

# Regulation Mechanism of miR-23a in *Oreochromis mossambicus* During *Aeromonas hydrophila* Infection

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## Abstract

This study used *Aeromonas hydrophila* infection and culture of tilapia (*Oreochromis mossambicus*) renal cells to analyze the time dependent expression of miR-23a in tilapia renal cells infected with *A. hydrophila*. The current results showed that the expression of miR-23a changed significantly at different time points of infection. Using RNAhybrid software to predict the target site of miR-23a and construct a plasmid, the binding ability of the target gene 3'-UTR to miR-23a was verified through dual luciferase reporter gene assay. We found that target genes were reverse regulated, and *tbk1*, *glut1* were identified as miR-23a target genes. Expression of *ldha*, *ldhba*, *pdha 1a* and *pdha 1b* was suppressed after overexpression of miR-23a. These results indicated that miR-23a was involved in the regulation of the immune response in tilapia kidney cells after *A. hydrophila* infection. *tbk1* and *glut1* are the target genes of miR-23a that can affect the expression of downstream genes by targeting GLUT1. The above results provide important insights into the role of miR-23a in regulating the immune response in bony fish and further understanding of miRNA-mediated multiple target genes to regulate innate immunity in fish.

## Introduction

Tilapia (*Oreochromis mossambicus*), as one of more production freshwater white meat fish, suffered from *Aeromonas hydrophila* infection (FAO, 2016; Luo et al., 2019). *A. hydrophila* is a class of gram-negative aquatic bacteria that is widely distributed in a variety of aquatic environments and aquatic animals. *A. hydrophila* is the main pathogen that causes bacterial diseases in a variety of fish, for example, *Ctenopharyngodon idella*, *Cyprinus carpio*, etc. The most common disease caused by *A. hydrophila* is active Aeromonas septicemia, which can manifest as

hemorrhage, ulcers, abscess, ascites, and anemia (Ordas et al., 2013). Every year, high mortality rate (68% of fish infected *A. hydrophila* not survived) caused billion USA dollars of economic losses in the aquaculture production industry in China (Qu et al., 2015; He et al., 2015). It has been observed that 21 of MicroRNAs (miRNAs) were differentially expressed in the spleen of susceptible and resistant tilapia after *A. hydrophila* infection, indicating that miRNAs may be involved in *A. hydrophila* infection and may play an important role in it (El-Magd et al., 2019).

MicroRNA (miRNA), as a novel gene expression regulatory molecule, is involved in the regulation of

various biological processes, including cell differentiation, apoptosis, and immune defense (Catalanotto et al., 2016). miRNA is an endogenous non-coding small molecule single-stranded RNA of 19–25 nucleotides in length that can bind to the 3'-untranslated regions (3'-UTR) of target genes to prevent its translation or degradation (Qu et al., 2015; He et al., 2015; Wheeler et al., 2009; Takacs & Giraldez, 2010). Inflammation is a complex biological process caused by various stimuli, such as tissue damage, chemical stimuli, microbial infection and immune cell infiltration (Kowalski & Li, 2017). Studies have shown that some miRNAs are closely involved in the regulation of the immune response following teleost viral and bacterial infections (Andreassen & Høyheim, 2017). For example, during the infection of *Plecoglossus altivelis* with *Vibrio anguillarum*, miR-155 promoted pro-inflammatory activity and enhanced apoptosis in monocytes/macrophages (Nie et al., 2019). And miR-148 (Chu et al., 2017a), miR-214 (Chu et al., 2017b) and miR-19a (Zhao et al., 2018) inhibited MyD88 expression, which is the canonical adaptor for inflammatory signaling pathways downstream of members of the Toll-like receptor (TLR) and interleukin-1 (IL-1) receptor families and negatively regulated the bacterial inflammatory response.

In addition, miR-23a is elevated in chronic inflammatory reactions such as hepatitis and periodontitis (Li et al., 2020; Rajendiran et al., 2022), and is closely related to the expression and secretion of inflammatory cytokine interleukin-6 (IL-6) (Sheng et al., 2021). Research has shown that under acute inflammatory conditions, macrophages reduce the expression and secretion of inflammatory cytokine IL-6 by inhibiting the expression of miR-23a, thereby preventing excessive activation of macrophages, Regulate the immune function of macrophages and alleviate immune damage of the body (Roufayel & Kadry, 2017; Ginckels & Holvoet, 2022; Sheng et al., 2021). Although several different types of miRNAs have been studied, the role and molecular mechanisms of specific miRNAs in inflammation remain to be further investigated.

