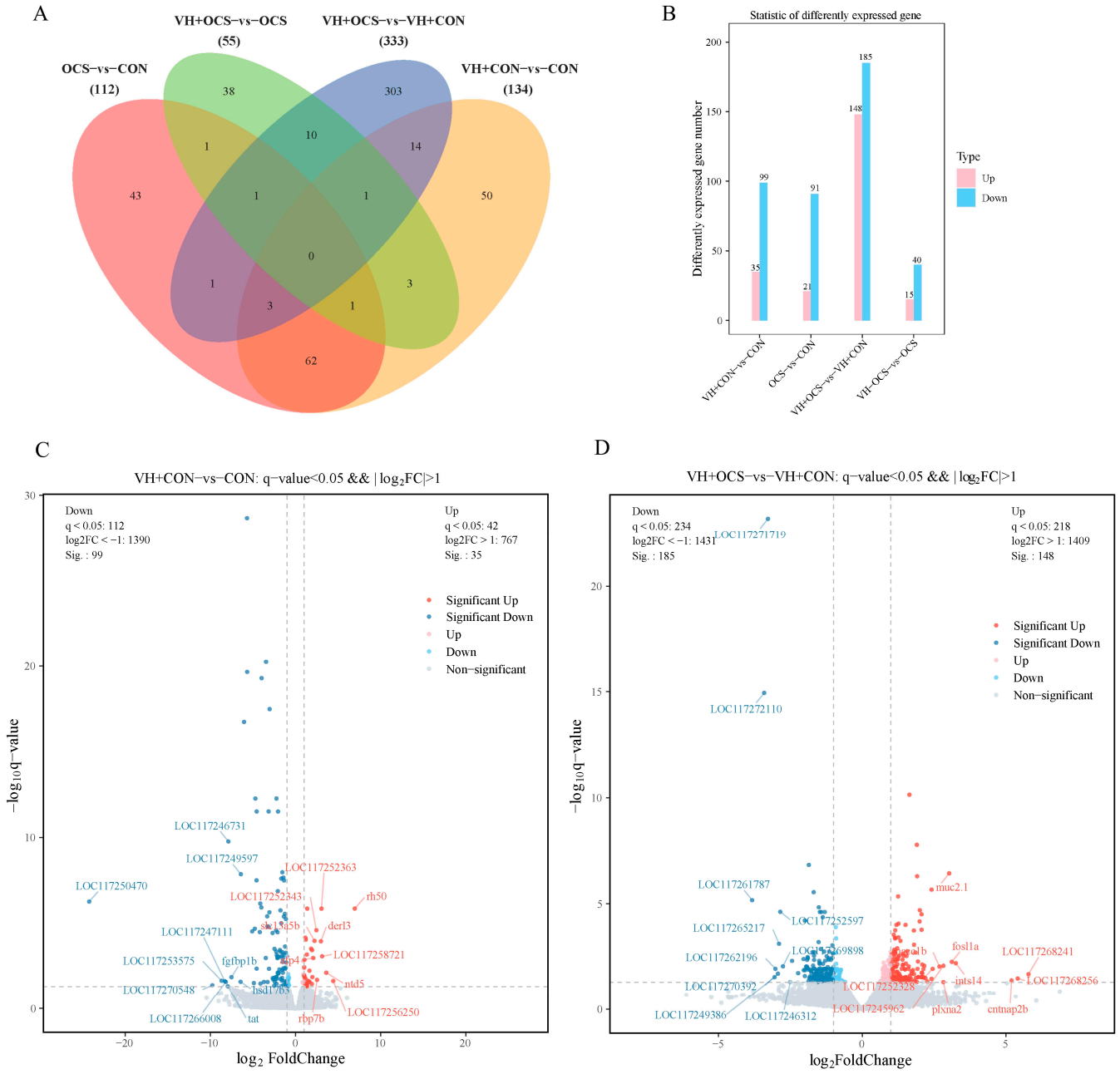

Figure 3. Overviews of DEGs identified in the intestines among different treatments. (**A**). Venn diagram of annotated DEGs between two different groups. (**B**) Number of differently expressed up-regulated and down-regulated genes. (**C**,**D**) Volcano plots of DEGs between VH + CON vs. CON (**C**) and VH + OCS vs. VH + CON (**D**).

