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The Oxytetracycline and Florfenicol Effect on the Immune System and Oxidative Stress Response of the SHK-1 Cell Line of *Salmo salar*

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Abstract: The aquaculture industry in Chile, as in the rest of the world, has rapidly grown, becoming a crucial economic sector. However, diseases pose a major threat, causing significant economic losses and environmental impacts. Various antimicrobials, particularly Oxytetracycline and Florfenicol, are used to combat these diseases, which has boosted production and mitigated economic losses. However, excessive antibiotic use has led to pathogen resistance, necessitating higher doses. This overuse can cause side effects in fish, including liver damage and immunosuppression. This study aimed to determine the impact of multiple doses of florfenicol and oxytetracycline on the SHK-11 cell line of *Salmo salar* by analyzing the expression of genes related to innate immunity and oxidative stress by qRT-PCR in addition to the quantification of immune system proteins via dot blot. The experimental treatments were the following: cells were stimulated with different concentrations of oxytetracycline (0.25, 0.5, and 1.5 µg/mL) and florfenicol (1, 10, and 20 µg/mL) for time kinetics of 0.5, 1, 3, 6, 12, 24, and 48 h. For both cases, controls consisting of cells without antibiotics were included. The expression of the immune system genes was mostly inhibited compared to the control. However, it was observed that TLR-1 and MyD88 present a joint activation pattern at different times and concentrations for both antibiotics. Regarding the expression of CAT and GPx, transcripts were increased in the early stages of stimulation with oxytetracycline and florfenicol, followed by a subsequent decrease in gene expression. This study provides relevant information to understand the effect of antibiotics at the cellular level in one of the most important species for global aquaculture, the Atlantic salmon.

Keywords: antibiotic; physiology; immune system; Atlantic salmon; fish

Key Contribution: The antibiotics are affecting the fish, modifying the immune system and oxidative stress response.

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1. Introduction

Aquaculture has been one of the fastest-growing and economically important activities in the world in recent years [1], bringing economic and social growth. However, with intensive production, the three main species of salmonids (salmon and trout) are farmed in Chile: Atlantic salmon (*Salmo salar*), by far the most farmed; Pacific salmon or coho salmon (*Oncorhynchus kisutch*); and rainbow trout (*Oncorhynchus mykiss*). These fish are affected by multiple factors, ranging from environmental factors to diseases [2–4]. In

Chile, disease is one of the main problems related to aquaculture activity [4]. This is why diseases pose a significant risk to aquaculture, as they appear at various stages of fish farming and are a leading cause of considerable economic losses. Furthermore, the dense populations within culture cages facilitate the rapid spread of infectious outbreaks caused by harmful microorganisms. The use of antimicrobials has made it possible to increase production considerably, thus avoiding economic losses [5]. These compounds are usually administered through food to both sick and healthy animals [6,7].

The pharmaceutical industry has biologically active chemical compounds that can inactivate and/or eliminate pathogenic microorganisms to keep disease-causing pathogens under control [8,9]. These encompass bactericides, which are characterized by causing permanent harm to bacteria, leading to their demise. Conversely, bacteriostatics do not kill the bacteria but inhibit their growth or metabolism, eventually causing them to die without reproducing. The leading causes of mortality in salmonids are different bacteria such as *Piscirickettsia salmonis* ('SRS'), *Renibacterium salmoninarum* (BKD), and *Tenacibaculum dicentrarchi* (Tenacibaculosis). However, the latter has been considered emerging since 2018 [4]. Pathogens are mainly dealt with using 'oxytetracycline (OTC)' and 'florfenicol (FLO)' [4]. Both antibiotics are broad-spectrum with bacteriostatic action. OTC is a compound that inhibits protein synthesis in the 30S subunit of the bacterial ribosome, and FLO inhibits protein synthesis in the 50S subunit of the bacteria, preventing their replication and then their death [10–12].

The use of antibiotics in Chile is quite elevated, with Chilean farms using a total of 463.4 tons of antimicrobials, and an annual consumption of 0.47 kg of antimicrobials per ton of harvested salmon [13], resulting in bacteria that are becoming more resistant. Bacteria possess various resistance mechanisms, such as acquired resistance through mutations, acquiring mobile genetic elements (plasmids or transposons), or producing enzymes that hydrolyze and inactivate the antibiotic, among other intracellular and extracellular mechanisms [14–16].