In this study, we detected the change in expression of miR-23a in kidney cells of *O. mossambicus* infected with *A. hydrophila*, determined the target genes of miR-23a using a dual-luciferase reporter system, and revealed the regulatory effect of miR-23a on downstream genes. The debaryomyces hansenii (*dha*), L-lactate dehydrogenase (*ldhba*), 7-L-lactate-hydroxysteroid dehydrogenase (*pdha1a*) and 3-L-lactate-hydroxybutyrate dehydrogenase (*pdha1b*) are genes downstream of glucose transporter 1 (*Glut1*) in the glucagon signaling pathway. Therefore, they were also measured in this study. The results would provide more theoretical basis for the further study of the regulatory mechanism of miRNAs on target genes and its role in inflammation.

## Materials and Methods

### Cell Culture and Bacterial Infection

The *O. mossambicus* kidney cell line was adherently growing cells and was purchased from the China Typical Culture Conservation Center (Beijing, China). The cells were cultured using M199 medium (Wolcavi biotechnology, Beijing, China) supplemented with 10% heat-inactivated bovine fetal serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and then the cells were incubated in a 2016BT thermostatic cell incubator (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) with 5% carbon dioxide and 28°C. Cells in the logarithmic phase went down to post-growth once every 3 d to 4 d. The *A. hydrophila* used in the bacterial infection experiment was AH10 strain, which was stored at the Aquatic Strain Conservation Center of Guangxi Academy of Fishery Sciences (No. AB2014155) and isolated from tilapia. Prior to the experiment, *O. mossambicus* kidney cells were seeded at  $2 \times 10^6$  cells/ml from 50 ml plates into 24-well plates for incubating 11.5 h. After washing with phosphate buffered saline (PBS) twice, the cells reached a growth density of 70–80% (Bao et al., 2022). M199 medium without antibiotics (containing 10% heat-inactivated bovine fetal serum) was added, followed by stimulation of the cells with 100 ml of *A. hydrophila* ( $10^3$  CFU/ml) (Feng et al. 2019). After 0.5 h of exposure, M199 medium supplemented with anti-serum was substituted. Infected *O. mossambicus* kidney cells were incubated and collected at 0, 6, 12, 24 and 36 h to analyse time-dependent expression of miR-23a, TANK binding kinase 1 (*tbk1*) and *glut1*. Three biological replicates were performed for each experimental group.

### Target Site Prediction and Plasmid Construction

The potential target genes of miR-23a were predicted using RNAhybrid software 2.1.2 (Boston, MA, USA). The 3'-untranslated regions (3'-UTR) fragments of the target gene containing the predicted miR-23a binding site were amplified using a T960 PCR (polymerase chain reaction) amplifier (Likang Biomedical Technology Holding Co., LTD, Shanghai, China). The amplicon was then inserted into the *Sac* I site of the pmirGLO luciferase reporter vector and the recombinant plasmid was synthesized using Seamless cloning kit (Biyun Biotechnology, Shanghai, China). All recombinant plasmids were verified by sequencing according to the method of Catalanotto et al. (2016).

### Dual-luciferase Reporter Assay

PmirGLO-*tbk1*, pmirGLO-*glut1*-luciferase reporter vector (Promega, USA) was co-transfected into tilapia kidney cells with miR-23a agomir (Genepharma Company, Shanghai, China) or a negative control (NC) (GMR-miR microRNA mimics FAM-labelled single-

stranded negative control, Genepharma Company). After 36 hours of transfection, tilapia kidney cells were lysed using a dual-luciferase reporter assay kit (Promega (Shanghai), Shanghai, China) to analyze luciferase activity according to detailed instructions, and this assay was repeated in triplicate for each treatment. The relative activity of luciferase activity (FL/RL) was obtained by standardizing firefly luciferase activity (FL) relative to sea pansy luciferase activity (RL) to verify the binding ability of the target gene 3'-UTR to miR-23a.

### Transfection Assay

The logarithmic growth phase of *O. mossambicus* kidney cells was inoculated into 24-well cell plates, when cell fluidity reached 70-80%, the cell was transferred to fresh Opti-MEM transfection medium (Gibco) and synthetic miR-23a agomir, antagomir (Genepharma company) and NC were transfected respectively into *O. mossambicus* kidney cells according to the instructions of Lipofectamine 3000 reagent (Thermo Scientific, USA). Three experimental replicates of each treatment were performed and cell samples were collected for 36 h after transfection.

