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Research on the Adaptive Response Mechanism of Bacterial Infection in *Triplophysa siluroides*

Yuanliang Duan, Ya Liu, Jun Ou, Feiyang Li, Xiaoyun Wu, Jianshen Lai, Jian Zhou and Mingjiang Song *

Fisheries Research Institute, Sichuan Academy of Agricultural Sciences (Sichuan Fisheries Research Institute), Chengdu 611731, China; ylduan@scsaas.cn (Y.D.); liuya@scsaas.cn (Y.L.); 18328042969@163.com (J.O.); lfeiyang@scsaas.cn (F.L.); wuxy@scsaas.cn (X.W.); laijiansheng@scsaas.cn (J.L.); zhoujian980@126.com (J.Z.)
* Correspondence: smj@scsaas.cn; Tel./Fax: +86-028-86106576

Abstract

Triplophysa siluroides, a unique species of plateau fish, holds significant economic value. However, its natural population has sharply declined due to overfishing and the construction of water conservancy projects. Investigating the various conditions necessary for its growth is a crucial prerequisite for successful artificial breeding. This study used *Edwardsiella tarda* as the pathogenic bacterium to determine the median lethal concentration following infection of *T. siluroides*, as well as to examine changes in tissues, organs, and gene expression. The study found that dead *T. siluroides* displayed symptoms such as abdominal distension, fluid accumulation, and a reddened anus, and the median lethal concentration of *E. tarda* for *T. siluroides* was calculated to be 1.00×10^6 CFU/mL. Following infection with *E. tarda*, the liver, intestine, gills, spleen, and kidneys exhibited varying degrees of lesions. Transcriptome sequencing identified a total of 54,667 genes. Compared to the blank control group, 192 genes were downregulated and 125 genes were upregulated in *T. siluroides* infected with *E. tarda*. In contrast, after infection with the poly(I:C) viral mimic, 225 genes were downregulated and 436 genes were upregulated. This study determined the median lethal concentration of *E. tarda* for *T. siluroides* via intraperitoneal injection under laboratory conditions. The results may contribute to disease prevention and control in the breeding of *T. siluroides*, as well as inform future risk assessments of infection in aquaculture water bodies.

Keywords: *Triplophysa siluroides*; bacterial challenge test; median lethal concentration; differentially expressed gene; histopathology

Key Contribution: This research provides fundamental data to support the prevention and control of diseases in *Triplophysa siluroides* during its farming process.

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Academic Editor: Giacomo Zaccone

Received: 16 April 2026
Revised: 20 May 2026
Accepted: 21 May 2026
Published: 22 May 2026

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1. Introduction

The bacterial challenge test holds significant importance in aquaculture, disease prevention and control, and scientific research. Its primary value lies in accurately analyzing the pathogenic mechanisms of bacteria, evaluating the effectiveness of prevention and control measures, and facilitating the transition of industries toward green and efficient development through controlled artificial infection experiments [1,2].

The bacterial challenge test accurately simulates pathogen infection by artificially introducing specific pathogenic bacteria, thereby elucidating pathogenic mechanisms [2,3]. In *Edwardsiella ictaluri*, intrinsic resistance to cationic antimicrobial peptides is mediated

by the Ugd enzyme, which exerts pleiotropic effects by influencing the synthesis, motility, aggregation, intestinal inflammation, and virulence of lipopolysaccharides [4]. Ten days after infection with *Flavobacterium psychrophilum*, transcript levels of the *SAA* and *IL-8* genes in the intestine of *Oncorhynchus mykiss* increased by more than 250-fold and 20-fold, respectively [5]. Observations of tissue pathology following the challenge reveal inflammatory responses and apoptotic characteristics in organs such as the liver and kidneys, providing targets for vaccine development [6]. After artificial infection, treatment with different antibiotics (such as cephalosporins and fluoroquinolones) allows assessment of bacterial inhibition and the evolution of drug resistance [7]. Bacterial challenge tests reveal interaction patterns between pathogens and hosts by detecting immune genes related to disease resistance in fish after viral challenge (involving innate and/or adaptive responses), highly disease-resistant species can thus be identified [8], providing molecular targets for disease-resistant breeding [9]. By simulating and investigating water environments, the impact of water quality parameters (such as ammonia nitrogen and dissolved oxygen) and microorganisms can be assessed, and can also evaluate the antibacterial efficacy of disinfectants (such as sodium hypochlorite and ozone) [10,11], guiding appropriate usage concentrations and frequencies to avoid excessive disinfection that disrupts ecological balance. Elevated temperatures have promoted the spread of *E. tarda* in farmed fish [12], a finding that can guide farmers to enhance management during winter. Additionally, bacterial challenge tests can guide the adjustment of feed additives to optimize compound feed formulations for fish [13]. Moreover, introducing Poly(I:C) to simulate viral infection in bacterial challenge tests can construct a composite immune model that more closely resembles real clinical infection scenarios [14], providing a crucial foundation for understanding host immune response mechanisms and developing novel prevention and treatment strategies. Ultimately, research involving bacterial challenge tests serves to reduce antibiotic use, lower production costs, and improve the quality of aquatic products.