Overusing antibiotics may lead to side effects like liver damage and a weakened immune response. According to Nakano et al. [17], excessive doses of OTC influence stress-related biomarkers and redox status in coho salmon, causing tissue damage, especially to the liver. Similarly, Rodrigues [18] considers that OTC can cause a potential health risk to aquatic organisms, reporting that OTC can interfere with biochemical pathways in *Oncorhynchus mykiss*, causing oxidative effects in tissues and, in turn, generating genotoxicity as an indirect result of oxidative stress.

Immunosuppression will affect the activation cascade of the innate immune system at the cellular level, which is carried out by a few PRRs (Pattern Recognition Receptors) that recognize the PAMPs (Pathogen-Associated Molecular Patterns) of invading microbes. The recognition and binding of PAMPs by specific TLRs (Toll-like receptors) activate cell signaling cascades through MyD88 (primary myeloid differentiation response 88)-dependent and MyD88-independent pathways. This MyD88 binds to the TIR domain of the receptor, is recruited, and activates cytoplasmic kinases, which leads to the activation of several cytosolic enzymes to induce the production of proinflammatory cytokines such as IFN, IL1β, IL6, IL12, and TNFα [19,20].

According to Nakano et al. [17], liver damage will affect oxidative processes, such as inducing oxidative stress as a result of a cyclic oxidation-reduction process (redox cycle) [21]. Antioxidants come in 'enzymatic' and 'non-enzymatic' forms. Among the primary enzymatic antioxidants are the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx); however, another series of specialized enzymes with indirect antioxidant functions must be taken into account, such as glutathione reductase (GR), whose function is to regenerate glutathione to its reduced form (GSH), and glutathione-S-transferase (GST), which is involved in the transport and removal of reactive components and the transport system of conjugates with glutathione [22,23].

Different research models encompass fish used in aquaculture, ornamental fish, and, though less frequently, wild fish. Certain species, like salmonids, exhibit significant adapt-

ability. Recently, the principles of 'animal welfare' and the 3Rs rule have aimed to enhance procedures. This optimization can be achieved through primary cell cultures or cell cultures, such as Atlantic salmon head kidney cells (SHK-1), which are highly effective for obtaining direct cellular responses [24,25].

Because of this, we set the following objective: 'to determine the innate immune and oxidative response of SHK-1 cells when challenged with different concentrations of OTC and FLO'. To monitor these responses, we will use qRT-PCR determinations of *TLR1*, *MyD88*, *IFNγ* and *NFκb*, catalase (*CAT*), and glutathione peroxidase (*GPx*) genes and the quantification of NFκb proteins by dot blot.

2. Materials and Methods

The cells were cultured at 18 °C in T-25 flasks (Corning, Sigma-Aldrich, Burlington, MA, USA) in Leibovitz L-15 medium supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Waltham, MA, USA). The confluent flasks were subcultured by distributing the medium uniformly between them and adding fresh medium to complete the volume, following the Pontigo and Vargas-Chacoff and Nualart et al. protocol [24,25].

The experimental stimuli were carried out at 0.5, 1, 3, 6, 12, 24, and 48 h and with 0.25, 0.5, and 1.5 µg/mL of OTC or 1, 10, and 20 µg/mL of FLO in supplemented L-15 medium. Phosphate-buffered saline (PBS) alone was used as a control. The OTC doses chosen are similar to those described by Tafalla et al. [26], and the FLO doses chosen are described by Martinsen et al. [27], following which the doses used were calculated from the pharmacokinetic study of OTC and FLO in Atlantic salmon in seawater using as a reference plasma levels of antibiotics [14,27]. The experiment was assayed in duplicates and by two technical replicates for RT-qPCR analyses.

