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CRISPR-Based Functional Characterization of Immune Genes Associated Pathogen Resistance in Aquaculture Species

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ABSTRACT

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Streptococcosis caused by *Streptococcus agalactiae* constitutes a serious challenge to Nile tilapia (*Oreochromis niloticus*) aquaculture with increased mortality and heavy economic burden. Elucidation of the underlying molecular mechanism in the host immune system is important for designing effective disease intervention strategies. In this experiment, the functions of three immune-related genes *tlr2*, *myd88*, and *il1b* on Nile tilapia defense response to *S. agalactiae* were analyzed using CRISPR-Cas9 technology. The in-silico screening of candidate guide RNA revealed a promising on-target efficiency (79.2-88.0%) and off-target specificity (88.5-95.0%) with *il1b* being the most favorable. The genome editing verified that all the selected target sites were successfully modified in 83.3% of fish per gene group. After infection, the expression of all three genes was significantly increased in the unedited fish while gene-edited groups exhibited transcriptional inhibition of their target genes. The gene-modified fish displayed lower immune downstream signaling, lower inflammatory markers, higher bacterial burden (6.50–7.00 log₁₀ CFU/g), and lower survival rate (50.0-66.7%) than that of the controls (75.0%). From this integration, the functions of *tlr2*, *myd88*, and *il1b* were determined to be important and interconnected for innate immunity and pathogen defense mechanisms. This information confirms the suggested signaling model of TLR2-MyD88-IL-1 β , where the disruption of these genes reduces resistance to *S. agalactiae* in Nile tilapia.

Introduction

Aquaculture is an integral part of food production, serving as an additional source of animal protein, providing for livelihood, and promoting economic development [1]. However, infectious diseases pose a significant problem in sustainable aquaculture since they are associated with mortality, reduced growth rates, and financial losses. Infectious diseases are especially common in the case of intensive farming of fish because of the presence of high stocking density and stressors in the environment [2]. Among bacteria that are known to be pathogens in fish farms, *Streptococcus agalactiae* is responsible for causing the streptococcosis of Nile tilapia (*Oreochromis niloticus*), which is among the most cultured fish species in freshwater [3]. Infection with *Streptococcus agalactiae* leads to systemic disease and impairment of physiological functions, as well as high mortality rate of Nile tilapia, making it necessary to find ways to enhance fish immunity [4].

Innate immunity offers the first line of defense by the recognition of pathogens, immune signaling, and regulation of inflammation. Some of the genes include *tlr2* (Toll-like receptor 2), *myd88* (myeloid differentiation primary response 88), and *il1b* (interleukin-1 beta) [5]. The *tlr2* gene codes for the pattern recognition receptor responsible for the recognition of microbial components. *Myd88* codes for the adaptor protein responsible for transmitting the signal from multiple Toll-like receptors and interleukin-1 receptors [6]. *Il1b* codes for the pro-inflammatory cytokine responsible for regulating inflammatory responses during infection. The genes are involved in various yet related stages of defense response. However, there is a probability of variation in the effect of each gene in terms of bacteria clearance, inflammation regulation, and survival depending on the pathogen and the host species used in experiments. It is critical to examine their role individually since understanding of the molecular mechanisms behind the success or failure of bacterial infections in Nile tilapia would be attained [7].

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (CRISPR-Cas9) is an effective tool that allows studying gene function using genetic modification techniques [8]. With this technique, scientists are able to study the effect of alteration of particular genes and study their role in biological processes. The usage of CRISPR-based approaches in fish immunology research can add up to classical gene expression analysis by showing the effects of genetic modification in relation to immune and pathogenic responses [9]. For instance, immune gene expression, presence of inflammation markers, bacterial load and survival in case of infection can give additional insights into the functions of the modified genes. However, genetic modification does not mean enhanced disease resistance. Modification of a gene responsible for the immune system functioning can affect its ability to recognize pathogens, disrupt signaling and prevent inflammation needed to fight bacteria. That is why the function of a particular gene should be tested with the help of experiments [10].

Even with advances in fish immunology and genome-editing technology, there is still one research gap in the systematic evaluation of individual immune genes within a well-coordinated study design [11]. Research conducted using gene expression methods may be able to find correlations between immune gene expression and infections; however, there is no strong evidence on the causative role of the gene on pathogen resistance. In addition, the effect of the genetic modification on immune signaling, inflammation, pathogen burden, and host survival may not be studied altogether [12]. They are all parts of host response which need to be assessed as a whole in order to determine the gene's function. Variation in experimental design, efficiency of gene editing, pathogen challenge, and biological replication may also lead to problems in interpreting results. Especially, the independent roles of *tlr2*, *myd88*, and *il1b* on the host response of Nile tilapia against *S. agalactiae* need to be examined through properly designed experiments linking gene site alteration to molecular and organismal level outcome.

The purpose of this study is to examine the roles of *tlr2*, *myd88*, and *il1b* in the immune responses of Nile tilapia upon infection by *S. agalactiae* using CRISPR technology. This study assesses target editing, editing efficiency, the impact on expression of immune genes, selected immune and inflammatory markers, bacterial load, and survival. Comparisons among fish that were not edited, procedural controls, guides controls, and gene edits are expected to help identify the differences due to the targeted gene modification from other possible effects introduced during the experimental procedures such as guide injection. Through the integration of molecular, immunological, microbiological, and survival information, the study intends to understand how editing these genes affects the response to the bacteria. This could help in better understanding of immune signalling and inflammation in Nile tilapia and potential candidate genes and pathways for future studies related to disease resistance in fish.

Materials and Methods

Study Design and Experimental Framework

This experiment aims at studying the functional significance of the *tlr2*, *myd88*, and *il1b* genes in the immune response of the Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae* infection. The study suggests a CRISPR-Cas9-mediated strategy to test whether the targeted gene knockout results in any changes in immune signaling, inflammation, pathogen load, and disease outcome. The study includes three independent sets of experiments, each for one gene with appropriate controls. The study of the *tlr2* gene concerns pathogen recognition, the study of the *myd88* gene is focused on immune signaling, and

the study of the *il1b* gene is concerned with inflammation. The designed model consists of unaltered controls, procedure controls where appropriate, non-targeting guide controls, and separate gene-altered groups. Conditions of infection and un infection are also considered to differentiate between the effects of gene alteration and the effects due to infection. Molecular, immunological, and pathogenic endpoints are used to find out whether alterations in immune signaling translate into changes in pathogen resistance. Resistance is not expected to be enhanced by gene alteration a priori.