Triplophysa siluroides is an endemic species found in the upper reaches of the Yellow River in China [15]. It exhibits two body color morphs: the typical brown and orange [16,17], and holds significant economic value. However, its wild population has declined markedly due to human activities, leading to its classification as a vulnerable species [18,19]. Current research on *T. siluroides* primarily focuses on resource and gene development, biology, breeding, and disease studies. Genetic research is the most extensive, encompassing chromosome karyotyping (25 chromosomes) [20]; mitochondrial genome analysis (including 13 protein-coding genes, 22 transfer RNAs, 2 ribosomal RNA genes, and a noncoding control region) [21]; molecular markers [22]; specific genes such as *Tyr*, *Mc1r*, *Agouti*, and *SLCs* [16]; and gene expression differences induced by environmental stress [23,24]. Studies on the genetic diversity of *T. siluroides* often utilize special genes such as mtDNA *D-loop*, *Cytb*, and *COI* [25–27]. As *Triplophysa* is a large genus, *T. siluroides* serves as a reference species for genetic research on other *Triplophysa* species [28,29]. Biological research mainly addresses growth and body size, particularly in wild populations [15,30,31], since fish body size largely reflects the structural composition of wild resources [32]. Due to the scarcity of *T. siluroides*, researchers and farmers have actively pursued artificial breeding, focusing on fry cultivation [33], the effects of stocking density [34], feeding frequency [35], and dissolved oxygen levels [36] on growth. Disease research primarily involves the isolation and identification of pathogens at lesion sites, including CMTV-like ranavirus [37], *Aeromonas sobria* [38], and *E. ictaluri* [39]. Additionally, studies have examined tissues such as skin, fins, and blood cells of *T. siluroides* [40,41]. Many factors influence meat quality; however, current research on *T. siluroides* meat quality mainly addresses body color and artificial breeding. Findings indicate that wild *T. siluroides* have superior meat quality compared to

artificially bred individuals, and the orange-skinned morph exhibits better quality than the typical brown morph [17,42].

Currently, only two studies have examined the association between *T. siluroides* and *Edwardsiella*: the isolation and identification of the sepsis pathogen (*E. ictaluri*) in *T. siluroides* [39], and the expression characteristics of the *LEAP-2* gene following *E. tarda* infection [23]. To date, no systematic research has investigated the response of *T. siluroides* to *E. tarda* infection. In this study, *E. tarda*, a common pathogenic bacterium of *T. siluroides*, was used as a stimulus to systematically explore its median lethal concentration (LC_{50}), as well as the histological and gene expression dynamic changes in *T. siluroides*.

2. Materials and Methods

2.1. Ethics Statement

All animal specimens utilized in this research were managed in strict adherence to relevant ethical guidelines. All protocols involving the handling and experimentation of fish were reviewed and authorized by the Animal Care and Use Committee of the Fisheries Research Institute, Sichuan Academy of Agricultural Sciences (Sichuan Fisheries Research Institute), with the official approval number 20250206008A.

2.2. Source of Experimental Organisms and Experimental Design

A total of 600 domesticated *T. siluroides* specimens, with an average body length of 16.14 ± 1.48 cm and an average body weight of 24.62 ± 5.70 g, were obtained from the Fisheries Research Institute, Sichuan Academy of Agricultural Sciences (Sichuan Fisheries Research Institute). Prior to the formal experiment, the fish were acclimated in recirculating aquaculture tanks maintained at a water temperature of 14 °C, with an oxygen pump providing continuous oxygen supply for 24 h. They were fed twice daily, in the morning and evening, at a rate of 1% of their body weight. Feeding was ceased one day before the experiment commenced, and no feeding occurred during the experiment. All other environmental conditions remained consistent with those during the acclimation period.

The LC_{50} study was conducted using four gradient concentrations of *E. tarda* (1.6×10^6, 3.2×10^6, 8×10^6, and 16×10^6 CFU/mL), designated as groups G1, G2, G3, and G4, respectively. Each group consisted of three replicates, with 10 fish per replicate. A blank control group, injected with physiological saline, also included three replicates of 10 fish each (G0). The treatment groups received an intraperitoneal injection of 200 μL of bacterial suspension at the corresponding concentration, while the control group received 200 μL of physiological saline via intraperitoneal injection. All injections were administered at 8:30 AM. Fish survival was recorded at 0, 12, 24, 48, 72, 96, 120, and 144 h post-injection for each group.

The study on the effects of *E. tarda* on tissue morphology was conducted using five gradient concentrations of the bacterium (1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 CFU/mL), designated as groups G1, G2, G3, G4, and G5, each with three replicates. A physiological saline blank control group (G0) with three replicates was also included. Each group contained 12 fish. All fish were intraperitoneally injected with 200 μL of the corresponding bacterial suspension or control solution. At 24 h post-injection, one fish was randomly sampled from each tank—resulting in three fish per treatment group—for histopathological analysis.