The pathway annotations and the different DEG abundances in the midgut of hybrid groupers in response to the different treatments are depicted in Figure 4. The Level 2 KEGG enrichment analysis for comparisons between VH + CON versus CON and VH + OCS versus VH + CON is shown in Figure 4A,B, revealing that the disease-related and organismal system pathways were significantly enriched in both comparison groups.

In particular, the bacterial infectious diseases within the disease pathways and the immune system within the organ system pathways were dominant, emphasizing their importance for the differential response between VH + CON and CON and between VH + OCS and VH + CON. The Level 3 KEGG pathway analysis identified common disease-related pathways between VH + CON versus CON and VH + OCS versus VH + CON. These common pathways include allograft rejection, graft-versus-host disease, inflammatory bowel disease, and Staphylococcus aureus infection (Figure 4C,D).

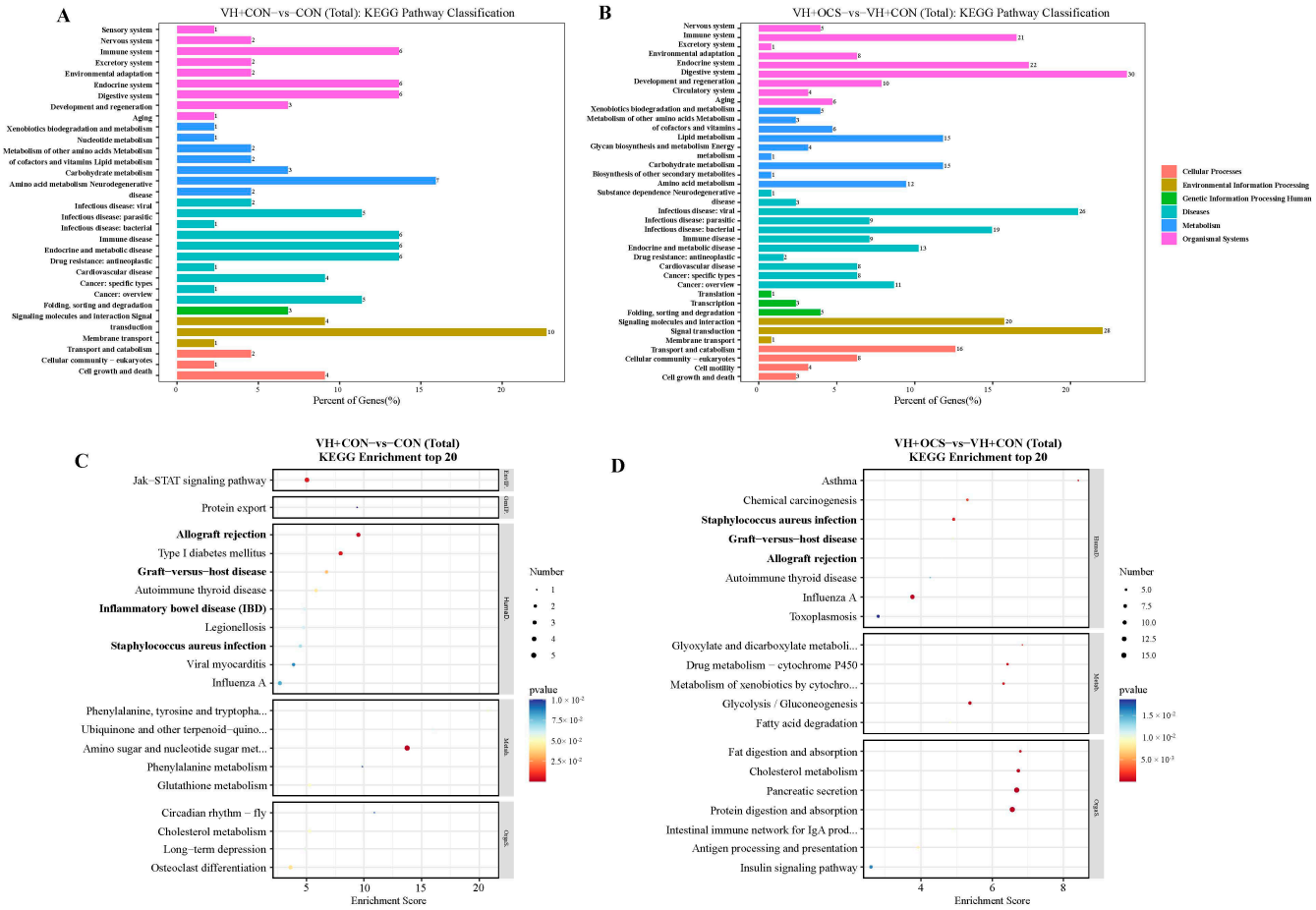