### RNA Extraction and Quantitative Real-time PCR (qPCR)

Total RNA from each sample was extracted according to the instructions of TRIzol reagent (Promega). Firstly, 1 ml TRIzol reagent was added to distribute the lysate evenly on the cell surface. The cell lysate was transferred to a 1.5ml EP tube. After then, immediately it was turned upside down with wrist force until the cells and tissue powder was evenly dispersed without lumps, and left at room temperature for 5 minutes to completely separate the nucleic acid-protein complexes. Addition of 200  $\mu$ l chloroform to shake vigorously for 15 seconds, and stilled at room

temperature for 2 minutes. After that, centrifuged at 13,000 rpm for 10 minutes, 600  $\mu$ l of supernatant was transferred to a new 1.5EP tube. Added 600  $\mu$ l of isopropanol to the above 600  $\mu$ l of supernatant, turned it upside down several times, and placed it at -20°C for 5 minutes. After centrifuged at 13,000 rpm for 10 min and discard edthe supernatant to save the bottom total RNA pellet. Added 1ml of 70% ethanol to each tube of the pellet to shake, then centrifuged at 13,000 rpm for 5 min. The bottom RNA pellet was saved. Repeat step 8 and wash again. Poured off the washing solution, centrifuged again for a short time for 10 seconds, absorbed the remaining washing solution with a 10  $\mu$ l tip, and placed it at room temperature to evaporate the ethanol (20min). Added 20-100 $\mu$ l TE Buffer or RNase Free H<sub>2</sub>O to each tube to dissolve total RNA. Total RNA concentration and quality were measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific), and an agarose gel electrophoresis instrument (2018 Bio-Rad mini-GT, Bio-Rad, USA) and the agarose gel concentration was 1.1%. cDNA was obtained by reverse transcription of RNA using an RNA reverse transcription kit (TaKaRa company, Tokyo, Japan) and Prime Script™ RT reagent Kit with gDNA Eraser kit (TaKaRa company). The qPCR reaction conditions for total RNA: 95°C and 30 s; 95°C and 5 s; 60°C and 30 s; 40 cycles. The qPCR reaction conditions for miRNA: 95 °C and 15 min; 94°C and 15 s; 55°C and 30 s; 70°C and 30 s; 40 cycles.

Melting curve analysis was performed after each assay to ensure the specificity of the amplified product. According to the report of Catalanotto et al. (2016), relative expression levels of miRNA and mRNA were performed using miR-101a and 18S rRNA as reference genes, respectively, and three parallel samples for each assay. Primers used for qPCR analysis are shown in Table 1.

**Table 1.** Sequences of primer used for qPCR

Primers name	Sequences
miR-23a	ATCACATTGCCAGGGATTTCCA
miR-101a	TACAGTACTGTGATAACTGAAG
<i>tbk1</i> -F	TTGGCACAGTTTGTCTTATCG
<i>tbk1</i> -R	CCTGTCTGGGTTTACTGTGA
<i>glut1</i> -F	CCTGTCTGGGTTTACTGTGA
<i>glut1</i> -R	GCAAGGCAAGAGCAATGGTC
<i>ldha</i> -F	ACAAGATAGTGCGGATAA
<i>ldha</i> -R	GTAACGGAAACGAGCAGA
<i>ldhba</i> -F	ATCGGCACAGATAAAGACG
<i>ldhba</i> -R	CACAGGGCAGACTCAGGTA
<i>pdha1a</i> -F	GGTGGTATCTGCCAGGAC
<i>pdha1a</i> -R	GATCTCACGAACAGTGCC
<i>pdha1b</i> -F	GGGACATCAGTGGAAACGA
<i>pdha1b</i> -R	CTAATCATGCGGTCTTC
18S rRNA-F	GGACACGGAAAGGATTGACAG
18S rRNA-R	CGGAGTCTCGTTCGTTATCGG

F, forward sequences (5'→3'); R, reverse sequences (5'→3')

## Statistical Analysis

All experiments were carried out in triplicate. The relative expression of the detected genes was calculated using the  $2^{-\Delta\Delta CT}$  method, and differences between treatments were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparisons. All data were expressed as mean  $\pm$  standard deviation (mean  $\pm$  SD) and analyzed using Origin 10.1 software. The \* $p < 0.05$  indicated a significant difference and \*\* $p < 0.01$  indicated a highly significant difference.