The study on the effects of *E. tarda* on gene expression was conducted using three groups: a *E. tarda* group with a concentration of 1.00×10^6 CFU/mL (designated as the B group), a poly(I:C) (Invitrogen, Carlsbad, CA, USA) viral mimic control group (5 mg/kg poly(I:C), designated as the P group), and a physiological saline blank control group (designated as the C group). Each group consisted of three replicates, with 12 fish per

replicate. All fish were intraperitoneally injected with 200 μL of the corresponding solution. At 48 h post-injection, one fish was randomly sampled from each tank—resulting in three fish per treatment group—for transcriptomic analysis.

2.3. Tissue Sample Collection

The collected *Triplophysa* specimens were euthanized using a high concentration of MS-222 (250 mg/L, Sigma-Aldrich, St. Louis, MO, USA) and disinfected with 75% ethanol. Under low-temperature conditions, dissections were performed on a sterile bench to collect intestinal, hepatic, splenic, renal, gill, and skin tissues. Tissue samples for histopathological analysis (intestine, liver, spleen, kidney, gill, and skin) were fixed in 4% paraformaldehyde, while splenic tissue intended for transcriptomic analysis was rapidly frozen and preserved in liquid nitrogen.

2.4. Calculation of Median Lethal Concentration

The LC_{50} was determined using the Karber method by combining the bacterial infection concentration gradient data with the mortality rates of *T. siluroides* over time.

The calculation formula is as follows:

$$\lg LC_{50} = (X_i + X_{i+1})(P_{i+1} - P_i)/2$$

where Xi represents the logarithm of the concentration, and Pi represents the mortality rate.

The 95% confidence limit of LC_{50} is calculated as:

LC_{50} 95% CL = $\lg^{-1}$ ($\lg LC_{50} \pm 1.96 \times Sm$)

Where $Sm = d\sqrt{\frac{\sum Pi - \sum Pi^2}{n}}$, Sm is the standard error, d is the logarithmic difference, and n is the number of experimental animals.

2.5. Histopathological Analysis

Fixed tissue samples were removed from the fixative and rinsed under running water for 30 min. After paraffin embedding, sectioning, deparaffinization, and staining, the tissue sections were analyzed microscopically. Embedding: tissues were dehydrated through a graded ethanol series (75% ethanol for 6 h, 85% ethanol for 10 h, 95% ethanol for 4 h, anhydrous ethanol I for 2 h, anhydrous ethanol II for 2 h), cleared in xylene (xylene I for 20 min, xylene II for 15 min), infiltrated with melted paraffin wax for 3 h, and finally cooled to form paraffin-embedded tissue blocks. Sectioning: tissues were cut into 5 μm-thick sections using an RM2235 microtome (Leica, Wetzlar, Hesse, Germany), floated on a warm water bath to flatten, mounted onto glass slides, and baked at 60 °C for at least 2 h to ensure firm adhesion. Deparaffinization and rehydration: sections were sequentially immersed in xylene I (20 min), II (20 min), ethanol (5 min), ethanol (5 min), distilled water (5 min) to remove paraffin and rehydrate the tissue. Staining: sections were stained with hematoxylin for 4 min, with running water for 3 min, in hydrochloric alcohol for 5 s, again for 3 min, with eosin for 20 s, rinsed once more for 1 min. Dehydration and sealing: sections were dehydrated in anhydrous ethanol I (5 min) and II (5 min), in xylene for 5 min, and finally sealed with neutral balsam.

2.6. Transcriptome Analysis

Approximately 1 g of tissue was ground under liquid nitrogen, and total RNA was extracted using an RNAqueous™ Total RNA Isolation Kit (Invitrogen, USA) according to the manufacturer's protocol. The integrity and concentration of the extracted RNA were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). mRNA was enriched from the total RNA, followed by fragmentation and reverse transcrip-

tion to construct the sequencing library. After quality and quantity control, transcriptome assembly and gene function annotation were performed for downstream analysis.

2.7. Data Analysis

SAS 9.4 and Excel software (Microsoft Excel 2010) were used for statistical analysis, while images were created using Adobe Photoshop CS4 and Excel. Tests for equality of variance and normality were performed using SAS 9.4 prior to the formal analysis. Multiple comparisons and one-way analysis of variance were then conducted. Results are presented as mean ± SD. A p-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Median Lethal Concentration Analysis

All *T. siluroides* survived the first 12 h following intraperitoneal injection of *E. tarda* but exhibited abnormal behaviors such as dispersion and reduced mobility, most notably in Group G4. Dead *T. siluroides* showed symptoms including abdominal distension, fluid accumulation, and anal redness. Mortality occurred in Groups G2–G4 within 24 h, with 1, 2, and 6 fish dying in G2, G3, and G4, respectively. In Group G1, one fish died within 48 h, and a second died by 144 h, totaling 2 deaths. Group G2 experienced 3 deaths within 48 h, with no further mortality thereafter. Groups G3 and G4 experienced mass mortality within 48 h, with a total of 9 fish dead in G3, and all fish in G4 dying within 96 h. The mortality details of *T. siluroides* are presented in Figure 1. No mortality was observed in the blank control group. Based on bacterial dose data and mortality records, the LC_{50} of *E. tarda* for *T. siluroides* was calculated using the Karber method to be 1.00×10^6 CFU/mL.