Figure 4. Pathway enrichments of all DEGs in the intestine of hybrid groupers. (**A**,**B**). KEGG classification in second level of the DEGs between VH + CON and CON (**A**) and VH + OCS and VH + CON (**B**). It shows enrichments of secondary KEGG terms in the background and differentially expressed genes. (**C**,**D**) The most enriched KEGG pathways in third level between VH + CON and CON (**C**) and VH + OCS and VH + CON (**D**).

The relative abundance of the DEGs co-occurring in the pathways, including allograft rejection, graft-versus-host disease, inflammatory bowel disease, and *Staphylococcus aureus* infection, is presented in the heatmap (Figure 5). In contrast to CON, the relative mRNA expression levels of several DEGs, including nucleotide-binding oligomerization domain 2 (*NOD2*), keratin-1 (*KRT1*), and complement factor D (*CFD*), significantly increased in VH + CON and decreased in VH + OCS compared to VH + CON. Unexpectedly, the relative mRNA expression levels of DEGs such as perforin 1 (*PRF1*), platelet-activating factor receptor gene (*PTAFR*), interleukin-22 (*IL-22*), Fc gamma receptor-1A (*FCGR1A*), major histocompatibility complex class II (*MHCII*), and interleukin-22 (*IL-12*) significantly decreased in VH + CON and increased in VH + OCS compared to VH + CON.