2.1. Gene Expression Analyses

RNA was isolated from cell pellets using TRIzol reagent (Invitrogen) according to the manufacturer's guidelines and then processed with amplification grade DNase I (1 U/µg RNA, Invitrogen). SuperScript III RNase H Reverse Transcriptase (Invitrogen) synthesized first-strand cDNA with oligo-dT18 primers and 1 µg of total RNA at 50 °C for 50 min. Experiments were conducted with an AriaMx Real-Time PCR System. For qRT-PCR analyses, cDNA diluted to 100 ng served as the template, and the Brilliant SYBR® Green qPCR Master Mix (Stratagene, La Jolla, CA, USA) was utilized. Primers were designed for TLR1, MyD88, INFδ, NFκb, catalase (CAT), glutathione peroxidase (GPx), and 18S (housekeeping gene) (Table 1). All reactions were performed in triplicate and in a total volume of 14 µL, which contained 6 µL SYBR® Green, 2 µL cDNA template, 1.08 µL of each primer, and 4.92 µL of PCR-grade water. The applied PCR protocol was as follows: 95 °C for 10 min, then 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and lastly 95 °C for 15 s. After each PCR, a melting curve analysis of the amplification products was conducted to verify the presence and amplification of only one product. TLR1, MyD88, IFNγ, NFκb, catalase (CAT), and glutathione peroxidase (GPx) were analyzed using the comparative Ct (ΔΔCt) method [28]. PCR products were visualized on a 2% agarose gel, purified using the E.Z.N.A Gel Extraction Kit (Omega Biotek, Norcross, GA, USA), and sequenced by Macrogen Inc (Seoul, Republic of Korea). The primers' efficiency was checked between 100 and 103% (Table 1).

Table 1. Primer sequences for expression analysis.

Primer	Nucleotide Sequences (5′→3′)	Efficiency (%)	GenBank No/Reference
TLR-1 Fw	TCCGGAGACGTTTCATCCCA	102.91	MF945984
TLR-1 Rv	GAGGTTCAGCGCTAACAGCA		
MyD 88 Fw	CTTTCACAACCACCGAAGCC	101.6	Martinez et al., 2018 [29]
MyD 88 Rv	TACAAACCGAAACCGCTCCT		
INF-γ Fw	GCCGTGTGTTGGTTTTTGATTT	100.1	Martinez et al., 2018 [29]
INF-γ Rv	GTGTCTGTCTGACTGATGGTGA		
NFκ-b Fw	AAAGTGCCAGTACCAAGCCC	102.5	Martinez et al., 2018 [29]
NFκ-b Rv	CATGCTGATGAGCTACTGTTGTT		
CAT Fw	CTGCCAGCAACCCAGATTAT	101.9	NM_001140302.1
CAT Rv	ACCAAACCTTGGTGAGATCG		
GPx Fw	GAACTGCAGCAATGGTGAGA	102.7	Pedro et al., 2019 [30]
GPx Rv	CATGAGAGAGATGGGGTCGT		
18S Fw	GTCCGGGAAACCAAAGTC	102.1	Pedro et al., 2019 [30]
18S Fw	TTGAGTCAAATTAAGCCGCA		

2.2. Immunodetection of NFκb in SHK-1 Cells (Dot Blot for NFκb)

Total protein extraction was performed from SHK-1 cells and subsequently quantified using the PierceTM BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). NFκb was immunodetected using the dot blot technique, loading 30 µg of total protein per spot. The membrane was blocked with 6% albumin and incubated with primary anti-NFκb (Santa Cruz, Starr County, TX, USA) and anti-Actin (Cell Signaling, Danvers, MA, USA) antibodies. It was then incubated with a secondary anti-mouse antibody (Cell Signaling) coupled to the HRP enzyme for its subsequent detection. Images were captured on the G: BOX unit using Genesys Software 4.3.8.0 (SynGene, model Chemi XRQ) and quantified with ImageJ Software 1.45.

2.3. Statistical Analyses

All data are expressed as the mean ± standard error of the mean (S.E.M.). PCR efficiency was assessed through linear regression analysis of sample data using LinRegPCR. Gene expression was evaluated by conducting a two-way ANOVA, with various stimuli and time serving as factors for variance. The data were previously checked for normality and homoscedasticity. Tukey's HSD test was carried out after ANOVA as a posterior analysis test.