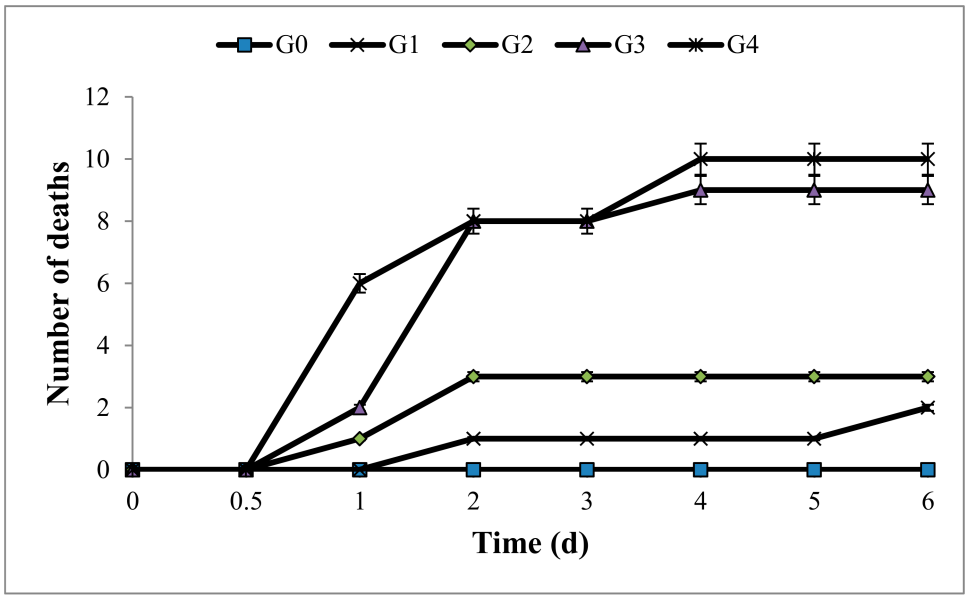


Figure 1. Mortality of *Triplophysa siluroides* following infection with bacteria at varying concentrations. Note: G1–G4 represent Groups G1–G4 injection with bacterial suspensions at concentrations of 1.6×10^6, 3.2×10^6, 8×10^6, and 1.6×10^7 CFU/mL, respectively. G0 represents blank control group.

3.2. Histopathological Analysis

Tissue samples exposed to bacterial infection exhibited varying degrees of pathological lesions, including cellular necrosis and sloughing; these changes became more pronounced with increasing bacterial concentration (Figures 2 and S1–S5). In the liver, bacterial invasion caused hepatocyte swelling, irregular cytoplasmic vacuolation, and occasional nuclear pyknosis and hyperchromasia. Additionally, some sinusoids were dilated, accompanied

by a slight increase in lymphocyte count compared to other tissues. In the intestinal tissue, infection induced sloughing of the mucosal epithelium, edema in the submucosal layer, and infiltration of inflammatory cells. At higher bacterial loads, epithelial necrosis and detachment, structural disruption, villi fragmentation and dissolution, as well as intensified submucosal edema and lymphocytic infiltration, were observed. Gill tissue exhibited acute diffuse necrotizing gill disease, characterized by damage, necrosis, and sloughing of the gill lamella epithelium. With increasing infection severity, the bases of the gill lamellae showed hyperplasia and fusion, leading to shortening or complete loss of gill lamellae, accompanied by a marked reduction or disappearance of gill lamella cells. The spleen displayed loose tissue architecture, reduced lymphocyte numbers, and vacuolar degeneration, with dilated and congested or hemorrhagic sinusoids. In the kidney, tubular epithelial cells showed swelling and degeneration, with focal necrosis, nuclear pyknosis or fragmentation; basement membrane separation occurred, macrophage infiltration increased in the interstitium, and mild necrosis was observed in the renal hematopoietic tissue.

