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Thymomodulin enhances vaccination responses against *Streptococcus agalactiae* in Nile tilapia

Timomodulina melhora as respostas vacinais contra Streptococcus agalactiae em tilápia-do-Nilo

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ABSTRACT

Streptococcosis caused by *Streptococcus agalactiae* is a major economic problem for Nile tilapia *Oreochromis niloticus* production worldwide. Inactivated vaccines are considered an efficient method for controlling streptococcosis. However, during the vaccination process, stress during fish handling could affect the immune response. Immunomodulators, such as thymomodulin, act on the immune system and can improve vaccination responses. Thus, the aim of this study was to assess the immunomodulatory effect of thymomodulin in *O. niloticus* vaccinated against *S. agalactiae*. For this, fish were distributed in a factorial design (2x2x6), corresponding to two levels of thymomodulin supplementation (0.0 and 0.3%), two treatments (sterile phosphate buffered saline and inactivated *S. agalactiae* vaccine). Initially, fish received thymomodulin supplementation for 30 days. Next, fish were vaccinated by injection intraperitoneal (i.p.) of heat inactivated *S. agalactiae* vaccine. 15 days after vaccination, all fish were challenged i.p. with *S. agalactiae* and blood samples were collected at 7, 14, 21 days post infection (DPI). Results revealed that fish supplemented with thymomodulin vaccinated or not developed better protection against *S. agalactiae* than vaccinated fish without thymomodulin. Furthermore, fish vaccinated and supplemented with thymomodulin presented high levels of antibodies after 14 and 21 DPI, suggesting that thymomodulin can promote the immune response in Nile tilapia.

KEYWORDS: fish vaccines; immunostimulant; thymic extract; streptococcosis.

RESUMO

A estreptococose causada pelo *Streptococcus agalactiae* é um grande problema econômico para a produção de tilápia-do-nilo *Oreochromis niloticus* em todo o mundo. As vacinas inativadas são consideradas um método eficiente para o controle da estreptococose. No entanto, durante o processo de vacinação o estresse durante o manejo dos peixes pode afetar sua resposta imune. Imunomoduladores como a timomodulina, atuam no sistema imunológico e podem melhorar as respostas vacinais. Assim, o objetivo deste estudo foi avaliar o efeito imunomodulador da timomodulina em *O. niloticus* vacinado contra *S. agalactiae*. Para isso, os peixes foram distribuídos em esquema fatorial (2x2x6), correspondendo a dois níveis de suplementação de timodulina (0,0 e 0,3%), dois tratamentos (solução salina tamponada com fosfato estéril e vacina *S. agalactiae* inativada). Inicialmente, os peixes receberam suplementação de timomodulina por 30 dias. Em seguida, os peixes foram vacinados por via intraperitoneal (i.p.) da vacina de *S. agalactiae* inativada pelo calor. 15 dias após a vacinação, todos os peixes foram desafiados i.p. com *S. agalactiae* e amostras de sangue foram coletadas aos 7, 14, 21 dias após a infecção (DPI). Os resultados revelaram que os peixes suplementados com timomodulina vacinados ou não desenvolveram melhor proteção contra *S. agalactiae* do que os peixes vacinados sem timomodulina. Além disso, os peixes vacinados e suplementados com timomodulina apresentaram altos níveis de anticorpos após 14 e 21 DPI, sugerindo que a timomodulina pode promover a resposta imune em tilápia-do-nilo.

PALAVRAS-CHAVE: vacinas para peixes; imunoestimulante; extrato tímico; estreptococose.

INTRODUCTION

Nile tilapia is considered the third most farmed fish species in the world, with commercial production capacity of 2.6 million tons in 2020 (FAO 2020). However, infections caused by *Streptococcus* spp., especially *Streptococcus agalactiae* and *Streptococcus iniae*, can cause large mortalities and economic losses to the industry of aquaculture (WANG et al. 2020). *Streptococcus agalactiae* is widely distributed worldwide. In Brazil, it was first isolated from Nile tilapia in the Northern Region of Parana State (SALVADOR et al. 2003). This bacteria has been associated to meningoencefalitis and systemic infection in Nile tilapia of several Brazilian states and is considered the main infectious disease for this fish (DELPHINO et al. 2019, RAMOS-ESPINOZA et al. 2020a, b).