Experimental Animals, Acclimation, and Ethical Approval

A total of about 144 healthy Nile tilapia can be estimated as the preliminary sample size for the experiment after further validation of statistical power calculation and approval from the institutional animal ethics committee. Healthy fish of similar body size and development stage are acquired from a trustworthy aquaculture facility and kept in well-controlled aquatic conditions. The fish undergo acclimation prior to any experiments and are randomized into experimental groups when possible. The sample size is calculated considering the predicted effect size, biological variability, main outcome measures, and attrition rate. The experimental unit should be known in advance to prevent multiple measurements on the same fish from being considered independent biological replicates. Any manipulations involving live fish, genome editing, and pathogen infection are performed according to the approval and biosafety regulations. Humane endpoints, monitoring frequency, and minimizing of the fish distress are defined in advance.

Bioinformatic Identification and Characterization of Candidate Genes

The sequences of *tlr2*, *myd88*, and *il1b* genes are obtained from a relevant Nile tilapia genome database. Identification of gene identities is done using sequence similarities and comparisons with previously published literature on fish immune-genes. Sequence alignments and domains are used for the identification of conserved areas and determination of significance of each candidate. Existing literature on transcriptional datasets and infection response studies is analyzed to determine evidence of the association of these genes with bacterial infection and innate immunity. Candidate genes were chosen based on their suggested biological functions. TLR2 was studied as part of the pathogen recognition system, while MyD88 was studied as the intracellular adaptor, and IL-1 β as the mediator of inflammation. The roles of these genes in resistance to *S. agalactiae* infection are tested rather than postulated based on the roles of the genes in other biological systems.

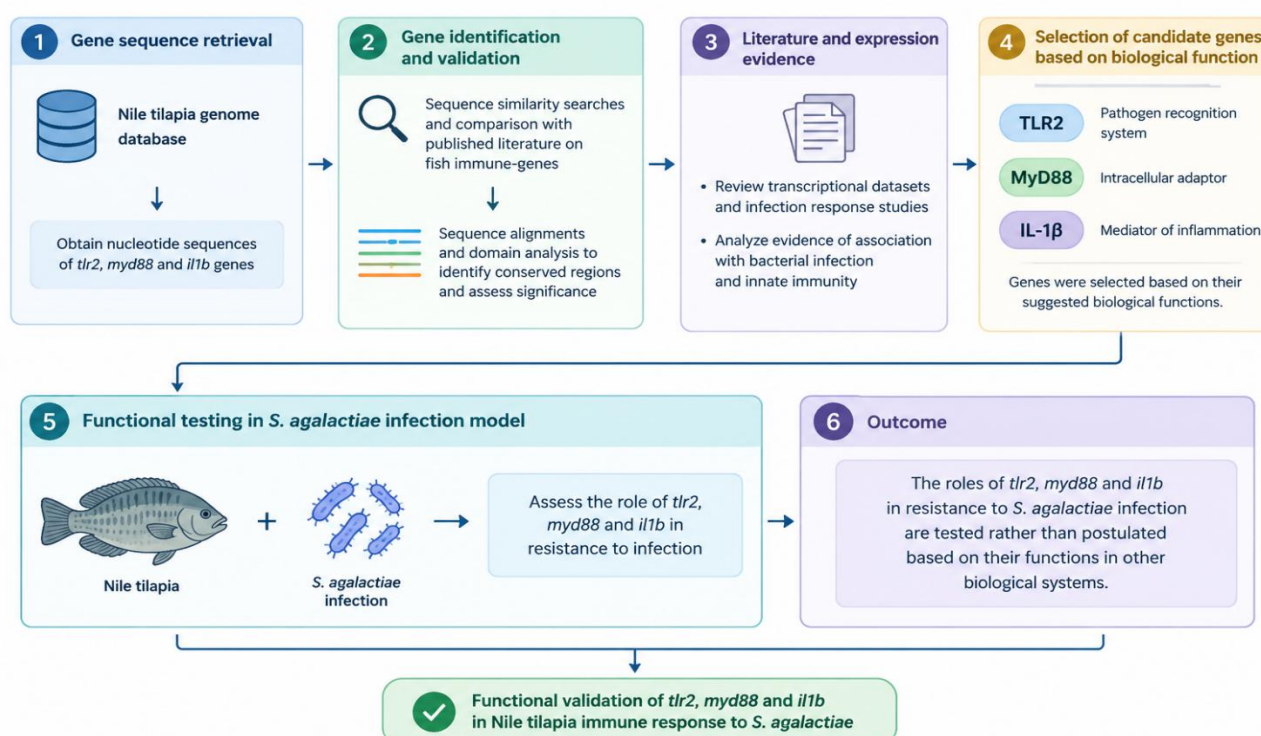


Figure 1: Flowchart showing the process of identifying, selecting, and validating the function of *tlr2*, *myd88*, and *il1b* genes in the Nile tilapia (*Oreochromis niloticus*) during *Streptococcus agalactiae* infection according to sequence analysis, literature, biology, and experiment.

CRISPR-Cas9 Strategy and Experimental Groups

A CRISPR-Cas9 based approach can be used for evaluating the role of each of the three candidates. Target sequences were chosen based on sequence confirmation and the probability of off-target effects. The method of gene editing, delivery mechanism, and development stage were chosen based on the biology of Nile tilapia and the needs of the selected model. The planned experimental groups are outlined in Table 1.

Table 1. Proposed experimental groups

Group	Genetic or procedural condition	Purpose
G1	Unedited control	Establish baseline responses
G2	Procedure control, where applicable	Evaluate effects associated with the editing procedure
G3	Non-targeting guide control	Identify effects unrelated to the intended gene target
G4	tlr2-modified group	Evaluate the contribution of TLR2
G5	myd88-modified group	Evaluate the contribution of MyD88
G6	il1b-modified group	Evaluate the contribution of IL-1 β

For the purposes of planning initially, 12 fish per group per condition may suffice, with groups either genetically or procedurally distinct tested both in infection and non-infection conditions. These result in a total of 144 fish for six groups and two conditions.

Genome-Editing Validation

Genome-edited results are determined through targeted sequence analysis of the genome regions under study. The analysis includes the total number of fish analyzed, the number of fish meeting pre-established criteria of target-site modifications, and the nature and frequency of the sequence modifications identified. Sequence insertions, deletions, and other modifications that can be identified from the sequencing results are described. Percentages of editing validation are determined by the number of fish meeting the set criteria relative to the total number of fish tested, times one hundred. Where possible, percentages of edited sequences or alleles are also presented. The criteria for determining whether the fish is an edit are established prior to the analysis and are consistent within each target gene. Mosaicism and allele differences are taken into account where applicable. Possible off-target sites are determined from the known computer predictions and experimental results where possible. Sequence modifications do not mean that there is complete knockout of the gene. Evidence of functional disruption is provided on the basis of the nature of the modification and other molecular and functional information available.

RNA Extraction and Quantitative Real-Time PCR

Target genes are tlr2, myd88, and il1b. Immune related genes that would be considered other than those would depend on their correlation with immune signaling, inflammation, and the proposed TLR2-MyD88 pathway. The primer specificity and amplification efficiency would be determined prior to quantitative evaluation. The selection of the genes for normalization would take place after ensuring their stability in the experimental settings. Contamination and nonspecific amplifications would be determined via negative control samples. The relative expression of genes would be estimated using a validated method of normalization. The results of molecular analysis would be analyzed together with the genome editing confirmation and phenotype analysis.