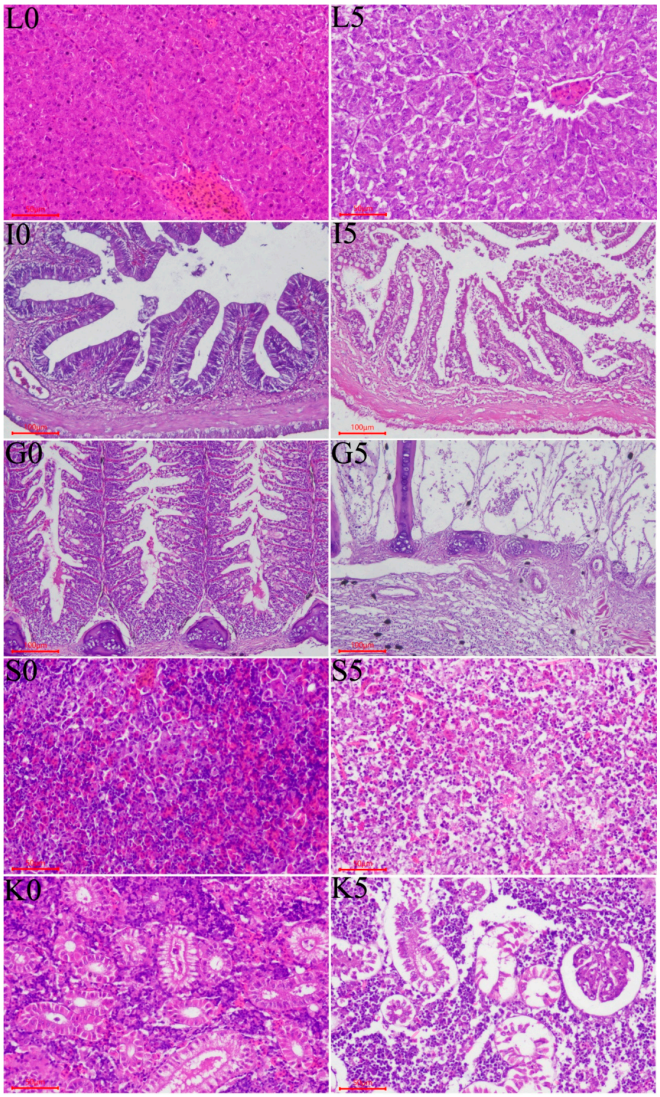


Figure 2. Histological changes in *Triplophysa siluroides* under bacterial infection at different concentrations. Note: L, I, G, S, and K represent liver, intestine, gill, spleen, and kidney, respectively. 0 represents the blank control group, and 5 represents the treatment group with a bacterial concentration of 1×10^8 CFU/mL.

3.3. Transcriptome Analysis

3.3.1. Quality Control Analysis

Through high-throughput sequencing, 37,015,471 ± 4,174,811 clean reads were obtained in the C group, while the number of clean reads in B group and P group (Table 1). The Q20 value for all groups was ≥97.8%, the Q30 value was ≥93.29%, and the GC content was approximately 44%.

Table 1. Statistical analysis of quality control data.

Group	Clean Reads	Q20(%)	Q30(%)	GC Content(%)
C	37,015,471 ± 4,174,811	98.08 ± 0.29	94.17 ± 0.68	43.70 ± 0.55
B	34,735,874 ± 383,195	97.89 ± 0.25	93.98 ± 0.71	44.37 ± 1.16
P	33,978,526 ± 476,865	97.80 ± 0.34	93.29 ± 1.43	43.52 ± 2.05

Note: C, B and P represent the physiological saline blank control group, the *Edwardsiella tarda* group with a concentration of 1.00×10^6 CFU/mL and the poly (I:C) viral mimic control group (5 mg/kg poly (I:C), respectively.

3.3.2. Gene Annotation Analysis

As shown in Table 2, a total of 54,667 genes were identified in this study. These genes were annotated using seven databases: NR, NT, KO, SwissProt, PFAM, GO, and KOG. Among them, the NT database had the highest annotation rate at 55.84%, while the KOG database had the lowest, at only 18.48%. Only 5848 genes were annotated across all seven databases, representing an annotation rate of 10.69%. A total of 41,389 genes were annotated in at least one database, corresponding to an annotation rate of 75.71%. The petal diagram in Figure 3 clearly illustrates the gene annotations from the NT, NR, KOG, GO, and Pfam databases. The number of unique genes in each database is 7094, 3042, 0, 0, and 0, respectively. Additionally, 7317 genes have been successfully annotated across all five databases.

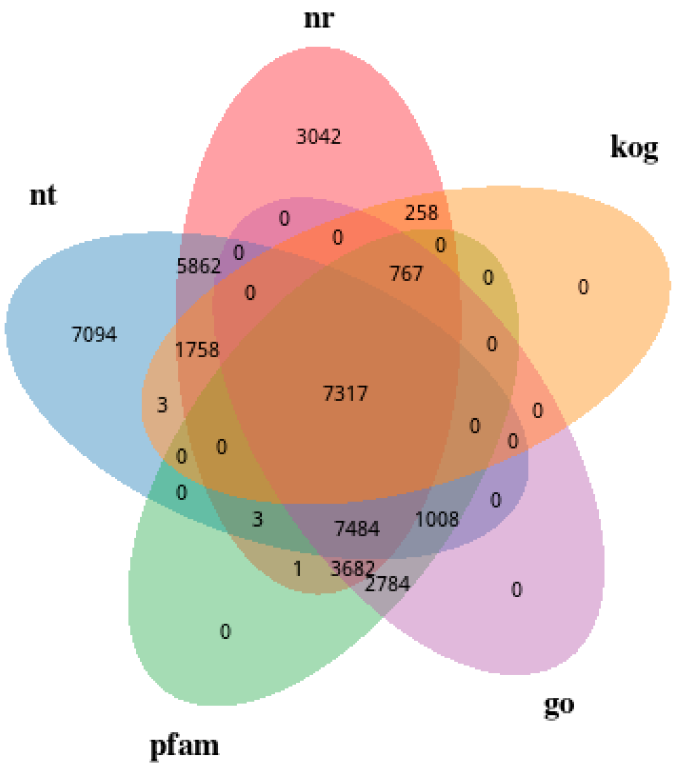


Figure 3. The petal chart of the annotation data statistics of the gene database. Note: The numbers in different regions represent the number of genes contained in that region.