## Results

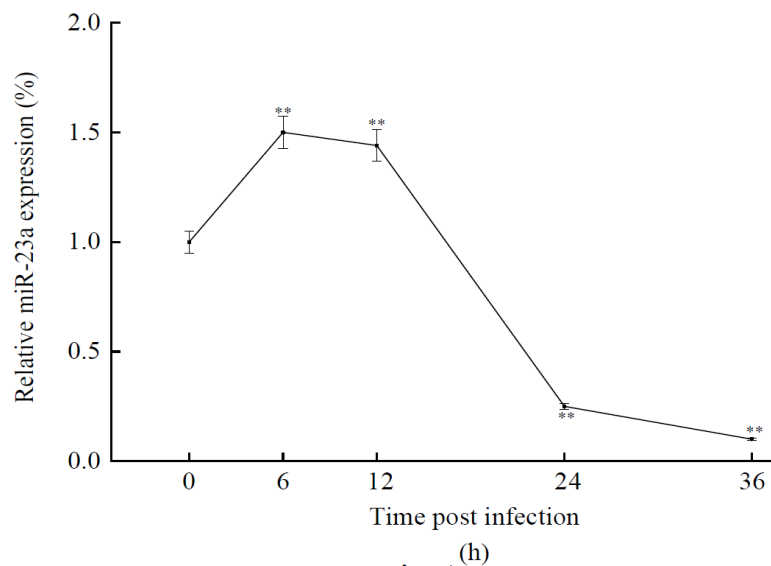
### Effect of *A. hydrophila* Infection on miR-23a in *O. mossambicus* Kidney Cells

MiR-23a expression after infection of tilapia kidney cells was determined by qPCR. The results showed that miR-23a expression levels were initially up-regulated and then decreased ( $p < 0.01$ ) (Figure 1). After infection for 6 h and 12 h, the expression levels of miR-23a was

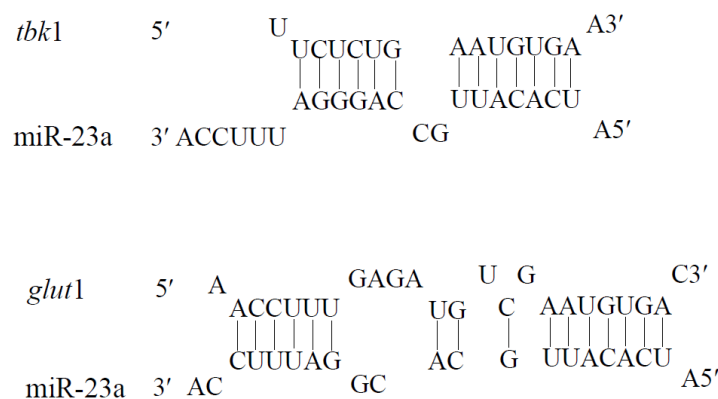
significantly higher than controls ( $p < 0.01$ ). At 24 h and 36 h, miR-23a expression levels was significantly lower than that of baseline (0 h) ( $p < 0.01$ ). These results indicated that expression levels of miR-23a were significantly affected during the *A. hydrophila* infected *O. mossambicus* kidney cells.

### Identification of the miR-23a Target Genes

According to the previous reports, *tbk1* and *glut1* were predicted as target genes for miR-23a using the bioinformatics software RNA hybrid. The binding sites of miR-23a in the 3'-UTR of *tbk1* and *glut1* are shown in Figure 2. The qPCR data indicated that the expression of *tbk1* and *glut1* was first up-regulated and then down-regulated in *A. hydrophila*-stimulated *O. mossambicus* kidney cells, which negatively correlated with miR-23a expression (Figure 3). A 3'-UTR containing the miR-23a binding site of *tbk1* and *glut1* was cloned downstream of the luciferase reporter vector to obtain a pmirGLO reporter plasmid named pmirGLO-*tbk1* and pmirGLO-*glut1*. Luciferase activity in the co-transfected miR-23a



**Figure 1.** Expression level of miR-23a in tilapia kidney cells upon *A. hydrophila* infection. Error bars indicate the mean and standard deviation ( $n=3$ ). \*\* represents very significant difference ( $p < 0.01$ ).



**Figure 2.** Prediction of miR-23a target genes. Schematic diagram of the binding site of miR-23a to 3'-UTR of *tbk1* and *glut1*.

agomir treatment was significantly reduced. The pmirGLO-*tbk1* and pmirGLO-*glut1* luciferase activity was reduced by approximately 64.3% and 55.5%, respectively ( $p < 0.01$ ) (Figure 4). The above data suggested that miR-23a can be targeted to complementary sites on the 3'-UTR of *tbk1* and *glut1*, thus inhibiting its expression.