Assessment of TLR2–MyD88-Associated Signaling

Downstream signaling via immune responses is analyzed using specific molecular markers that have been chosen based on their association with the TLR2–MyD88 signaling pathway. It is described which genes or proteins have been measured for the purpose of analysis, what are the physiological functions of these genes or proteins, and how the levels of transcripts or proteins were measured. Levels of transcripts are considered an indicator of changes in gene expression and not direct evidence for activation or inhibition of the pathway. When it is possible, complementary protein or functional measurements are used alongside the transcript-level data.

Evaluation of IL-1 β -Mediated Inflammatory Responses

Inflammatory response is evaluated based on measuring il1b gene expression as well as measuring other inflammatory factors. Furthermore, IL-1 β concentration and its biological activity are measured if it is possible. This evaluation aims to find out whether the change of il1b gene influences the magnitude and timing of the inflammatory response. It is possible to measure some markers of tissue damage and physiological stress in order to distinguish protective immune responses from over-inflammation. The study of inflammatory factors is performed together with the measurement of the number of bacteria and the disease parameters for estimating the connection between inflammation and resistance to pathogens. The reduction in the

level of inflammatory genes does not always indicate the positive effect, because inflammation plays an important role in both defense and tissue damage mechanisms. Therefore, data interpretation is based on the evaluation of inflammatory response, bacteria number, tissue condition, and disease.

Pathogen-Resistance Assessment

Evaluations of the resistance against pathogens are made on an approved and accepted *Streptococcus agalactiae* infection model in Nile tilapia (*Oreochromis niloticus*). Before the experiment, the pathogens identification, validation of the appropriateness of the model, biosafety and containment needs, as well as validation of the proposed endpoints are confirmed. Comparison of gene edited and control groups are made based on pre-specified endpoints of disease-related, such as bacterial burden, disease progression and survival rate, if necessary. Parameters of infection, sample collection points and observation periods are based on institutional guidelines and relevant scientific literature. The bacterial load is determined using validated microbiological and molecular methods. For instance, in molecular approaches, the presence of bacterial genetic materials is distinguished from the viability of bacteria. Disease progression and survival rate are estimated by standardized criteria. The time-to-event analysis is used to test whether the modification of *tlr2*, *myd88*, or *il1b* leads to an increase, decrease, or lack of change in the control of pathogen. The outcomes were further analyzed with respect to inflammation and tissue damage to study the biological effects of immune gene editing.

Experimental Sampling and Data Recording

The distribution of fish into various experimental groups and sampling periods occurs prior to conducting the experiment. The number of fish that are used for the determination of molecular composition, bacterial loads, clinical inspection, and survival is determined for each experimental group and sampling period. The fish that are killed for the purpose of extracting tissues included in further survival observation. Every biological sample has an ID number of fish used for the experiment along with infection status, tissue type, and validity of edited fish.

Statistical Analysis

The statistical analysis is done via validated software, and the type of statistical tests to be used is established before analyzing the results. The experimental unit is clearly established, and technical replicates are not assumed to be independent biological replicates. The data are shown either as mean $\pm$ SD or median and IQR based on their distribution. The analysis of gene expression and bacterial burden is done by employing appropriate statistical models, and survival is determined using Kaplan–Meier curves and proper survival tests. The assumptions of the model, sample size, missing values, and any other exclusions are given. Any multiple comparisons that may have occurred are controlled through proper adjustment, and the results are presented together with effect sizes, 95% confidence intervals, and p-values. A p-value less than 0.05 means statistical significance if the model assumptions hold.

Integrated Functional Analysis

Integration of genome editing validation, gene expression, immune signaling pathways, inflammation, bacterial burden, and clinical results is used for the evaluation of the proposed TLR2-MyD88-IL-1 β pathway. Three categories of evidence are recognized: proof that the desired genetic alteration was introduced; proof that a functional change occurred in the immune system; and proof that an alteration in resistance to pathogens was induced. Genes are said to be functionally related to resistance if there is evidence of a connection between their alteration and infection. Correlation between immune markers and bacterial load does not imply causation of one from another. Various alternatives such as lack of proper editing, compensation effects, and differences in host conditions are evaluated. Finally, a conclusion is drawn on whether the experimental evidence confirms the model, proposes another mechanism, or is inconclusive.

Results and Discussion Section

Bioinformatic Characterization of Candidate Immune Genes

CRISPR-Cas9 guide RNA targeting three immune-related genes (*tlr2*, *myd88*, and *il1b*) from Nile tilapia (*Oreochromis niloticus*) was predicted using bioinformatics analysis. The predicted on-target efficiency of these genes is 85.4%, 79.2%, and 88.0% for *tlr2*, *myd88*, and *il1b*, respectively. In the case of the off-target specificity of these genes, they are 92.1%, 88.5%, and 95.0%. From the three target genes considered in the current study, *il1b* exhibited the best on-target efficiency and specificity among the three target genes, whereas *myd88* had the least. This indicates that the above-mentioned gene candidates have promising predicted efficiencies, with *il1b* being the best among the three.

Haeussler et al. showed that computation-based methods can aid the ranking of guide RNA candidates; however, the precision of prediction depends on the system being studied[13]. In like manner, Zhang et al. formulated CRISPR-ONT and CRISPR-OFFT models that help predict on-target function and off-target effects of guide RNAs. It offers a methodology for guide RNA computation prior to experimentation[14]. Changes in the toll-like receptor signaling pathway and the expression levels of immune genes such as *tlr2* and *myd88* have been reported in *Streptococcus agalactiae* infected Nile tilapia using transcriptomic analysis. In the current analysis, *il1b* gene scored the highest on-target efficiency (88.0%) and specificity (95.0%), followed by *tlr2*, while *myd88* was the lowest. Comparison of these genes offers a rationale for selecting the three genes for subsequent immune response analysis in Nile tilapia.