Vaccination is an important strategy for protecting tilapias from *S. agalactiae* infection. Currently, several antigen delivery systems have been tested against *S. agalactiae* including inactivated whole cell, subunit, extracellular vaccines, live attenuated, heterologous live vector and DNA vaccines (MUNANG'ANDU et al. 2016). Inactivated vaccines account for the largest proportion of vaccines developed against *S. agalactiae* in tilapias (MUNANG'ANDU et al. 2016). Results obtained in Nile tilapia after immunization with inactivated *S. agalactiae* vaccine showed high efficacy with relative percent survival (RPS) values of 83.6 and 96.4% for single and booster immunization with IP vaccines (PRETTO-GIORDANO et al. 2010).

Immunostimulants are used to modulate the immune system by increasing the host resistance against diseases (GANNAM & SCHROCK 1999, BRICKNELL & DALMO 2005). Receptors on the target immune cells recognize the immune-stimulatory substances as high-risk molecules and induce defense pathways (KIRON 2012). Immunomodulators are generally included as dietary supplements during stressful aquaculture operations and management, such as grading, transfer or vaccination (BRICKNELL & DALMO 2005, KIRON 2012). The use of immunostimulants in combination with fish vaccines can increase the protective capabilities of fish by enhancing innate immune parameters, as well as primary and secondary antibody responses (GALINA et al. 2009). Although, some studies have reported that high doses of immunostimulants in combination with vaccines can be immunosuppressive (LOGAMBAL & MICHAEL 2000).

Some immunostimulatory peptides have been extracted from the thymus gland and have been used to enhance the resistance to pathogens or tumors and retardation of immune senescence in animal models (RAA 1996, OLMOS et al. 2006). Thymomodulin is a calf thymus acid lysate derivative, composed of several peptides with a molecular weight range of 1-10KD, with capacity to modulate bone marrow stem cell proliferation and differentiation (KOUTTAB et al. 1989), enhance the phagocytosis in rat macrophages and human polymorphonuclear cells *in vitro* (BRAGA et al. 1993). Thymomodulin has evidenced therapeutic action to counter the alterations in cytokine expression induced by an immunodeficiency model in rats (OLMOS et al. 2006). Nevertheless, up to date, thymomodulin potential as immunostimulant for fish has not been addressed.

Thus, the aim of this study was to evaluate the effects of dietary thymomodulin supplementation on vaccination response against *S. agalactiae* in *O. niloticus*.

MATERIAL AND METHODS

Ethics Committee on the Use of Animals

The project was carried out based on the ethical principles proposed by the Brazilian College of Animal Experimentation and was approved by the Ethics Committee in the Use of Animals of the Faculty of Agricultural and Veterinary Sciences of UNESP (nº 14507/15).

Fish and experimental conditions

Two hundred and eighty Nile tilapia, *O. niloticus,* fingerlings (35 ± 2.0 g, mean ± SD) obtained from a commercial fish farm were transferred to 100 L glass tanks with dechlorinated tap water, continuous aeration by blowers and air stones, mechanical filter, and thermostat for acclimatization during a 14 days period. During this time, fish received commercial feed (Nutripiscis, 28% crude protein and 4000 $kcalkg^{-1}$ crude energy) at 3% body weight, two times a day (8:00 and 16:00). The remaining feed and feces were removed daily by siphoning 30 min after feeding and water renewal was applied up to 15% total volume. Water quality was monitored using multiparameters probes (YSI® model 55 and model 63 - YSI, Yellow Springs, OH, USA). Water quality values were kept as it follows: temperature = 28.4 ± 1.5, dissolved oxygen = 5.3 ± 0.7, pH = 7.6 ± 0.3, and electrical conductivity = 118.0 ± 10.1 μScm^{-1}. Parameters remained within the adequate range for this species of fish.