### Changes in miR-23a targets *tbk1* and *glut1*

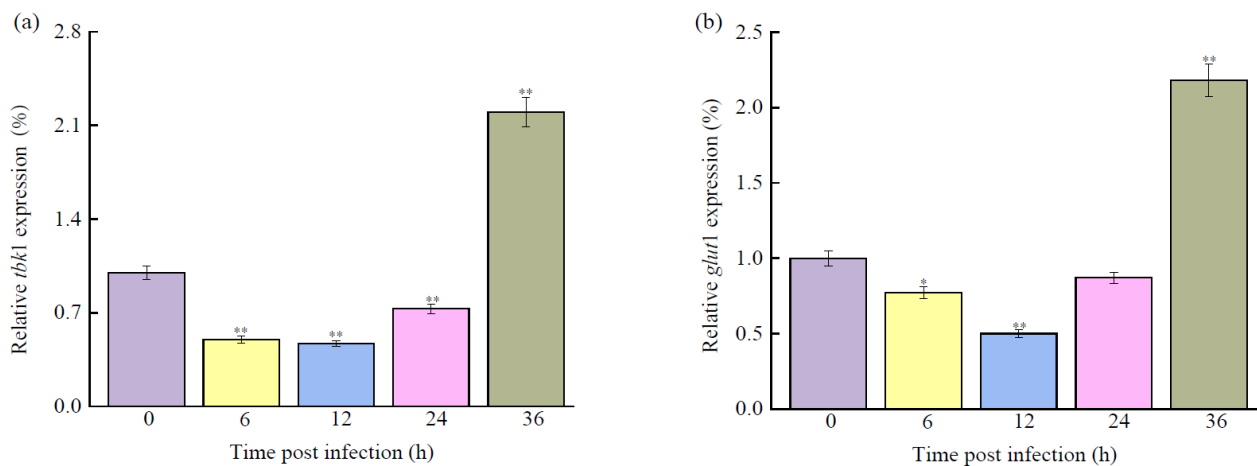
After overexpression of miR-23a, its relative expression ( $120.04 \pm 15.33$ ) was significantly increased compared to NC ( $1.00 \pm 0.04$ ) ( $p < 0.01$ ) (Figure 5a), indicating that miR-23a agomir transfection was effective and could specifically up-regulate the miR-23a expression in *O. mossambicus* kidney cells. The relative expressions of the target genes *tbk1* and *glut1* were determined from 18S rRNA used as a reference gene (Figure 5b). The result showed that the relative expression of *tbk1* and *glut1* in miR-23a agomir treatment was significantly inhibited compared to NC treatment, with expression values of  $0.44 \pm 0.04$  and

$0.33 \pm 0.01$ , respectively. When miR-23a was inhibited, the relative expression of miR-23a was  $0.04 \pm 0.00$ , which was significantly lower than the NC expression of  $1.00 \pm 0.04$  ( $p < 0.01$ ) (Figure 5c), indicating that miR-23a antagomir was efficiently transfected and was able to specifically down-regulate miR-23a expression in *O. mossambicus* kidney cells.

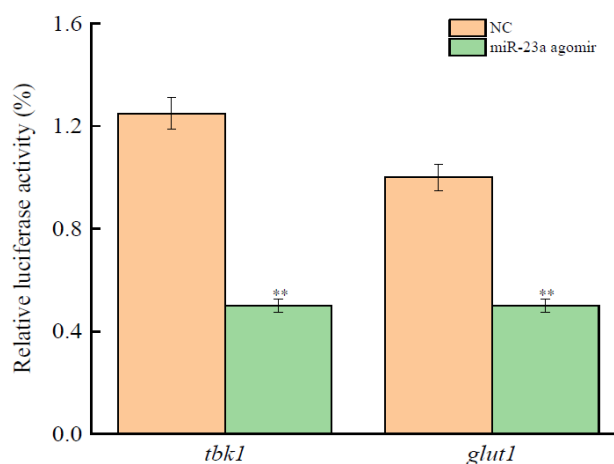
The relative expression of the target genes *tbk1* and *glut1* was detected as a reference gene on 18S rRNA (Figure 5d). As compared to NC, the expression levels of target genes *tbk1* and *glut1* in miR-23a antagomir transfections showed an up-regulation, with expression values of  $1.81 \pm 0.19$  and  $1.76 \pm 0.11$ , respectively.