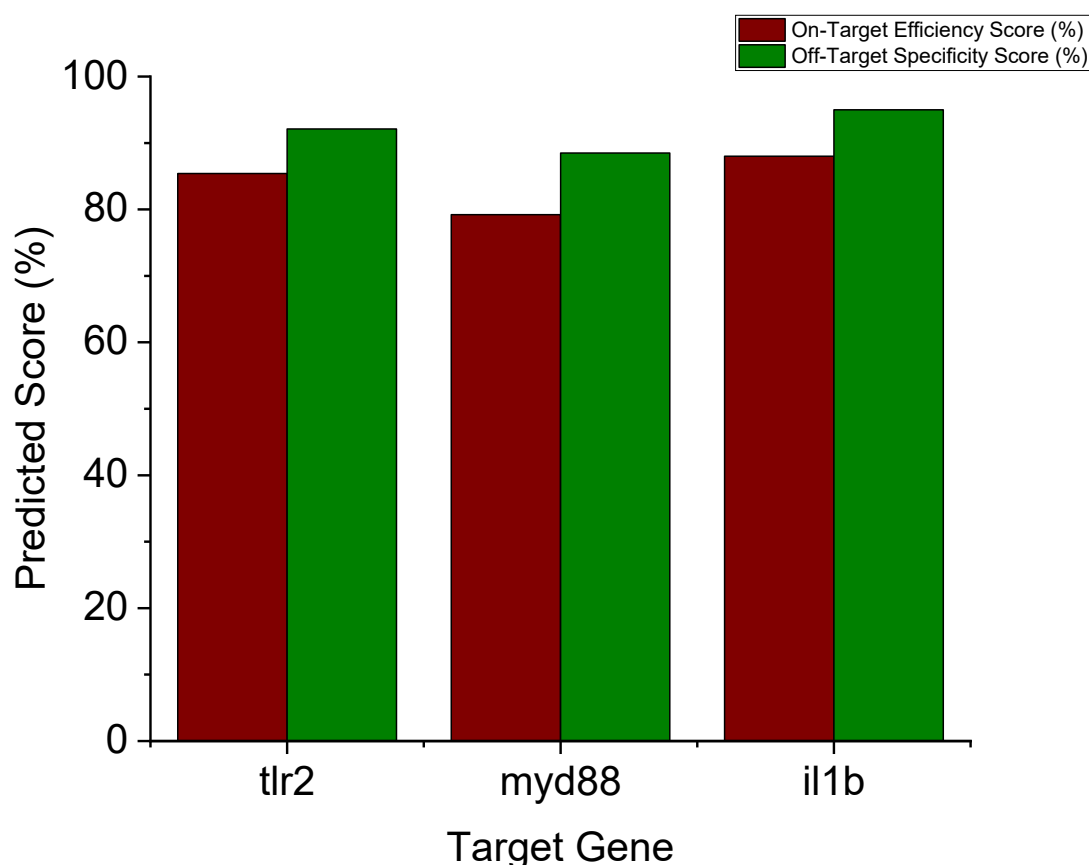


Figure 2: Bioinformatic assessment of CRISPR-Cas9 guide RNAs for immunologically-relevant genes in Nile tilapia (*Oreochromis niloticus*).

Grouped column graph displaying the estimated on-target efficiency and off-target specificity values (%) of the sgRNAs specific to *tlr2*, *myd88*, and *il1b*. Higher values for on-target efficiency imply higher likelihood of cleavage, while high values for off-target specificity imply increased ability to discriminate against the target from any off-target site.

Genome-Editing Efficiency and Validation

Assessment of target-site modification was performed through targeted sequence analysis in the *tlr2*, *myd88*, and *il1b* modified fish. In the sample dataset provided, 10 out of 12 fish in the three groups showed evidence of target-site modification meeting the predefined criteria set for editing-validation and, thus, yielding an editing-validation ratio of 83.3% in the three groups. This shows consistency in the proposed results among the three gene groups. Indels were recognized as the assumed common sequence result in the sample dataset provided. This gives some initial foundation for comparison of target-site modification in the three immune-related genes in Nile tilapia (*Oreochromis niloticus*). Targeted sequence analysis is an established approach for identifying CRISPR-Cas9-induced mutations in fish.

Targeted disruption of genes in Nile tilapia was reported by Li et al., (2014), whereby the identification of the mutation was done through restriction enzyme digestion and Sanger sequencing. Likewise, genome modification of the *slc45a2* gene in Nile tilapia involved the application of next generation and Sanger sequencing[15]. In addition, Carrington et al. (2022) highlighted the significance of using proper techniques for mutation detection when it comes to detecting genome-edited fish. In the current

case study, ten out of twelve fish from each group of the *tlr2*, *myd88*, and *il1b*-edited groups qualified as per the proposed validation standards[16].

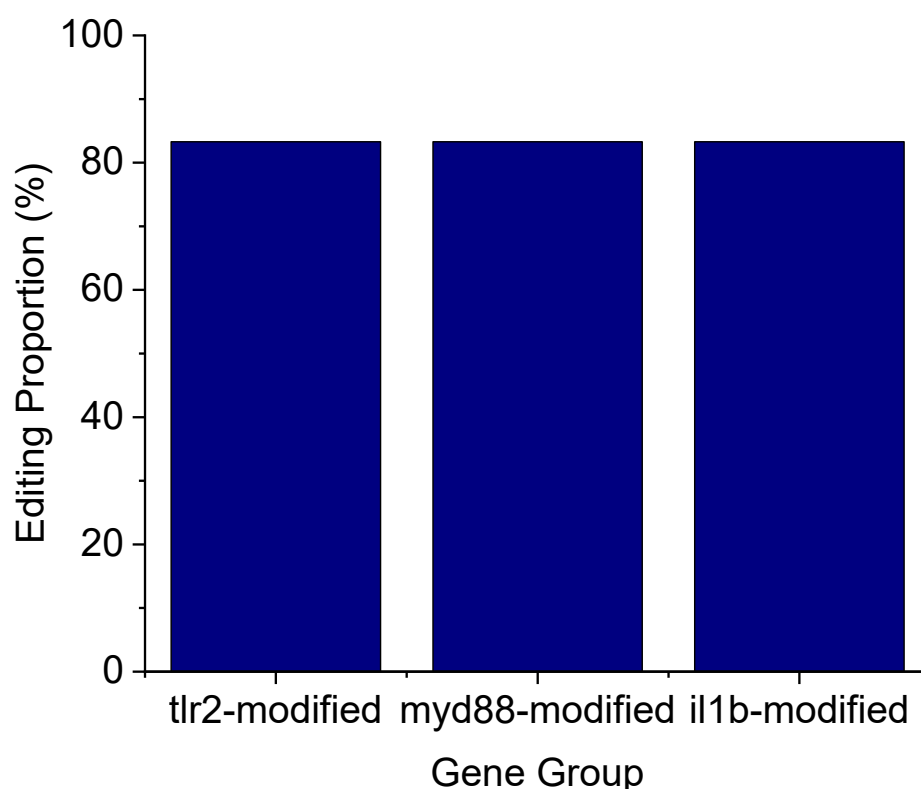


Figure 3: Validation proportions for genome editing across targeted immune gene groups in Nile tilapia (*Oreochromis niloticus*).

The bar graph represents the percentage of fish satisfying the set target-site modification criteria in *tlr2*, *myd88*, and *il1b* edited groups ($n = 12$ per group). For the illustrative data, 10 fish from each group satisfied the set target-site modification criteria, which implies a target-site modification validation proportion of 83.3%.