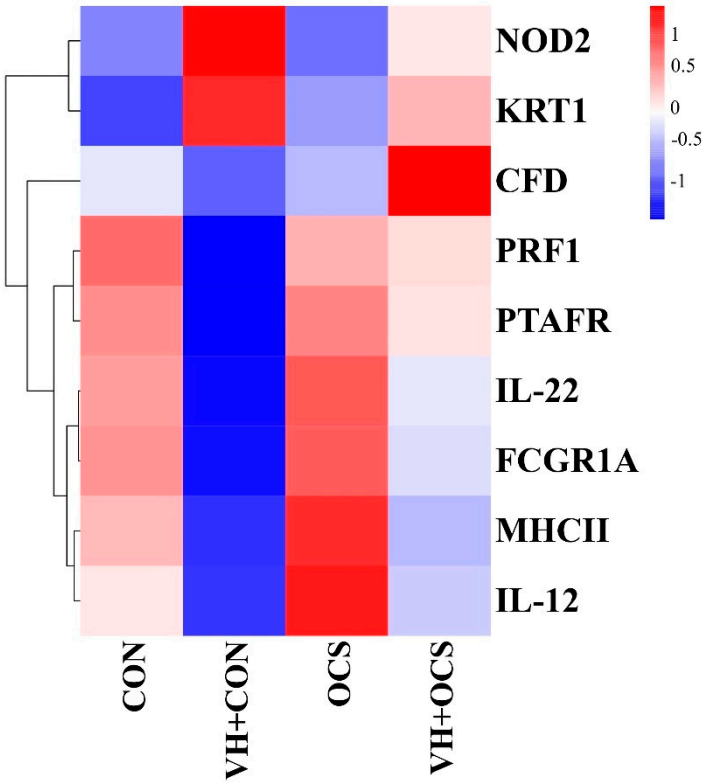

Figure 5. Heatmap of relative abundances of the 9 different DEGs in the midgut of hybrid groupers among four groups, according to the KEGG pathways. *NOD2*, nucleotide binding oligomerization domain 2; *KRT1*, keratin-1; *CFD*, complement factor D; *PRF1*, perforin 1; *PTAFR*, platelet-activating factor receptor gene; *IL−22*, Interleukin−22; *FCGR1A*, Fc gamma receptor−1A; *MHCII*, major histocompatibility complex class II; *IL−12*, Interleukin−12.

3.4. Changes in Immune Responses

To evaluate the impact of *V. harveyi* infection on the immune system and the immunomodulatory effects of oligochitosan, we quantified the mRNA expression levels of key immune-related genes in the intestinal tissue of hybrid groupers by qRT-PCR (Figure 6). Compared to CON, VH + CON exhibited a significant downregulation in the mRNA expression of pro-inflammatory cytokines, such as *IL-1β* and *IL-10*; conversely, the relative expression of *IL-10* significantly increased in VH + OCS compared to VH + CON (Figure 6A–C). Antioxidant-related genes, including *CAT* and *GPx*, significantly decreased in VH + CON compared to CON. However, the relative expression of *CAT* was significantly decreased in VH + OCS compared to VH + CON (Figure 6D,E). In terms of tight-junction proteins, the relative gene expression, including *CLDN-3α*, *OCLN*, *ZO-2*, and *ZO-3*, significantly decreased in VH + CON compared to CON. Furthermore, the relative expression of *OCLN* significantly increased in VH + OCS compared to VH + CON. However, the relative expression of *CLDN-3α* and *ZO-3* significantly decreased in VH + OCS compared to VH + CON (Figure 6 F–I).

To elucidate the impact of *V. harveyi* infection and the restorative effects of oligochitosan on intestinal cell apoptosis, we assessed TUNEL signals across different gut compartments of hybrid groupers (Figure 7). Fluorescence data in Figure 7A revealed a significant upregulation of TUNEL signals in the foregut and midgut of VH + CON groupers compared to CON, indicating heightened apoptosis. Conversely, a significant reduction in TUNEL signals was observed in the foregut and midgut of VH + OCS groupers relative to VH + CON, suggesting a protective effect of oligochitosan. No significant differences were noted in the hindgut among treatment groups. Relative fluorescence intensity analysis corroborated these findings, demonstrating that *V. harveyi* infection significantly

induced intestinal apoptosis in hybrid groupers, which was mitigated by oligochitosan supplementation (Figure 7B).