### The miR-23a Regulates the Downstream Genes

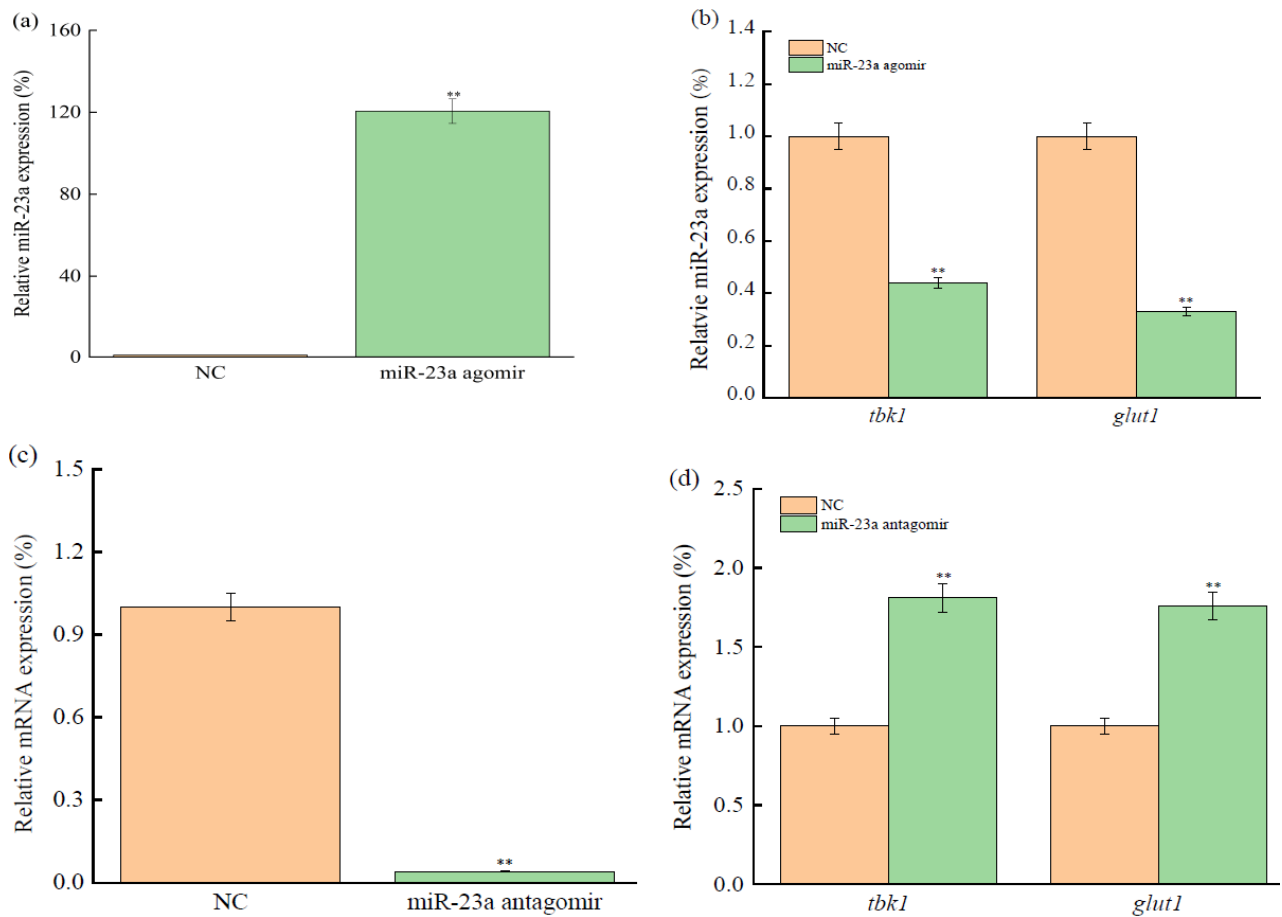
The *dha*, *ldhba*, *pdha1a* and *pdha1b* are genes downstream of *Glut1* in the glucagon signaling pathway. The qPCR data showed that overexpression of miR-23a significantly reduced the expression of these downstream genes *ldha*, *ldhba*, *pdha1a*, and *pdha1b* ( $p < 0.01$ ) (Figure 6).