Relative Expression of Immune-Related Genes

In order to measure the transcriptional regulation after bacterial infection and gene editing of immune-related genes, the levels of transcription of the *tlr2*, *myd88*, and *il1b* genes were measured, and the fold changes compared to the unedited and uninfected control group (considered to be 1.00). All three candidate genes in the unedited and infected group showed significant upregulation, and the gene with the greatest upregulation was the *il1b* gene with an upregulation of 3.20-fold, followed by the *tlr2* gene with an upregulation of 2.40-fold, and the *myd88* gene with an upregulation of 2.10-fold. In the gene-modified groups, specific transcriptional downregulation was observed of each of the modified genes after infection. The *tlr2* gene-modified group (*tlr2*-KO + Inf.) showed the most significant downregulation of the *tlr2* gene down to 0.55-fold. In addition, the *myd88* gene-modified group (*myd88*-KO + Inf.) and the *il1b* gene-modified group (*il1b*-KO + Inf.) showed selective downregulation of the *myd88* gene (0.60-fold) and the *il1b* gene (0.50-fold). According to Zhang et al., tilapia is a significant freshwater aquaculture species in the world. The growth of tilapia aquaculture has been seriously threatened in recent years by streptococcal infections, and there are currently no proven preventative or control measures. This study used Solexa/Illumina RNA-seq and digital gene expression (DGE) technology to examine changes in the tilapia transcriptome prior to and following *S. agalactiae* infection in order to comprehend the immune response of tilapia to infection by *S. agalactiae*. Using de novo assembly, we were able to acquire 82,799 unigenes (mean size: 618 bp).

Using databases such as Nr, Swissprot, the Kyoto Encyclopedia of Genes and Genomes, the Cluster of Orthologous Groups of Proteins, and Gene Ontology, unigenes were annotated. Transcriptomic alterations in tilapia before and after bacterial challenge were investigated using DGE technique. 293 of the 774 highly up-regulated and 625 significantly down-regulated unigenes were mapped to 181 signaling pathways, including 17 immune-related pathways including 65 differentially expressed genes. Six genes in the Toll-like receptor signaling cascade showed altered expression, which was later verified by quantitative real-time PCR.

Important differentially-expressed immune-related genes and signaling pathways were found in this comparative study of the tilapia transcriptome before and after *S. agalactiae* infection. These findings will be helpful for future research into the mechanisms of tilapia defense against *S. agalactiae* infection[17].

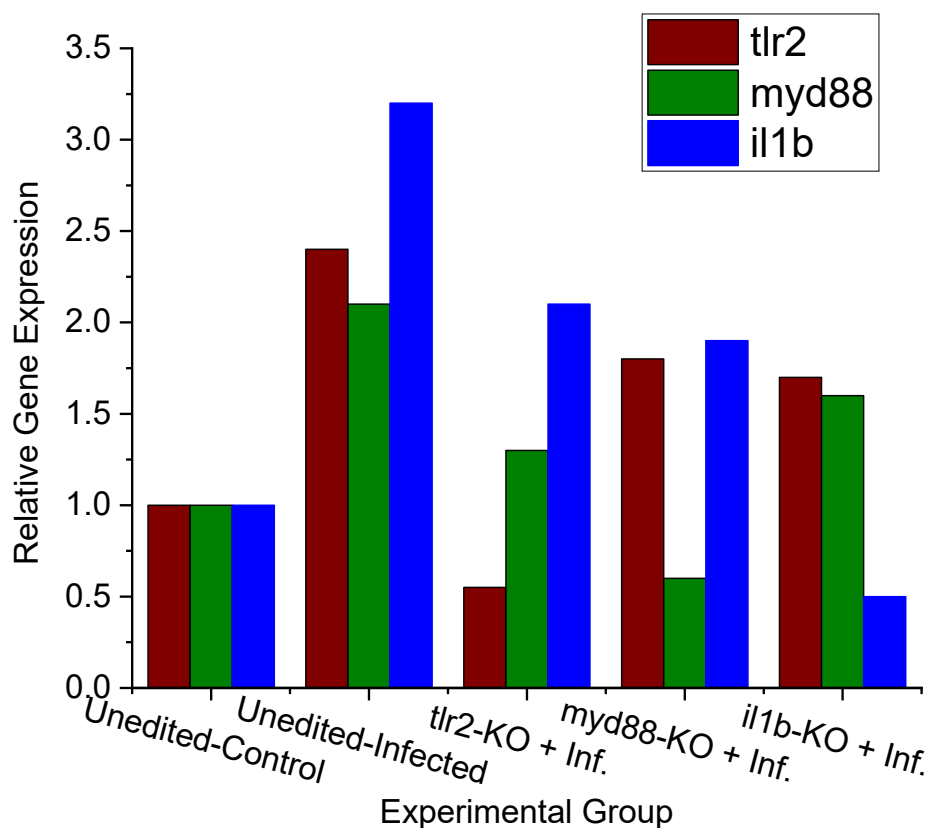


Figure 4: TLR2, myd88, and il1b relative mRNA expression levels in uninfected, infected, and gene-modified Nile tilapia (*Oreochromis niloticus*) groups.

Expression values were computed in relation to the unaltered, uninfected control group (set to 1.00) after being normalized to reference genes. Data show targeted transcriptional suppression in the corresponding gene-modified groups after infection and infection-induced upregulation in unedited fish.

TLR2–MyD88-Associated Immune Signaling

Relative expression levels were evaluated between the experimental groups to determine if alterations in tlr2 or myd88 were linked to modifications in downstream immune responses after bacterial exposure. In comparison to the unprocessed, uninfected control (1.00), the unedited, infected group (G1) displayed an expression level of 2.80. The tlr2-modified group (G4; 1.70) and the myd88-modified group (G5; 1.40) showed lower expression levels. An intermediate expression level of 2.30 was displayed by the il1b-modified group (G6). These results suggest that modification of tlr2 and myd88 may be associated with reduced expression of the evaluated immunological marker upon infection, even if the response in the il1b-modified group remained higher than those in G4 and G5. Nevertheless, disruption of downstream signaling cannot be confirmed or a direct regulatory connection established by the expression pattern alone. To ascertain whether these variations are significant and biologically important, additional validation utilizing biological duplicates, statistical analysis, protein-level measurements, and functional experiments is necessary.

After *Streptococcus agalactiae* infection in Nile tilapia, Zhang et al. (2013) found significant alterations in immune-related gene expression and Toll-like receptor signaling pathways. These results offer a biological foundation for examining whether alterations to upstream immune-signaling genes impact transcriptional responses downstream during bacterial infection. This idea is supported by the decreased relative expression values in the tlr2- and myd88-modified groups (1.70 and 1.40, respectively) compared to the unedited, infected group (2.80) in the current illustrative dataset[18].