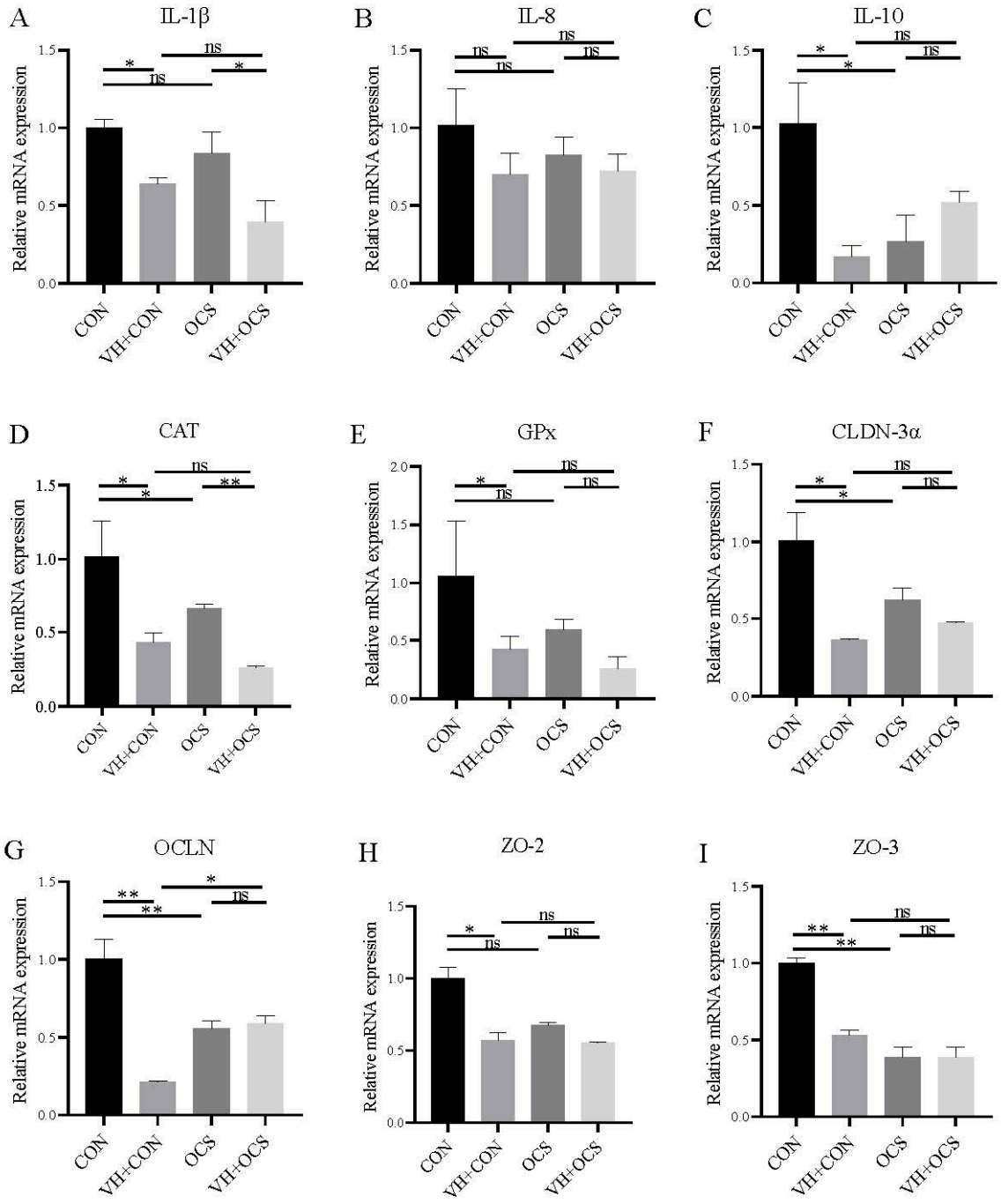

Figure 6. Immune genes' expression levels in the midgut of hybrid groupers monitored by quantitative real-time PCR (qRT-PCR). The relative expression of the immune genes is as below: *IL-1β*, Interleukin-1β (**A**); *IL-8*, Interleukin-8 (**B**); *IL-10*, Interleukin-10 (**C**); *CAT*, Catalase (**D**); *GPx*, Glutathione peroxidase (**E**); *CLDN-3α*, Claudin-3α (**F**); *OCLN*, Occludin (**G**); *ZO-2*, Zonula Occludens 2 (**H**); *ZO-3*, Zonula Occludens 3 (**I**). The different symbols show significant differences (**, $p < 0.01$; *, $p < 0.05$; or ns, not significant).