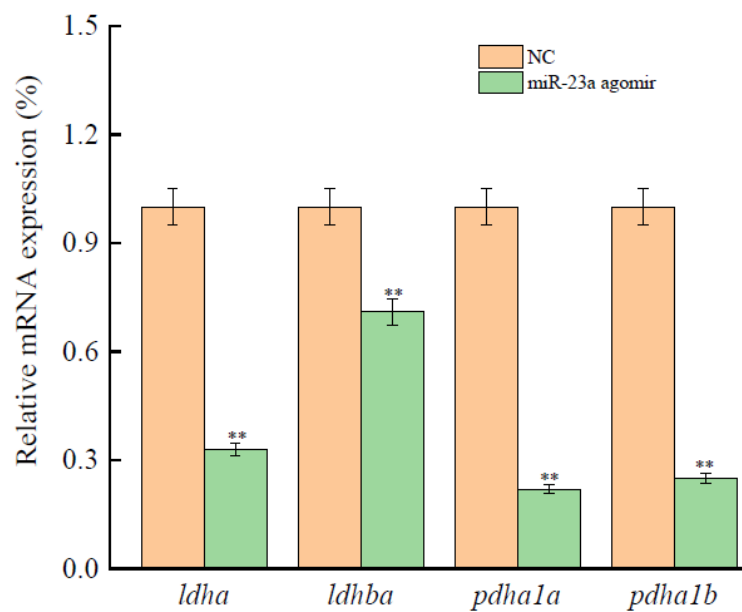
**Figure 3.** Expression level of *tbk1* (a) and *glut1* (b) in tilapia kidney cells upon *A. hydrophila* infection. Error bars indicate the mean and standard deviation ( $n=3$ ). \* represents significant difference ( $p < 0.05$ ), \*\* represents very significant difference ( $p < 0.01$ ).



**Figure 4.** Dual luciferase reporter system validates the targeting of miR-23a on *tbk1* and *glut1*. Dual luciferase activity assay after miR-23a agomir and recombinant plasmid (pmirGLO-*tbk1*, pmirGLO-*glut1*) were transfected to tilapia kidney cells. Error bars indicate the mean and standard deviation ( $n=3$ ). \*\* represents very significant difference ( $p < 0.01$ ).



**Figure 5.** miR-23a targets *tbk1* and *glut1*. Expression of miR-23a (a) and target genes *tbk1* and *glut1* (b) by qPCR after miR-23a agomir was transfected to tilapia kidney cells, expression of miR-23a (c) and target genes *tbk1* and *glut1* (d) by qPCR after miR-23a antagonist was transfected to tilapia kidney cells. Error bars indicate the mean and standard deviation (n=3). \*\* represents very significant difference (p<0.01).



**Figure 6.** Regulatory effect of miR-23a on downstream genes. Expression level of downstream genes *ldha*, *ldhba*, *pdha1a* and *pdha1b* by qPCR after miR-23a agomir was transfected to tilapia kidney cells. Error bars indicate the mean and standard deviation (n=3). \*\* represents very significant difference (p<0.01).

## Discussion

The inhibitory action of the inflammatory response and the activation level of inflammatory factors reduces the negative effect of infection on the body, so that improving the body's tolerance to bacterial sepsis (Wheeler et al., 2009; Takacs & Giraldez, 2010). But the negative regulatory inflammatory response has not developed in time (Qu et al., 2015; He et al., 2015). Therefore, in order to suppress the inflammatory dysfunction that occurs during bacterial septicemia, it is crucial to investigate how to prevent the over activation of inflammatory signaling pathways and the irregular expression of associated inflammatory factors. Recent studies continue to reveal the involvement of miRNA in the regulatory process of the body's inflammatory response. In a related *Arabidopsis* study, miRNA was found for the first time to be involved in host resistance to bacterial infection. miR-393 can inhibit the auxin signaling pathway and thus influence the pathogenic process in *Pseudomonas syringae*-infected *Arabidopsis* (Navarro et al., 2006). Reinhart et al. (2000) has been found that miRNA regulated the immune response by targeting mRNA, which both increased the inflammatory response to pathogen invasion and Abdel-Wahab et al. (2021) reported that attenuated the inflammatory response to protect the body. However, the mechanism of action of miRNA in regulating bacterial invasion and intracellular pathogen infection remains to be investigated.

Toll-like receptors (TLRs) are important receptors that recognize various types of pathogenic molecules, as exposed to different ligands, TLRs can trigger the activation of different signaling cascades, resulting in the production of inflammatory cytokines or interferons (IFNs) and initiating cell survival or proliferation pathways (Brown et al., 2011). The miR-23a was involved in the TLR-mediated innate immune response and revealed the functional activity of miR-23a in immune cells (Peng et al., 2015). The expression of miR-23a was reduced when macrophages were stimulated with TLR ligands (Rathore et al., 2012; Peng et al., 2015). The previous results show that miR-462 regulates *cx32.2*, *slc9a3.1* and *tbk1* (Wang et al., 2015), miR-21 regulates *jdk* and *ccr7* (Tao et al., 2019), miR-731 regulates *Gadd45a* (Fang et al., 2020), miR-23a-3p and miR-23a-5p regulate *CiGadd45ab* (Feng et al. 2019), and induce tilapia inflammation and apoptosis after *A. hydrophila* infection. This current study showed that miR-23a expression was significantly changed in *O. mossambicus* kidney cells infected with *A. hydrophila*, suggesting that miR-23a is involved in the regulation of the immune response.