Table 2. Statistical analysis of annotation data in gene databases.

Database	Number of Unigenes	Percentage (%)
Annotated in NR	30,174	55.19
Annotated in NT	30,529	55.84
Annotated in KO	14,969	27.38
Annotated in SwissProt	26,036	47.62
Annotated in PFAM	23,046	42.15
Annotated in GO	23,042	42.14
Annotated in KOG	10,103	18.48
Annotated in all Databases	5848	10.69
Annotated in at least one Database	41,389	75.71
Total Unigenes	54,667	100

3.4. Gene Expression Levels Analysis

The number of reads after quality control for the C group was 74,030,941 ± 8,349,623, with 59,631,052 ± 7,262,018 reads mapping to the reference sequence, accounting for 80.05%. For the B group, the number of reads after quality control was 69,471,747 ± 766,389, with 53,395,666 ± 3,325,666 reads mapping to the reference sequence, accounting for 76.85%. For the P group, the number of reads after quality control was 67,957,051 ± 953,730, with 54,487,879 ± 1,359,300 reads mapping to the reference sequence, accounting for 80.17%. More detailed information can be found in Table 3.

Table 3. Statistical analysis of data on gene localization to reference sequences.

Group	Total Reads	Total Mapped	Total Mapped (%)
C	74,030,941 ± 8,349,623	59,631,052 ± 7,262,018	80.50 ± 0.89
B	69,471,747 ± 766,389	53,395,666 ± 3,325,666	76.85 ± 4.43
P	67,957,051 ± 953,730	54,487,879 ± 1,359,300	80.17 ± 0.89

Note: C, B and P represent the physiological saline blank control group, the *Edwardsiella tarda* group with a concentration of 1.00×10^6 CFU/mL and the poly (I:C) viral mimic control group (5 mg/kg poly (I:C), respectively. Total reads: The number of clean reads after quality control of sequencing data. Total mapped: The number of reads mapped to the reference sequence. Total mapped (%): The proportion of sequencing reads that can be located on the reference sequence.

3.5. Differentially Expressed Genes Analysis

Compared to the control group, infection with *E. tarda* resulted in the downregulation of 192 genes, including 111 genes that were downregulated by more than 20-fold; 125 genes were upregulated, with two genes showing more than a 20-fold increase (Figure 4). Following infection with poly(I:C) viral mimic, 225 genes were downregulated, of which 100 exhibited more than a 20-fold decrease; 436 genes were upregulated, including 317 genes with over a 20-fold increase (Figure 4). A Venn diagram comparing C vs. P and C vs. B identified 60 overlapping differentially expressed genes, of which 41 were upregulated and 19 were downregulated (Figure 5).

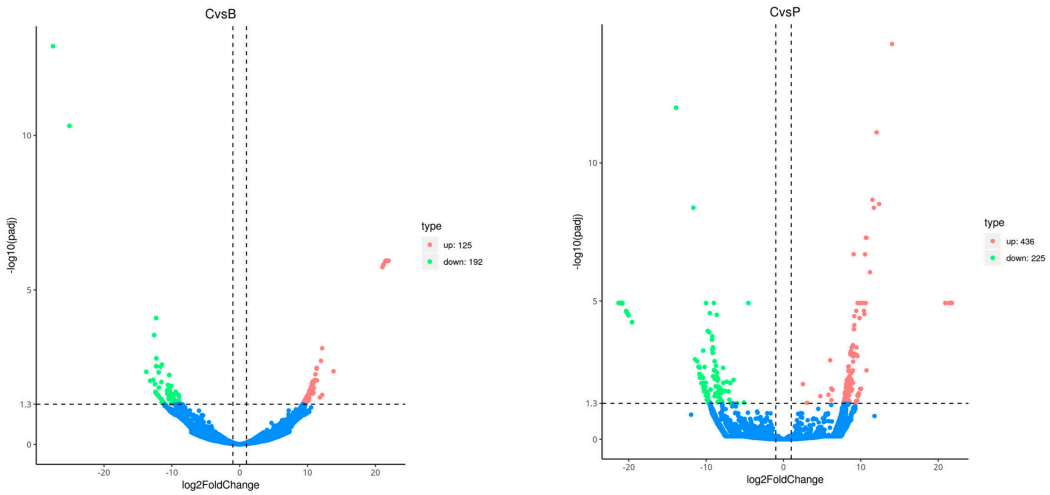


Figure 4. The upregulation and downregulation of gene expression in *Triplophysa siluroides* following infection with *Edwardsiella tarda*. Note: The left side (CvsB) represents the comparison between the bacterial infection group and the control group. The right side (CvsP) represents the comparison between the viral infection group and the control group. Red indicates upregulation, green indicates downregulation, and blue indicates no significant difference.