Bacteria

A pathogenic *S. agalactiae* strain isolated from a natural outbreak of streptococcosis in Nile tilapia in the state of Parana, Brazil, showing meningoencephalitis was used for the present study (SALVADOR et al. 2003). The same strain was previously used to tests several inactivated vaccines for Nile tilapias (RAMOS-ESPINOZA et al. 2020a, b). The samples were cultured in brain heart infusion agar (BHI, Difco Laboratories, Sparks, MD) enriched with 5% defibrinated ovine blood and incubated at 28°C for 48h. Identification of the isolated was by Gram coloration, morphology of the colonies, biochemical tests and PCR amplification (CHEN et al. 2012). Stock suspensions in BHI broth supplemented with 15% glycerol were maintained in ultra-freezer at -80 °C until further use.

Median lethal dose ($LD_{50\%}$)

Prior to the main experiment, Nile tilapia fingerlings, from the same batch, were randomly distributed into four experimental groups (10 fish/group), and maintained in 100 L aquaria, as described in section "Fish and experimental conditions". Fish were anesthetized with benzocaine (100 mgL^{-1}) and injected IP (0.2 mL) with three decreasing concentrations of *S. agalactiae* dissolved in sterile PBS ($1x10^8$, $1x10^6$, and $1x10^4$ CFU mL^{-1}); the remaining group was injected with the same volume of sterile PBS. The bacterial concentrations were determined by correlations between McFarland scale and spectrophotometry. Mortality after injection was monitored daily for 14 days, and the $LD_{50\%}$ was calculated as $1x10^8$ $CFUmL^{-1}$.

Vaccine production

Streptococcus agalactiae frozen cultures were grown onto BHI agar (Difco) and incubated at 28°C for 48h. After incubation, cultures were transferred to BHI broth and incubated under the same conditions in an orbital shaker at 3 G. Then, bacteria was heat inactivated by exposing the bacterial suspensions to 60°C for 30 min in a bacteriological incubator. Next, inactivated bacteria were centrifuged at 4000 G for 20 min at 4ºC. The supernatant was removed and bacterial pellets washed with sterile PBS (pH 7.2), the procedure was repeated twice. The bacterial concentration was confirmed prior to inactivation at 1.8×10^9 $CFUmL^{-1}$, the dose was chosen from prior studies with *S. agalactiae* vaccines in our laboratory (RAMOS-ESPINOZA et al. 2020a, b). To confirm that bacteria were correctly inactivated, samples (100 µL) were plated onto five BHI plates and incubated at 28°C for up to seven days. No visible grown was observed during this period. Finally, the inactivated bacterial solution was stored at 4°C for two days prior to vaccination.

Experimental design

Nile tilapia fingerlings were randomly distributed in 24 glass aquaria (ten fish each), including four treatments, six replicates, as it follows: Group C, fish fed commercial diet and non-vaccinated. Group V, fish fed regular commercial diet 30 days prior to vaccination with heat inactivated vaccine against *S. agalactiae*. Group L, fish fed commercial diet supplemented with thymomodulin (Leucogen®) and non-vaccinated. Group VL, fish fed diet supplemented with thymomodulin (Leucogen®) and vaccinated. After the feeding trial, fish were anesthetized in benzocaine solution (100 mgL^{-1}), and were IP injected with 0.2 mL of heat inactivated *S. agalactiae* vaccine (antigen dose $1.8x10^9$ $CFUmL^{-1}$). After inoculation, all fish were returned to their aquaria, and were maintained as previously described, but without supplemented feed. Two weeks after vaccination, fish were infected with 0.2 mL of a homologous *S. agalactiae* strain through IP route (bacterial dose $1.2x10^8$ $CFUmL^{-1}$). Finally, five fish from three replicates were anesthetized in a benzocaine solution (100 mgL^{-1}) at 7, 14 and 21 days post-infection (DPI) for blood collection to assess serum agglutinating activity and biochemical parameters. The remaining three replicates were used to assess cumulative survival up to 21 days post-challenge.