3. Results

3.1. Oxytetracycline (OTC)

The expression of innate immune genes like *TLR-1* was highest at 6 and 12 h at 1.5 µg/mL and 1.0 µg/mL after 3 h of exposure, compared to the control group (Figure 1A). *MyD88* gene expression was highest at 0.5 and 6.0 h at 1.5 µg/mL, and at 3 h at 0.25 and 0.5 µg/mL. During the last hours of the experiment, 24 and 48 h, the expression was downregulated (Figure 1B). Meanwhile, *INFγ* presented upregulation at 12 h in 0.25 µg/mL (Figure 1C). *NFκb* gene expression showed upregulation at 6 h with 1.5 µg/mL (Figure 1D).

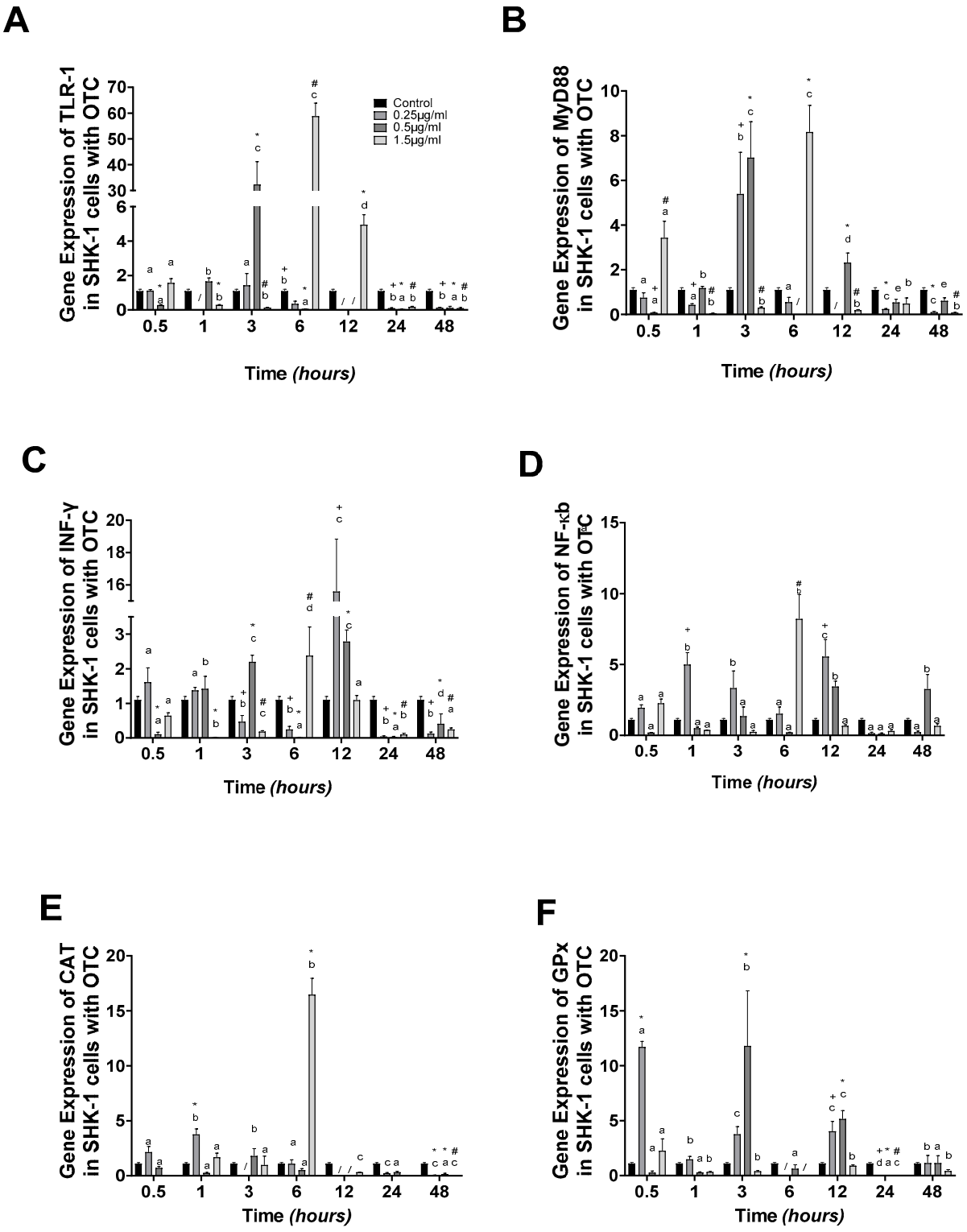

Figure 1. SHK-1 cells treated with oxytetracycline: gene expression. (**A**) *TLR-1*, (**B**) *MyD88*, (**C**) *INFγ*, (**D**) *NFκb*, (**E**) *CAT*, and (**F**) *GPx* in SHK-1 cells treated with oxytetracycline (OTC). The relative expression of genes was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, /, #, and +) over the bars indicate statistical differences among treatments (control and different doses of antibiotic) at the same time. Two-way ANOVA followed by Tukey's test ($p < 0.05$).