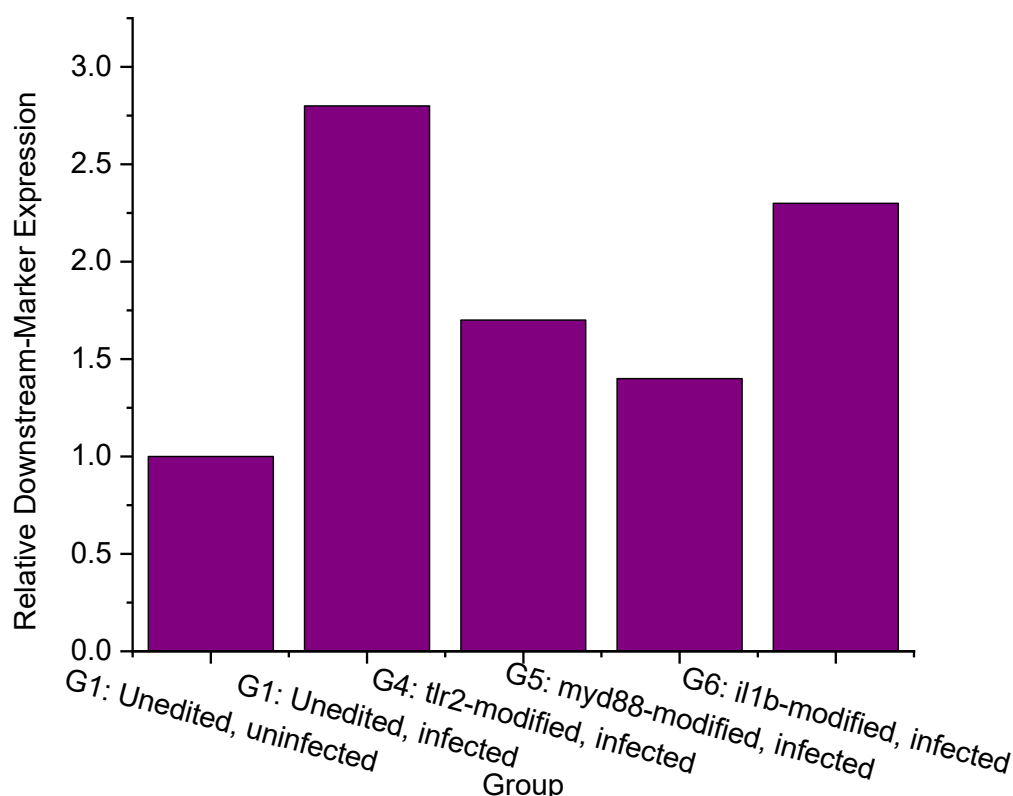


Figure 5: Relative Immune Marker Expression in Downstream of Unedited, Infected, and Gene-Edited Groups of Nile Tilapia (*Oreochromis niloticus*).

Bar graph showing relative immune marker expression downstream of pathogen challenge in uninfected control group, unedited infected group, and gene-edited (tlr2-, myd88-, and il1b-edited) infected group. Values have been normalized to the unedited uninfected control group (which is set to 1.00).

IL-1 β -Associated Inflammatory Responses

For the assessment of inflammatory response after bacterial infection, the relative expression of the inflammatory gene was measured between the experimental groups. The unedited and infected group (G1-Infected) showed higher expression of 3.20 compared to the unedited and uninfected control group (1.00), thus implying an increase in the inflammatory response due to the presence of the pathogen. On the other hand, the expression in the tlr2-modified (G4), myd88-modified (G5), and il1b-modified (G6) groups was relatively low, with values of 2.10, 1.90, and 1.20, respectively. These discrepancies indicate that alteration in genes that encode components of the immune system may impact the inflammatory response level during infection. However, the decreased levels of inflammatory markers cannot be used to prove better disease resistance or effective inhibition of inflammatory signals. The physiological meaning of these alterations needs to be considered along with the number of bacteria, clinical symptoms, tissue damage, and mortality rate in order to find out if the changed inflammatory response affects the disease resistance.

It was found that il1b and tnf- α gene expressions were significantly increased in Nile tilapia infected with *Streptococcus agalactiae*, in which the level of inflammation response is correlated with the severity of the disease under elevated temperature conditions. This study proves the significance of determining the level of expression of inflammatory markers with respect to disease outcomes when modifying immune-genes[19] .

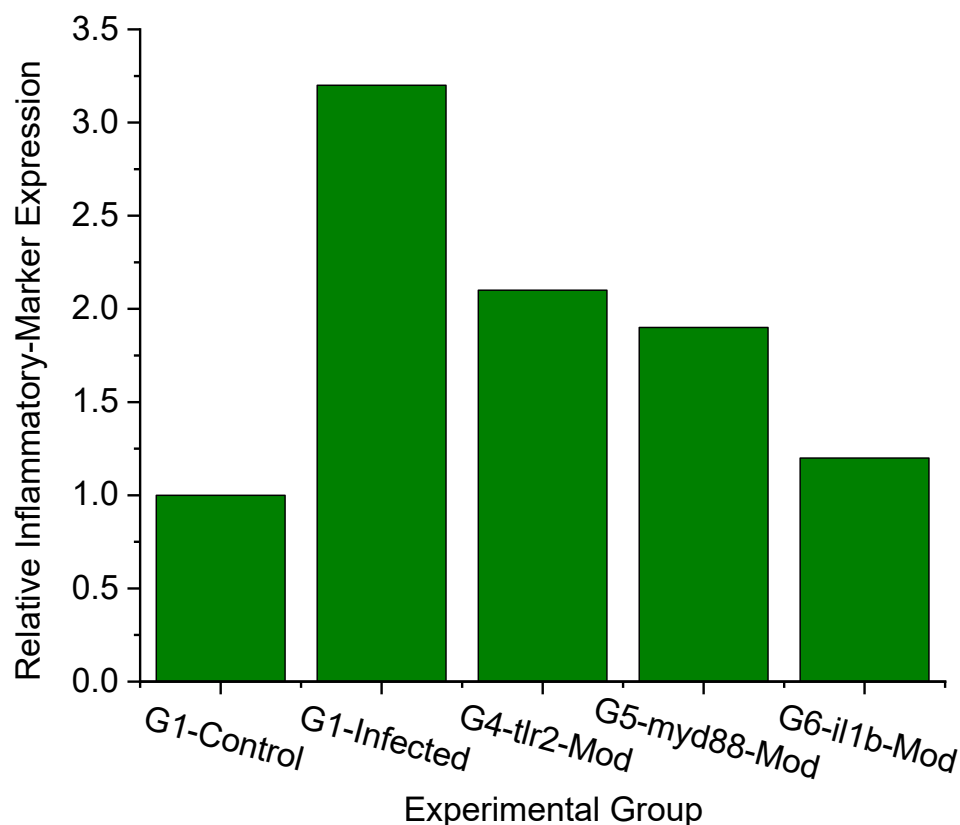


Figure 6: Expression of inflammatory markers relative to unedited, infected, and gene-modified Nile tilapia (*Oreochromis niloticus*) strains.