During bacterial infection of *O. mossambicus* kidney cells, miR-23a expression increased significantly at 6 h and 12 h, but decreased significantly after 24 h of infection. Hence we speculated that miR-23a may be involved in the early regulatory response to bacterial infection in tilapia. Similar observations were made in

other fish where miRNA expression patterns produced significant changes following bacterial infection (Guan et al., 2020). However, the regulatory mechanism of the miR-23a immune response in *O. mossambicus* infected with *A. hydrophila* has not been deeply explored. Bioinformatic RNA hybrid prediction software suggests that *tbk1* and *glut1* may be potential target genes for miR-23a. The TBK1 is a class of family I B serine/threonine protein kinases involved in various innate immune responses, including cell proliferation (Sarraf et al., 2019), apoptosis (Yu & Cleveland, 2018) and autophagy (Kumar et al., 2019). The GLUT1 is the first cloned glucose transporter, and activated T cells and effector T cells can selectively target to T cell glucose metabolism to prevent inflammatory responses and promote tolerance and immunosuppression (Macintyre et al., 2014). In present study, the expression patterns of *tbk1* and *glut1* were negatively correlated with changes in miR-23a. Analysis of the dual luciferase reporter system confirmed that *tbk1* and *glut1* are target genes of miR-23a. The results of miR-23a agomir and antagomir transfection assay showed that the expressions of *tbk1* and *glut1* were also down-regulated, and up-regulated respectively due to overexpression and inhibition of miR-23a, further demonstrating the negative regulatory relationship between miR-23a and *tbk1*, miR-23a and *glut1* and also indicating that *tbk1* and *glut1* are target genes of miR-23a (Michael et al., 2014; Sarraf et al., 2019).

Furthermore, the expression of *glut1* downstream genes *ldha*, *ldhba*, *pdha1a* and *pdha1b* was also repressed after miR-23a agomir transfection, indicating that miR-23a was able to target *glut1* to affect downstream gene expression (Kowalski & Li, 2017; Kumar et al., 2019). LDHA and LDHBA belong to the lactate dehydrogenase family, and LDHA may be a potential therapeutic target in autoimmune diseases through its anti-inflammatory effect in macrophages, as inhibited LDHA produces an anti-inflammatory effect (Song et al., 2019; Yi, 2017; Ginckels & Holvoet, 2022). PDHA1A and PDHA1B are pyruvate dehydrogenases that regulate glucose metabolism and cardiac AMPK signaling, and deficiency of PDHA1A and PDHA1B can aggravate hypertrophy, inflammation and fibrosis in myocardial cells (Sun et al., 2016). Thus, the present study reported that miR-23a further contributed to the progression of the inflammatory response by regulating the expression of downstream genes *ldha*, *ldhba*, *pdha1a* and *pdha1b*.

## Conclusion

MicroRNA (miRNA) plays an important role in *A. hydrophila* infection and regulates the immune response by targeting mRNA, including increasing the inflammatory response to pathogen invasion and attenuating the inflammatory response to protect the body. Although many types of miRNAs have been studied, the role and molecular mechanisms of specific

miRNAs in inflammation still need to be further investigated. This study showed that the expression pattern of miR-23a was significantly altered in *O. mossambicus* kidney cells after *A. hydrophila* infection, revealing its involvement in the immune response. At the same time, *tbk1* and *glut1* were identified as target genes of miR-23a. In addition, miR-23a could target *glut1* to affect downstream gene expression. The results provided a new idea to study the regulation of miR-23a in bony fish and further understanding of miRNA-mediated multiple target genes to regulate innate immunity in fish.

### Ethical Statement

Not applicable

### Funding Information

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### Author Contribution

Huawei Ma and Zhide Ruan conceived and managed the project, Shiya Ya and Hui Gan obtained funding, designed the study, and drafted the manuscript, done experiment and revised manuscript. Zhichan He operated data analysis and done experiment. Chuanyan Pan and Xu Luo done experiment and sampling. Min Lv and Wenmin Zhang edited and revised manuscript.

### Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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