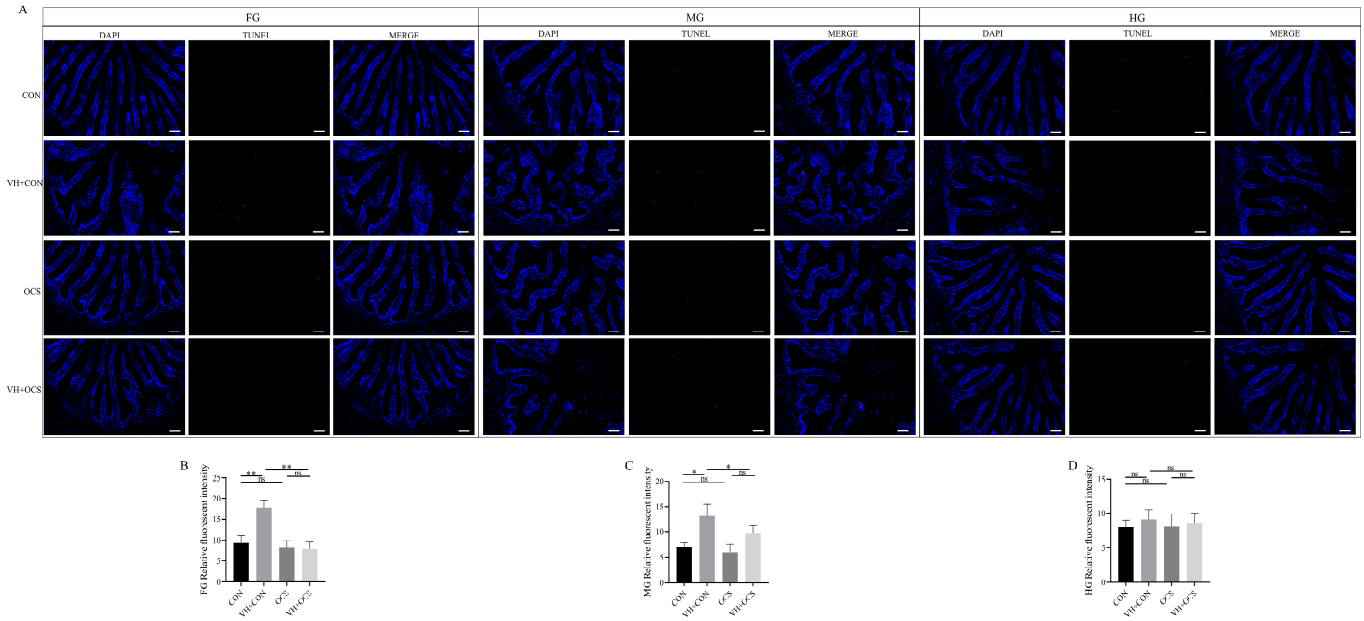

Figure 7. Apoptosis analyses in the midgut tissues of hybrid groupers. (**A**) TUNEL staining, showing apoptosis in red fluorescence. Scale bar, 50 μm. (**B**–**D**) Comparative analysis of fluorescence intensity within the foregut, midgut, and hindgut in various experimental treatments. The different symbols show significant differences (**, $p < 0.01$; *, $p < 0.05$; or ns, not significant).

4. Discussion

Vibrio species infections pose major challenges to aquaculture, causing colitis, increased mortality, and impaired growth [3,17,18,23]. Feed additives, such as oligochitosan, are widely used due to their metabolic and physiological benefits [6,24]. Our prior research demonstrated that oligochitosan-supplemented diets enhance growth, immune responses, and gut microbiome balance in hybrid groupers, potentially improving resistance to *V. harveyi* infections [19]. Thus, this study provides a detailed analysis of immune response dynamics and the modulating effects of oligochitosan under low-dose *V. harveyi* infection in hybrid groupers.

The inclusion of oligochitosan in the diet is reported to significantly enhance the growth performance in various fish species, such as pompano (*Trachinotus ovatus*) [25], loach (*Paramisgurnus dabryanus*) [26], and tiger puffer (*Takifugu rubripes*) [27]. Our results indicated that while hybrid groupers faced reduced growth performance and survival against *V. harveyi* infection, oligochitosan supplementation in their diet partially mitigated these effects by enhancing the body weight, suggesting a protective effect against the pathogen. Challenge tests with pathogens are commonly employed to assess the health status of fish in nutritional studies [28]. Numerous studies have indicated that dietary oligochitosan, regardless of the dosage, can significantly enhance resistance to pathogenic bacteria [7,29,30] and parasites [31]. Duy, et al. reported that oligochitosan reduced mortality rates in striped catfish (*Pangasianodon hypophthalmus*) [32] and olive flounder (*Paralichthys olivaceus*) [33] infected with *Edwardsiella ictaluri*. However, our study did not determine any significant effects of oligochitosan on hybrid groupers under low-dose *V. harveyi* infection, revealing that the efficacy of oligochitosan may be contingent upon the dosage of the pathogen and the susceptibility of the host species. This highlights the complexity of host–pathogen interactions and the need for tailored dietary interventions in aquaculture to optimize fish health and resistance to infections.