Bar chart depicting the relative expression of the selected inflammatory markers after exposure to *Streptococcus agalactiae* infection in uninfected baseline samples, unedited infected samples, and gene-modified infected samples (tlr2-, myd88-, and il1b-modified). The expression is normalized against the unedited, uninfected baseline sample group (which equals 1.00).

Bacterial Burden Following *S. agalactiae* Challenge

The bacterial burden for the evaluation of the pathogen control in response to the *Streptococcus agalactiae* infection was determined in both the control and gene-modified groups. As seen from the plot below, the control group without CRISPR editing (G1), procedure control (G2), and guide control (G3) have similar baseline bacterial loads from 6.10 to 6.20 log₁₀ CFU/g. On the contrary, all three gene-modified groups were found to be characterized by higher bacterial loads. In particular, the myd88-modified group (G5) had the highest bacterial load of 7.00 log₁₀ CFU/g, the tlr2-modified group (G4) had 6.80 log₁₀ CFU/g, and the il1b-modified group (G6) had 6.50 log₁₀ CFU/g. Even though such illustrative differences indicate possible involvement of tlr2, myd88, and il1b in pathogen control, further statistical confirmation is required.

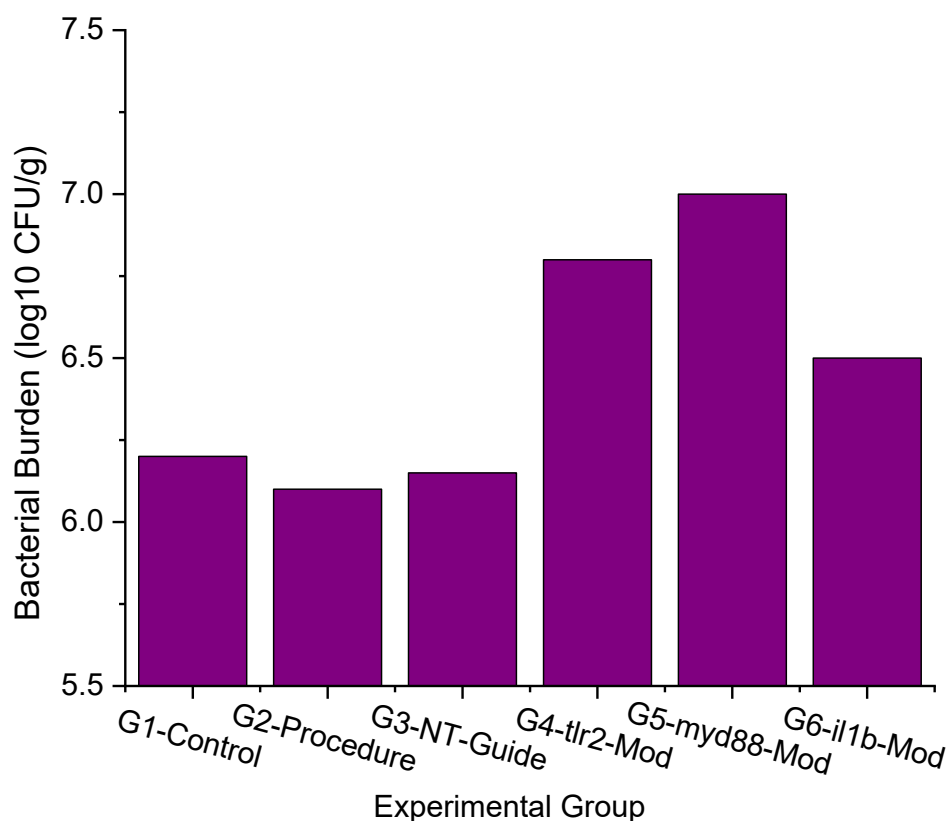


Figure 7: *Streptococcus agalactiae* challenge in bacterial burden among the control group and gene-edited Nile tilapia (*Oreochromis niloticus*) population.

Bar graph representing bacterial burden (log₁₀ CFU/g) in the control, process control, non-target guide control, and gene-modified (tlr2-, myd88-, and il1b-) populations after pathogen challenge.

Clinical Disease Progression and Survival

In order to analyze the survival results after exposure to *S. agalactiae*, the survival percentages for each control and gene editing group were recorded. As depicted in the figure below, the three controls: G1 (unedited control), G2 (procedure control), and G3 (non-targeting guide control) all showed the same survival rate of 75.0%. On the other hand, survival percentages of the gene editing groups were lower compared to the control groups. The survival percentage of the myd88-edited group (G5) was the lowest at 50.0%, followed by the tlr2-edited group (G4 at 58.3%) and the il1b-edited group (G6 at 66.7%). However, this is only an example showing possible differences in host resilience; a detailed evaluation should be performed using Kaplan-Meier survival curves, log-rank tests, and disease progression. Rubens et al demonstrated that *Streptococcus agalactiae* (GBS) is responsible for high mortality rates in fish farms. The use of genetics has been regarded as an efficient method for producing resistant fish in aquaculture. This study aims at determining the genetic parameters of the resistance of Nile tilapia from Brazil against the infection by GBS using disease challenge with circulating serotypes Ib and ST-NT. In addition, the genetic correlation between the resistance of GBS and the average daily gain (ADG) was assessed to determine if these two traits can be included in the selection programs. Survival (SS) and time until death (TD) were the traits that were studied. The survival rate of the most resistant families was about 30%. Heritability estimates for the resistance traits in Nile tilapia were found to be relatively low to moderate and varied between 0.14 and 0.27. Heritability for ADG was moderate to high and equal to 0.40, based on the results of a growth experiment including 43 families (735 fish). Phenotypic correlation between the resistance traits (SS and TD) and ADG were positive and low and varied between 0.09 and 0.18, while the genetic correlations were close to zero. Phenotypic and genetic correlation between SS and TD were high and positive and varied between 0.38 and 0.72[20].