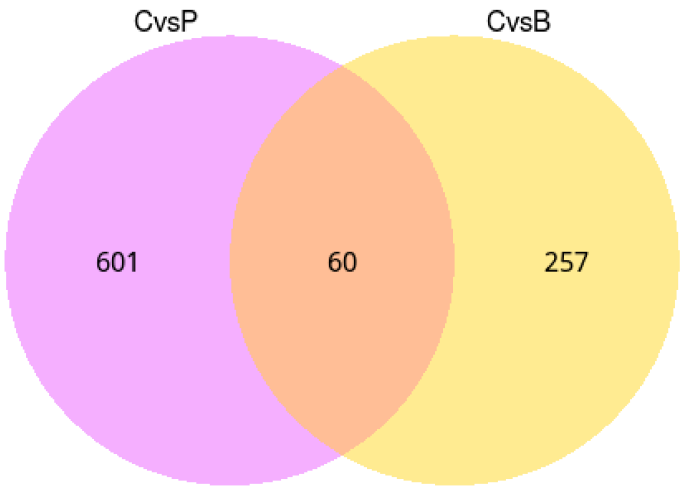


Figure 5. The Venn diagram of genes differentially expressed in *Triplophysa siluroides* after infection with *Edwardsiella tarda*. Note: CvsB represents the comparison between the bacterial infection group and the control group. CvsP represents the comparison between the poly(I:C) viral mimic infection group and the control group.

4. Discussion

The genus *Triplophysa*, native to specialized aquatic environments such as those in northwest and southwest China and the upper reaches of the Yellow River, holds invaluable significance in disease prevention and safety assessment through bacterial challenge tests. These tests enable precise determination of infection thresholds and pathogenic patterns of common bacteria, such as *Edwardsiella* [23,39], across different *Triplophysa* species. This provides a quantitative foundation for evaluating the impact of water pollution and habitat degradation on their populations. Bacterial challenge tests are also essential for screening probiotics and immune enhancers: by comparing survival rates of fish larvae fed with various spore-forming bacteria, xylooligosaccharides, and other supplements, optimal immune enhancement protocols can be identified [43,44]. This study established the LC_{50} for *T. siluroides*; when water pollution leads to pathogenic bacterial proliferation, this value can assist managers in implementing timely measures to prevent disease outbreaks in high-

density aquaculture, thereby ensuring the sustainable development of specialty fisheries. At the fundamental research level, the unique physiological adaptability of *Triplophysa* makes it an ideal model for studying the evolution of the fish immune system. Combining bacterial challenge tests with gene expression analysis reveals immune regulatory mechanisms in extreme environments such as hypoxia. For example, by simulating TRIM protein immune response studies similar to those conducted in *Oncorhynchus mykiss* [45], researchers can explore the functions of novel immune-related genes in *Triplophysa*, enriching the theoretical understanding of innate immunity in fish.

In the field of aquaculture, harmful bacterial infections pose a major threat to fish health and significantly constrain industry development. The most common pathogenic bacteria include species of *Edwardsiella* [46] and others. When these harmful bacteria invade fish, they damage various tissues through multiple mechanisms, triggering a series of pathological changes. In severe cases, infections can cause mass fish mortality, resulting in substantial economic losses for fish farmers. In natural environments, pathogenic bacteria typically enter fish through the digestive tract, body surface wounds, or gills, thereby causing disease. In this study, *E. tarda* was introduced into *T. siluroides* via intraperitoneal injection, minimizing interference from other pathogens. *Edwardsiella* is a common genus of pathogenic bacteria that poses serious threats to both marine and freshwater fish. Following *E. tarda* infection, the marine fish *Scophthalmus maximus* exhibited clinical symptoms including liver whitening, redness and swelling of the intestines with varying degrees of congestion, and the abdominal cavity filled with a small amount of pale red or light yellow mucus [47]; the migratory *Anguilla rostrata* developed both acute and chronic inflammation; during acute infection, multiple necrotic foci were observed in liver tissue, along with hepatocyte degeneration and necrosis [48]; Zhou et al. [49] reported that *Pelteobagrus fulvidraco* exhibited behavioral changes such as solitary swimming and sluggish responses following infection, and Gao et al. [50] found that *P. fulvidraco* developed redness and swelling around the anus, abdominal enlargement, and varying degrees of lesions in the liver, kidneys, intestines, and gills. Different fish species exhibited symptoms similar to those observed in this study after infection with *E. tarda*. These symptoms included an initial onset of sluggish movement, followed by abdominal distension and redness around the anus. Other *Edwardsiella* species may also cause symptoms comparable to those reported here. For example, infection with *E. ictaluri* can induce splenomegaly in hybrid tilapia [51]. Wang et al. [52] reported that *Micropterus salmoides* developed swelling in organs such as the kidneys and liver following infection with *E. piscicida*. Additionally, Xu et al. [53] observed that *P. fulvidraco* exhibited ascites in the abdominal cavity after infection with *E. ictaluri*.