The antioxidant and digestive enzymes serve as biomarkers for assessing both oxidative stress and nutritional status in fish, as evidenced by previous studies [34,35]. Environmental factors can trigger oxidative stress in aquatic life forms, resulting in the formation of reactive oxygen species (ROS) [36]. Cellular antioxidant defenses, comprising

enzymes such as SOD, GSH-Px, and CAT, protect against ROS-induced tissue damage, with enzyme deficiencies signaling cellular distress [37]. MDA, a toxic byproduct of lipid peroxidation, functions as an oxidative stress biomarker, signifying cellular damage levels. Our results determined that *Vibrio* infection significantly reduced the CAT activity in VH + CON compared to CON. This aligns with the view that ROS accumulation can overwhelm the cellular antioxidant defenses [38]. However, T-SOD activity significantly decreased in VH + CON, highlighting the vulnerability of this enzyme to infection-induced stress, with no significant changes observed in other groups. This may indicate differential responses to *Vibrio* infection and oligochitosan treatment. Moreover, MDA, a toxic byproduct of lipid peroxidation and a reliable biomarker for oxidative stress [39], was significantly elevated in VH + CON compared to CON, indicating increased lipid peroxidation and cellular damage. The reduction in MDA levels in VH + OCS compared to VH + CON further supports the protective role of oligochitosan against oxidative damage. Digestive enzymes, including AMS and LPS, play a crucial role in the breakdown of nutrients within the intestinal tract, a process that is essential for the absorption of these nutrients by the host body [40]. Our study found that LPS activity increased in VH + CON compared to CON and also showed an increase in VH + OCS compared to OCS, suggesting a potential upregulation in response to infection and treatment. This aligns with Awad, et al., who emphasized the importance of digestive enzymes in nutrient digestion and absorption [41]. These findings highlight the complex interplay between infection, antioxidant defense, and digestive function in fish and highlight the potential of oligochitosan as a dietary supplement to ameliorate the impacts of *Vibrio* infection on the antioxidant and digestive enzyme activities in hybrid groupers.

Immune and metabolic factors significantly shape the transcriptome profiles, which are vital for preserving the host's internal balance [42]. Prior investigations have emphasized the pivotal role of the intestinal transcriptome in aquatic species, influenced by both the immune response and the gut microbiome, which is critical for the preservation of the host's equilibrium [43]. In line with this, our mRNA sequencing analysis revealed a significant number of DEGs between treatment groups, indicating the dynamic regulation of gene expression in response to *Vibrio* infection and oligochitosan treatment. Previous comparative transcriptome analyses of immune responses in Chinese tongue sole (*Cynoglossus semilaevis*) under *V. harveyi* infection have revealed the involvement of various immune-related genes [15]. Notably, VH + OCS resulted in a significant number of DEGs compared to both VH + CON, indicating that oligochitosan may modulate the expression of genes linked to the immune response and metabolism. Our KEGG enrichment analysis revealed that the disease-related and organismal system pathways were significantly enriched, with bacterial infectious diseases and the immune system identified as dominant pathways. This aligns with prior research highlighting the significance of these pathways in modulating the differential response to bacterial infections in fish [44,45]. The relative abundance of DEGs co-occurring in these pathways is presented in a heatmap, revealing significant changes in the mRNA expression levels of key genes such as *NOD2* and *KRT1*. The intracellular sensor *NOD2*, responsible for recognizing muramyl dipeptide found in bacterial peptidoglycan, appears to be non-critical for the innate immune system's macrophage response to *Yersinia enterocolitica* [46]. *KRT1*, a major cytoskeletal component of the epithelium in the gastrointestinal tract of mammals, maintains the intestinal barrier by upregulating tight-junction proteins in ulcerative colitis [47]. These genes are known to play crucial roles in immune response and inflammation. Our findings suggest that oligochitosan may exert its modulating effect by altering the expression of these genes, thereby influencing the host's immune response to *V. harveyi* infection.