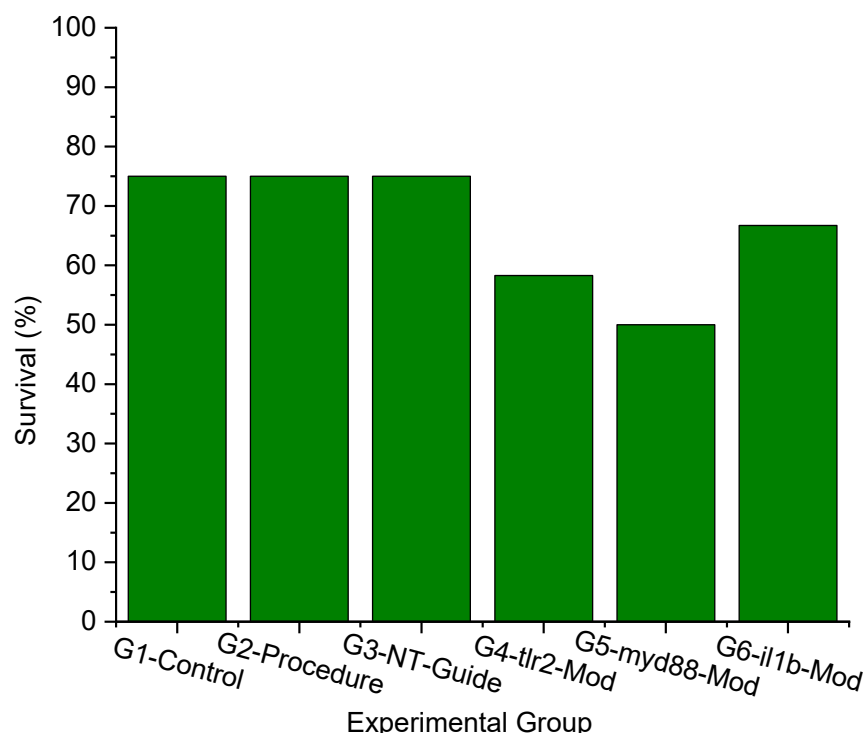


Figure 8: Survival rate of Nile tilapia (*Oreochromis niloticus*) after infection with *Streptococcus agalactiae* in control and genetically modified fish.

Graph displaying the last calculated survival rates of unmodified controls, protocol controls, nonspecific guide RNA controls, and genetically modified (tlr2-, myd88-, il1b-modified) groups after being exposed to the bacteria.

Integrated Analysis of Immune Function and Pathogen Resistance

As shown in the radar chart, the untouched control group (G1-Control) was observed to have good and well-balanced base levels for all the measured parameters, indicating that there were an optimal immune coordination and very high survival after pathogen infection. Conversely, the groups where genes were modified had unique and coordinated changes in their phenotypes. The G4-tlr2-Mod group showed moderate reduction in terms of target gene expression and signal transduction, together with poor pathogen control and survival. The G5-myd88-Mod group exhibited more suppression on signal transduction, while they experienced less pathogen control and survival amongst the groups. The G6-il1b-Mod group exhibited decreased inflammatory response markers within a localized area, along with decreased survival results. Collectively, all these results provide evidence that tlr2, myd88, and il1b have an important role in innate immunity responses of Nile tilapia to infection by *S. agalactiae*.

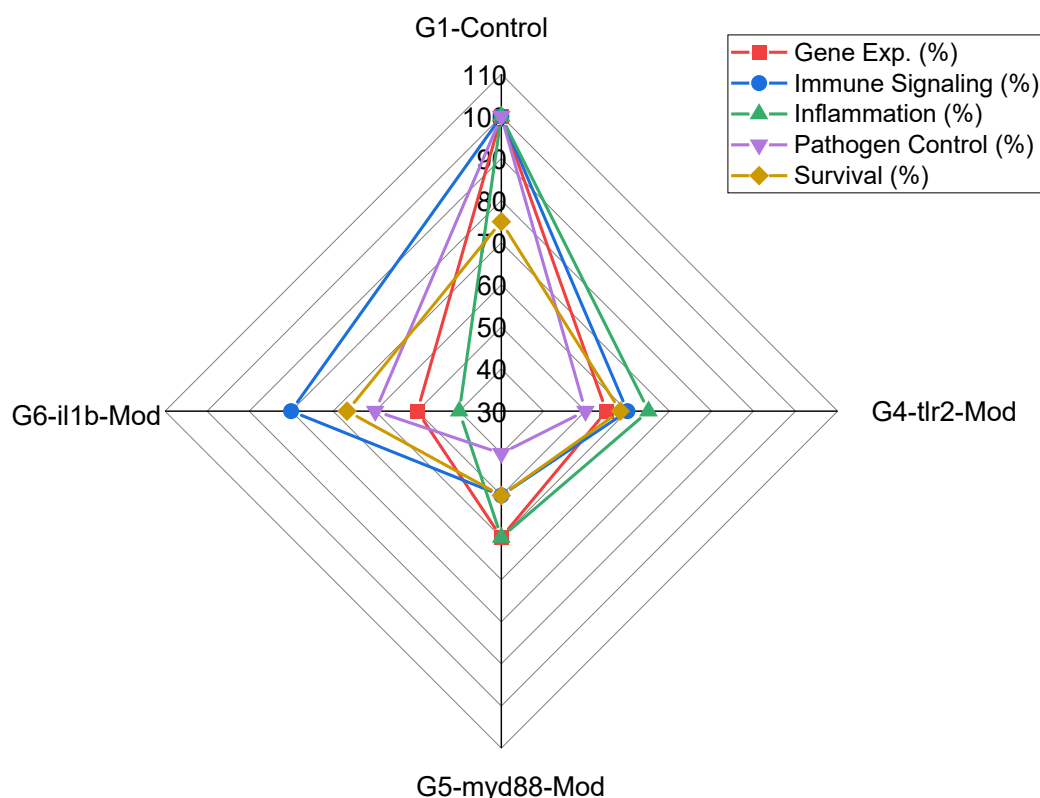


Figure 9: Multi-parameter profile integration that demonstrates the relationship between genetic modification, immune signaling, inflammation, pathogen elimination, and survival in Nile tilapia (*Oreochromis niloticus*).

Radar chart that plots the standard values of gene expression, immune signaling, inflammatory response, pathogen elimination, and survival ratios for unmodified and modified genes (tlr2, myd88, and il1b) in response to *Streptococcus agalactiae* infection.

Conclusion

In this experiment, it was shown that the modification of tlr2, myd88, and il1b by CRISPR-Cas9 resulted in impairment of the innate immune system of Nile tilapia against *S. agalactiae* infection through decreased expression of the target genes, decreased activation of immune pathway signaling downstream, and decreased responses of the inflammatory markers. It also caused an increased bacterial load (6.50-7.00 log₁₀ CFU/g) and decreased survival rate (50.0-66.7%) compared to the controls (75.0%). The controlled comparisons between unedited group, procedural group, non-targeting guide group, and gene-modified group enabled to differentiate the effects caused by target genes from the effects caused by procedures themselves. These results show that tlr2, myd88, and il1b are key factors in the immunity of the host, which proves the suggested model of TLR2-MyD88-IL-1 β signaling pathway. Future experiments have to include larger sample size and longer observation period.

Further studies need to evaluate compensatory mechanisms of the immune system, gene interactions in the immune system, as well as potential new candidate genes that might affect disease resistance. Testing different strains of *S. agalactiae*, varying environmental conditions, and partial editing will contribute to the understanding of the biological significance of these results. Replication in different laboratories and different genetic backgrounds will increase reproducibility of the experiments. The final step in applying these results is to integrate omics data into selective breeding programs in order to implement molecular findings into disease resistance improvement programs for Nile tilapia and other aquaculture species. This will allow to ensure sustainable fish production, avoid economic losses, and enhance food security worldwide.

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