Our research thoroughly investigated the oxidative stress response, as determined by the gene expression of catalase (*CAT*). We found that *CAT* was upregulated at 6 h in 1.5 µg/mL, almost 9-fold compared to the control group. After this time, the expression was downregulated at 12, 24, and 48 h (Figure 1E). In the case of the *GPx* transcript, upregulation was observed at 0.5 and 3 h in 0.25 and 0.5 µg/mL, respectively (Figure 1F).

The dot blot results of NFκb proteins provide further evidence of the reliability of our data. Compared to the control group, protein concentrations decreased at 1, 6, and 48 h (Figure 2A,B).

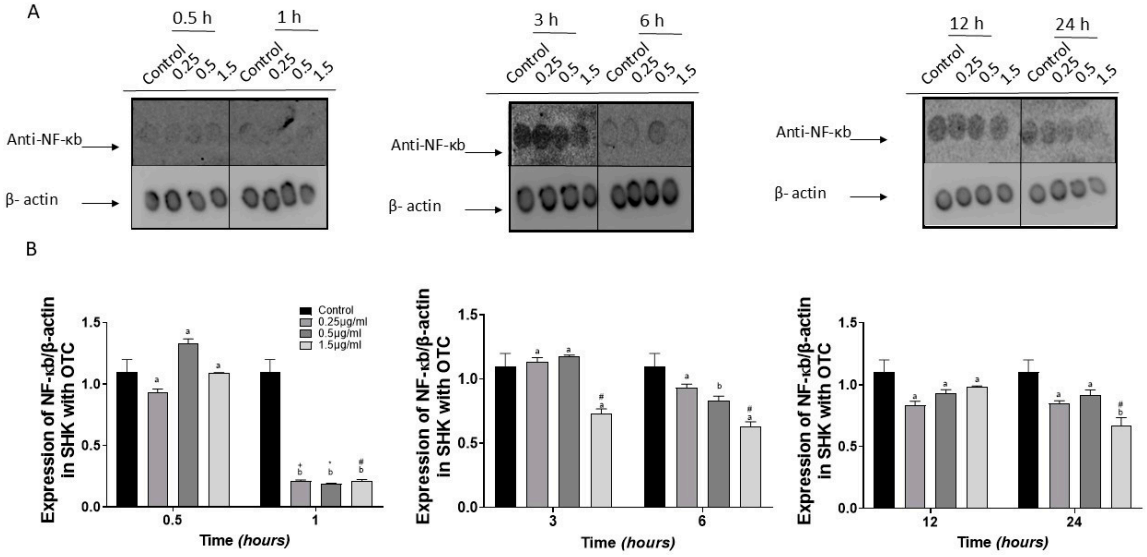

Figure 2. Immunodetection of NFkb in SHK-1 cells treated with oxytetracycline (OTC). (**A**) a representative image of the dot blot assay and (**B**) its respective graph. The quantification of expression of NFκb used b-actin as the internal reference control. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, #, and +) over the bars indicate statistical differences among treatments (control and different doses of antibiotic) at the same time. Two-way ANOVA followed by Tukey's test ($p < 0.05$).

3.2. Florfenicol (FLO)

The gene expression results of *TLR-1* indicated that it was in line with OTC concentration, with the highest gene expression at 20 µg/mL at 0.5 h. At 1 h, it presented the highest levels of expression at 1 and 10 µg/mL, whereas at 20 µg/mL, it was similar to the control group. At 12, 24, and 48 h, the experimental groups presented downregulation compared to the control group (Figure 3A). *MyD88* gene expression was highest at 0.5 and 12 h at 20 µg/mL and 1 µg/mL, respectively (Figure 3B). Meanwhile, *INFγ* showed upregulation at 12 and 48 h at 10 µg/mL (Figure 3C). The *NFκb* gene expression presented upregulation at 0.5 h at 10 and 20 µg/mL, but after 3 h of experimentation, the *NFκb* gene expression was downregulated (Figure 3D).