In nature, hosts often encounter not a single pathogen but co-infections or sequential infections by both bacteria and viruses [54]. For example, simultaneous infection of *Oncorhynchus mykiss* with infectious hematopoietic necrosis virus (IHNV) and *Flavobacterium psychrophilum* resulted in a high mortality rate (76.2–100%), whereas infection with the same dose of a single pathogen led to a lower mortality rate (5–20%). Additionally, compared to fish infected with *Flavobacterium psychrophilum* alone (6 days) or IHNV alone (5 days), the onset of death in the co-infection group occurred earlier (3–4 days) [55]. Poly(I:C), an artificially synthesized double-stranded RNA analogue [14], precisely mimics the natural double-stranded RNA produced during viral infection, triggering an immune response highly similar to that of natural viral infections. This enables the reproduction of a complex immune microenvironment for combined infections in a laboratory setting. In this study, the number of differentially expressed genes in the poly(I:C) viral mimic control group was more than twice that in the *E. tarda* group, indicating that Poly(I:C)-induced host transcriptional responses were more extensive and specific, potentially activating a more complex antiviral immune network. In contrast, the changes in gene expression induced by *E. tarda*

were relatively limited, which may be attributed to the low-temperature environment inhibiting the activity of *E. tarda*. Meanwhile, the antiviral immune pathway of the *T. siluroides* remained active at low temperatures and was able to mount an effective immune response to Poly(I:C). Furthermore, Poly(I:C) itself is a potent immune adjuvant [56], enhancing antigen presentation and T cell responses. Adding Poly(I:C) to the bacterial challenge model allows assessment of whether it can improve the host's resistance to secondary viral infection. This study compared the bacterial challenge test with simulated poly(I:C) viral infection, which will provide a certain data basis for the subsequent research on combined infection models.

Moreover, the experimental infection was conducted via intraperitoneal injection, which does not replicate natural infection routes such as exposure through the gills, skin, or oral intake. This limits the ecological relevance and the interpretation of host–pathogen interactions. Performing infection experiments using waterborne or oral exposure to better simulate real-world infection scenarios and comparing these outcomes with those from intraperitoneal injection could provide deeper insights. The study focused primarily on acute responses—mortality up to 144 h and tissue and gene expression at 24 to 48 h—which may not capture chronic infection, recovery, or long-term immune adaptation. Extending the observation period to several weeks could offer a more comprehensive understanding of disease dynamics. Additionally, collecting samples at multiple time points (e.g., one week and one month post-infection) for histological and transcriptomic analyses would be beneficial. The study did not examine the impact of environmental stressors, such as temperature and water quality, on disease progression. Investigating these factors could reveal their effects on infection severity, immune response, and mortality. Future research should address these gaps to strengthen the findings.

5. Conclusions

Triplophysa siluroides, a unique fish species endemic to the plateau with high economic value, has experienced a significant decline in its natural population, necessitating the initiation of artificial breeding. Consequently, research related to its farming has become a prominent focus. This study determined the LC_{50} of *E. tarda* for *T. siluroides*, providing crucial baseline data for disease prevention and safety assessment in the highland minnow breeding process. Additionally, the results obtained from the bacterial challenge test with a sample size of $n = 3$, combined with Poly(I:C) simulation of viral infection, may establish an important experimental foundation for the future development of new vaccine adjuvants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes11060310/s1, Figure S1 Histological changes in *Triplophysa siluroides* under bacterial infection at different concentrations in the liver. Figure S2 Histological changes in *Triplophysa siluroides* under bacterial infection at different concentrations in the liver. Figure S3 Histological changes in *T. siluroides* under bacterial infection at different concentrations in the gill. Figure S4 Histological changes in *T. siluroides* under bacterial infection at different concentrations in the spleen. Figure S5 Histological changes in *T. siluroides* under bacterial infection at different concentrations in the kidney. The raw transcriptome data can be downloaded from the public database GSA using the data identifier CRA042955.

Author Contributions: Conceptualization, M.S. and Y.D.; methodology, Y.L.; software, J.O.; validation, Y.D., X.W. and M.S.; formal analysis, J.L.; investigation, M.S.; resources, Y.D.; data curation, F.L.; writing—original draft preparation, Y.D.; writing—review and editing, M.S.; visualization, J.Z.; supervision, Y.D.; project administration, M.S.; funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the earmarked fund for CARS (CARS-45); Sichuan Freshwater Fish lnnovation Team Construction Project of China Agriculture Research System (SCCXTD-2026-15); Technology Program of Sichuan Academy of Agricultural Sciences (1+9KJGG004).

Institutional Review Board Statement: All animal specimens utilized in this research were managed in strict adherence to relevant ethical guidelines. All protocols involving the handling and experimentation of fish were reviewed and authorized by the Animal Care and Use Committee of the Fisheries Research Institute, Sichuan Academy of Agricultural Sciences (Sichuan Fisheries Research Institute), with the official approval number 20250206008A and approval date 9 January 2025.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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