Experimental diet preparation

Throughout the experimental period, a commercial extruded feed was used. From the beginning of the feeding trial, groups C and V were fed commercial diet supplemented with commercial soybean oil (100 $mLkg^{-1}$ of feed). While, groups L and VL were fed the same commercial diet, plus soybean oil and thymomodulin (Leucogen®) (2 $mgkg^{-1}$ body weight). Soybean oil was used to dissolve the prebiotic in the feed. The prebiotic dose was chosen because it proved to be effective in restoring the depressed immune responses associated with neoplasia in humans (GARACI et al. 2000), and previous trials proved to be well tolerated by tilapias without inducing any mortality for up to two weeks (data not showed).

Blood sampling

Five tilapias per group were anesthetized in benzocaine solution (100 mgL^{-1}) and blood was collected by caudal venipuncture using 1 mL plastic syringes. An aliquot of blood was allowed to clot for 1h. The serum was collected by centrifuging at 2.800 G for 5 min at 4 °C. Serum samples were stored at -80°C until further analysis.

Serum agglutinating activity

The agglutinating activity of fish serum was determined by plate agglutination technique (SWAIN et al. 2007), using "U" shaped 96-wells plates. Briefly, 50 µL of PBS were dispensed in each well. Then, an equal volume of formalin killed *S. agalactiae* suspension was added to a well and two-fold dilutions from 1:2 to 1:4096 were prepared for each sample. The plates were then incubated overnight at room temperature (24 ºC). The titer was calculated as the highest dilution of serum showing complete agglutination of the bacterial cells.

Biochemical parameters

The biochemical analysis of serum samples was determined in a semi-automatic equipment for biochemical analyses (CELM®, Barueri, Brazil) using commercial kits (Labtest Diagnóstica, Minas Gerais, Brazil) according to the manufacturer's recommendations. Plasma glucose was estimated immediately after fish blood sampling by using OneTouch Ultra Mini™ (Johnson, Johnson Medical, Brazil). Total serum protein was determined by using the biuret method, albumin by bromocresol green method, globulin by subtracting albumin from total protein levels.

Statistical analysis

Data was tested for normality and homogeneity of variances using Shapiro–Wilk's and Levene's tests, respectively. Data were analysed by using analysis of variance (ANOVA) and Tukey's multiple pairwise comparison. The significance level was defined as $p<.05$. All data were expressed as mean ± (SEM). Finally, for graphic design and statistical analysis the software GraphPad Prism 5 (GraphPad Software, San Diego, USA) was used.

RESULTS

Cumulative survival

The results showed no significant differences in survival ($P > .05$) between groups at 7, 14 or 21 days post-infection (Fig. 1). Groups L and VL reached a cumulative survival above 80% (86.7% and 83.3%). While, cumulative survival for group V was 100% until Day 7, but decreased just above 80% at Day 14, and reached 70% at the last sampling time. Control group achieved a cumulative survival below 60% (57.7%).