The oxidative stress response was determined by *CAT* gene expression, which was upregulated at 0.5 h at 1 and 20 µg/mL almost 40-fold compared to the control group. After this time, at 1 h at 1 and 10 µg/mL, it showed upregulation, but 4-fold compared to the control group (Figure 3E). Meanwhile, *GPx* gene expression showed upregulation at 0.5 and 1 h at 1 and 10, and again at 10 µg/mL, respectively (Figure 3F).

The dot blot results of NFκb proteins show that at 1, 6, and 12 h, protein concentrations increased compared to the control group, but at different FLO concentrations (Figure 4A,B).

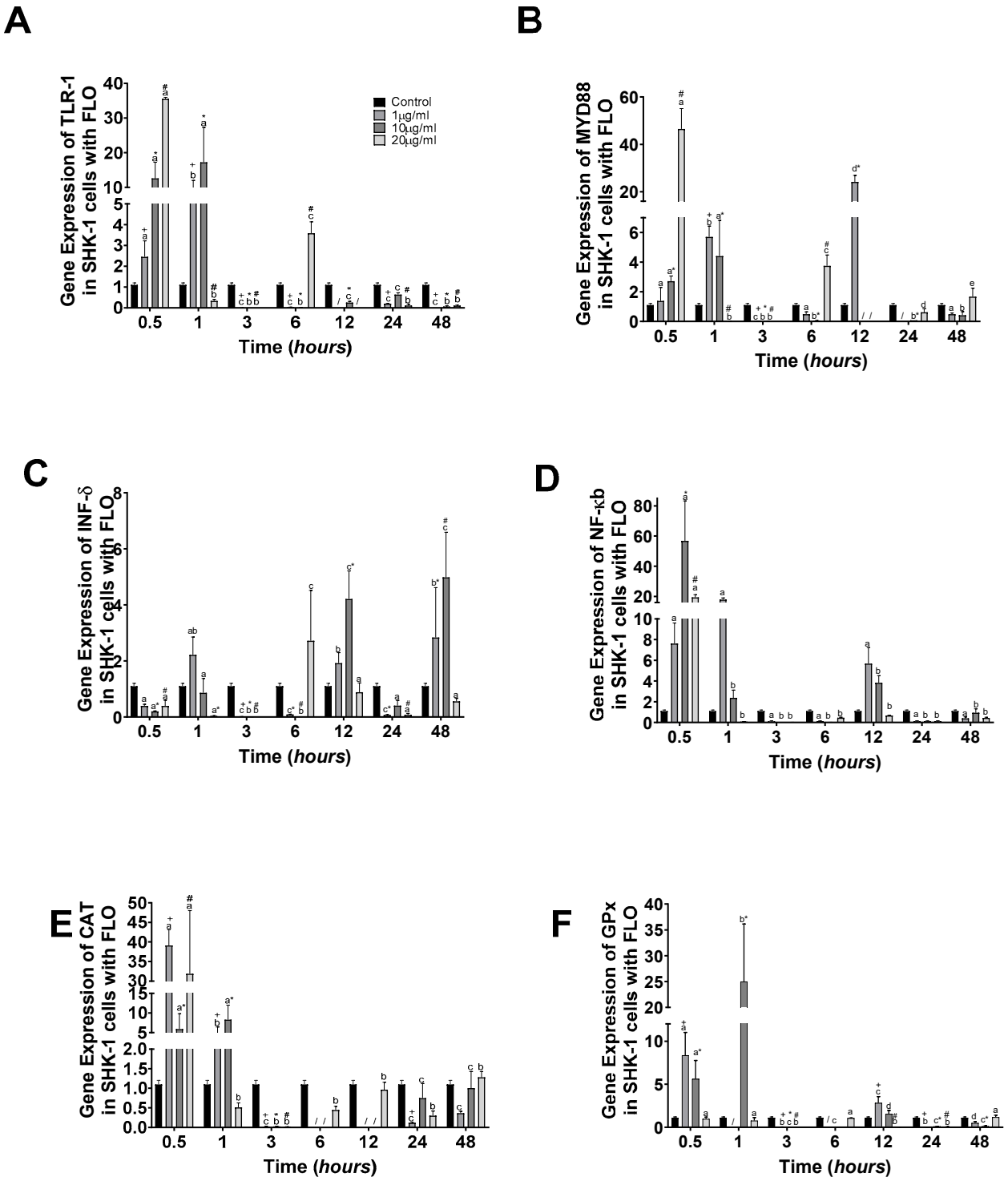

Figure 3. SHK-1 cells treated with florfenicol: gene expression. (**A**) *TLR-1*, (**B**) *MyD88*, (**C**) *INFγ*, (**D**) *NFκb*, (**E**) *CAT*, and (**F**) *GPx* in SHK-1 cells treated with florfenicol (FLO). the relative expression of genes was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, /, #, and +) over the bars indicate statistical differences among treatments (control and different doses of antibiotic) at the same time. Two-way ANOVA followed by Tukey's test ($p < 0.05$).

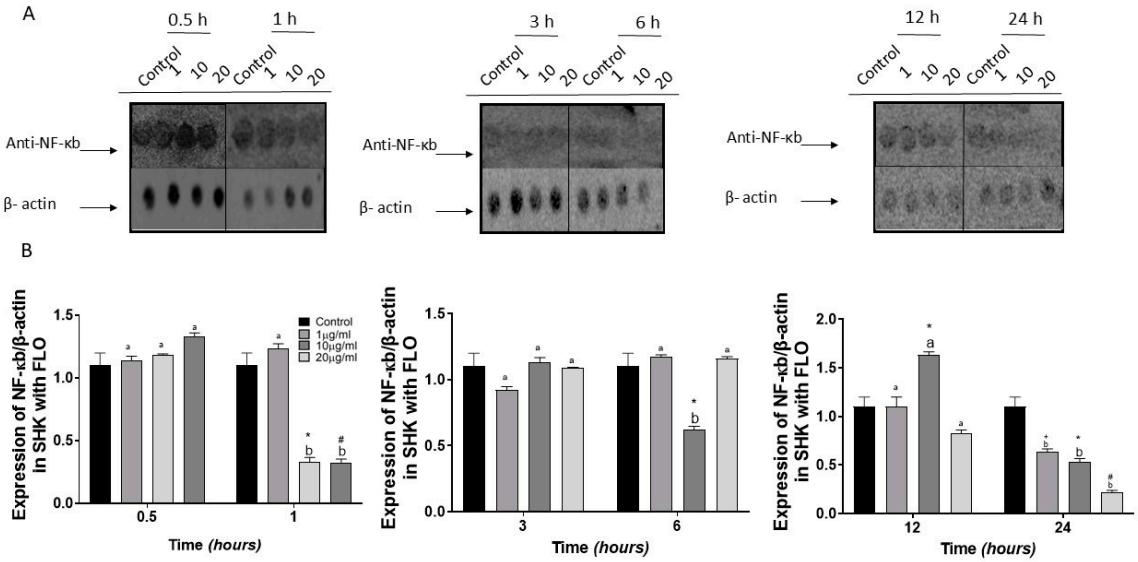

Figure 4. Immunodetection of NFkb in SHK-1 cells treated with florfenicol (FLO). (**A**) a representative image of the dot blot assay and (**B**) its respective graph. The quantification of expression of NFκb used b-actin as the internal reference control. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, #, and +) over the bars indicate statistical differences among treatments (control and different doses of antibiotic) at the same time. Two-way ANOVA followed by Tukey's test ($p < 0.05$).

4. Discussion

Antibiotics affect bacterial diseases, but their use and abuse modify resistance and alter the environment [9], influencing fish physiological responses, as Nakano et al. [17] indicated. OTC and FLO have therapeutic applications in Atlantic salmon culture, and their effects on the fish's different immune and oxidative stress parameters must be elucidated. Here, we studied the innate immune system and oxidative stress response. TLRs are type I transmembrane proteins with numerous LRRs (leucine-rich repeats) in their extracellular domain. They possess an intracellular signaling domain known as TIR (toll/interleukin-1 receptor), expected to both Toll and IL-1 receptors. This TIR domain can bind to and activate molecules such as MyD88 (myeloid differentiation factor 88) [31,32], while catalase and GPx are part of the detoxification process.