Inflammatory cytokines play a pivotal role in immune system balance and function, as they trigger inflammatory responses in fish that are vital for defending against pathogens [48,49]. We found that *V. harveyi* infection significantly downregulated the mRNA expression of pro-inflammatory cytokines, such as *IL-1β* and *IL-10*, in the intestinal tissue of hybrid groupers. This is in line with Lowa, et al., who reported that *IL-1β* can initiate inflammatory responses by stimulating the release of other cytokines or activating lymphocytes [50]. The pathogenic

factors associated with *V. harveyi* primarily include extracellular products, such as proteases, hemolysins, exotoxins, and lipopolysaccharides (LPS). Current research has shown that oligochitosan can suppress the LPS-induced activation of the mitogen-activated protein kinases, nuclear factor-kappa B, and adenosine monophosphate-activated protein kinase pathways, thereby reducing the release of downstream inflammatory factors and mitigating secondary inflammation-induced damage [51]. Fernandes, et al. found that oligochitosan shows anti-inflammatory properties by inhibiting cyclooxygenase, which results in decreased prostaglandin levels [52]. Tight-junction proteins, prevalent at the apical junctions of intestinal epithelial cells, are crucial for maintaining gut homeostasis [53,54]. Our study found that *V. harveyi* infection significantly decreased the relative expression of tight-junction proteins, including *CLDN-3α*, *OCLN*, *ZO-2*, and *ZO-3*, compared to CON. However, oligochitosan supplementation led to a significant increase in *OCLN* expression and a decrease in *CLDN-3α* and *ZO-3* expression compared to VH + CON. These results indicate that oligochitosan may help preserve the intestinal barrier by regulating the tight-junction proteins, potentially offering protection against bacterial infections.

Apoptosis, also known as programmed cell death, is a tightly controlled process essential for the development, differentiation, and preservation of interior homeostasis in fish and the elimination of unwanted or damaged cells [55]. We assessed TUNEL signals across different gut compartments, revealing a significant upregulation of TUNEL signals in the foregut and midgut of VH + CON groupers compared to CON, indicating heightened apoptosis. This agrees with previous research, which reports that *Vibrio* pathogens can induce apoptosis in host cells, leading to tissue damage and dysfunction [56,57]. Conversely, a significant reduction in TUNEL signals was observed in the foregut and midgut of VH + OCS groupers relative to VH + CON, suggesting the protective effect of oligochitosan. This is further corroborated by relative fluorescence intensity analysis, which revealed a decrease in apoptosis in the presence of oligochitosan supplementation.

5. Conclusions

In this study, infection with *V. harveyi* in hybrid groupers resulted in a significant reduction in the growth performance and survival rate, which was partially alleviated by oligochitosan supplementation. Oligochitosan also restored the balance of antioxidants, reduced oxidative stress, and modulated gene expression related to immune response and tight-junction proteins, thereby reducing apoptosis in the gut of infected groupers. These results highlight the potential of oligochitosan as a therapeutic feed additive to counteract the detrimental effects of *V. harveyi* infection on the growth, immune function, and gut health of hybrid groupers and emphasize its importance for sustainable aquaculture.

Author Contributions: F.S., Z.Q. and L.L. designed the study. F.S., L.M., Z.C. and H.Z. performed the experiments. F.S., L.M., Z.C. and C.Z. analyzed the data. F.S. and Z.Q. wrote the manuscript. L.L. reviewed the manuscript. All authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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