In our study, the OTC and FLO have different effects on the innate immune system; the OTC has a chained effect between 3 and 12 hours, where there was an increase in TLR1 (which is a broad-spectrum receptor), then MyD88, to then activate NFκb, which will then translocate to the nucleus to activate IFNγ, generating a "false positive" response because OTC is not a pathogen but can act as a PAMP. On the other hand, FLO has two responses: the first is the activation of TLR1, MyD88, and NFκb during the first minutes (0.5 to 1 h), while IFNγ increases its expression at 6 h of the experiment. Unlike OTC, FLO may be affected by an epigenetic modification from the binding to regimens that promote genes associated with the immune response with IFNγ, which stops immediate signaling (as in the case of OTC) to the nucleus through IFNγ, taking more than five hours of difference [33–36].

Rijker et al. [37] described that OTC dramatically suppresses the immune system in carp (*Cyprinus carpio*). Also, Guardiola et al. [38] found that the dietary intake of OTC leads to suppressed humoral and activated cellular innate immune responses in gilthead seabream (*Sparus aurata*). This also causes immune-related genes to be upregulated or downregulated, depending on the OTC concentration in the diet. The results of Guardiola et al. [38] are consistent with our results for both antibiotics, OTC and FLO, which depend on concentration and time (TLR1-MyD88-IFNγ and NFκb). On the contrary, Lundén et al. [39] showed that florfenicol does not significantly influence the im-

mune parameters in rainbow trout, while Tafalla et al. [26] observed the head kidney macrophage respiratory burst and phagocytosis were inhibited by the in vitro treatment in *Scophthalmus maximus*. They were dose-dependent on OTC; our dot blots of NFκb show similar results, where dose-dependent OTC and FLO inhibited protein production.

The innate immune system drives the activation of different cellular components such as leukocytes, phagocytes, macrophages, or nets, among others; if a false immune response is promoted against something that is not pathogenic or a PAMP, it causes these to promote the activation of oxidative stress, increasing reactive oxygen species, "ROS". In our study, as in the immune system, there are two responses according to the antibiotic, with OTC generating an increase in catalase at 6 h, while GPx had three peaks. This indicates the formation of peroxide that will be eliminated as water and oxygen. At the same time, GPx catalyzes the reduction of hydrogen peroxide (H_2O_2) or lipoperoxide (L-OOH), using reduced glutathione (GSH) as a reducing agent. Regarding FLO, like the immune system, it presented its highest expression at 0.5 and 1 h of treatment, indicating that both catalase and GPx are aligned with the immune system, helping to eliminate excess peroxide [40].

Antibiotics have been reported to activate the production of reactive oxygen species (ROS) and cause oxidative damage [41–43]. Caipang et al. [44] presented a differential transcription of the oxidative stress-related gene, catalase, in the blood during the oral administration of antibiotics in cod (*Gadus morhua*). They showed a significant upregulation of this gene in the oxolinic-fed group, while a downregulation of gene expression was observed in the florfenicol-fed group. There are two potential responses: "oxolinic acid can penetrate cells and tissue and produce oxidative stress" or "type of metabolites that are produced by each antibiotic that could result in downregulation of antioxidant defense in the fish" [45]. Meanwhile, other studies have demonstrated that the oral administration of OTC induces the production of ROS (reactive oxygen species) and DNA damage in different fish species.

These fish species include rainbow trout and silver catfish, *Rhamdia quelen* [18,46–48]. Our results show two responses: very high at the initial experiment with upregulation, but at the end of the experiment, there was downregulation, indicating the incapacity to eliminate the antibiotics or metabolites, as was mentioned before.

5. Conclusions

Our results indicate that both antibiotics affect the innate response from transcriptional expression until protein production. Meanwhile, the oxidative stress markers presented two responses, "upregulation" and "downregulation", at the beginning and end of the experiment, with dose-dependency and time as change factors. More studies are needed to clarify the effect of antibiotics on physiological response, achieve a holistic view, and improve tools in aquaculture handling.

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