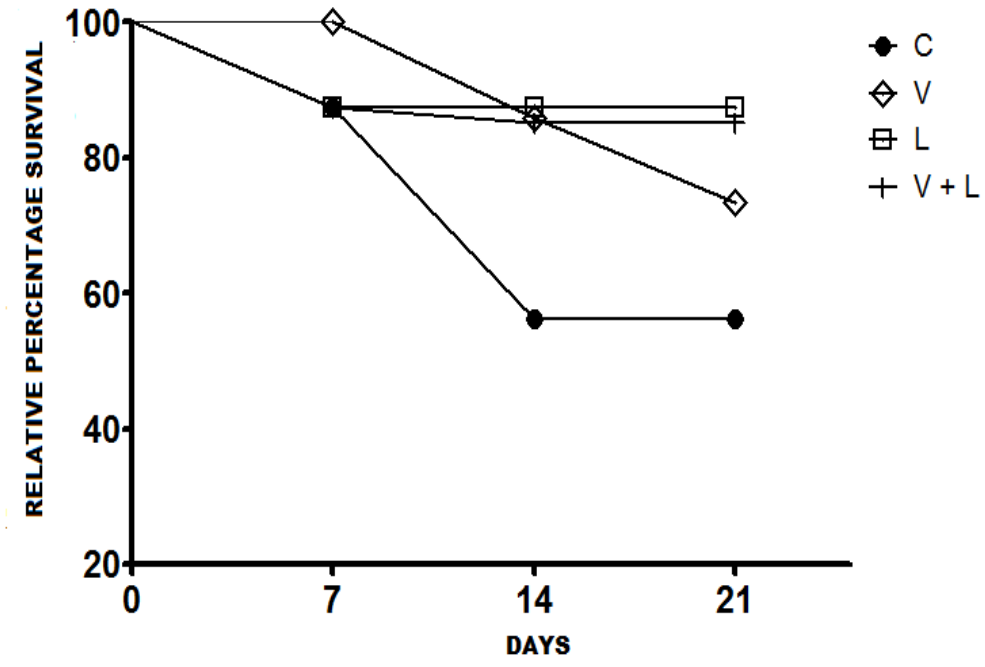

Figure 1. Cumulative percent survival of Nile tilapia after supplementation with thymomodulin and vaccination against *S. agalactiae* 21 days post challenge with *S. agalactiae*.

Serum agglutinating antibodies

Serum antibody titers were determined by agglutination assay at Days 7, 14 and 21 after challenge. The results showed several significant differences ($p<.05$) among groups at Day 7 (Fig. 2), with group V exhibiting higher values than VL, VL higher than C, and C higher than L. In addition, at 14 days post-infection (DPI), values from groups V and VL were significantly higher ($p<.05$) than groups C and L. Finally, at 21 DPI,

groups V and VL had significantly higher values than (p<.05) both Group C and L. Time course analysis showed that the antibody titers significantly increased from Day 7 to Day 14 in groups L and VL, while the antibody titers in groups C and V did not show significant differences between sampling periods.

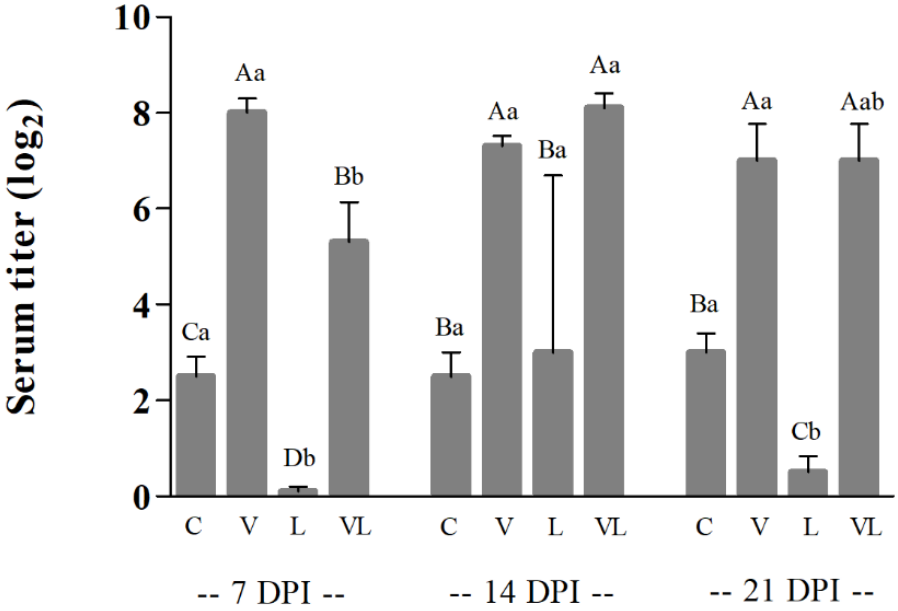

Figure 2. Serum agglutinating activity (mean ± SEM) of Nile tilapia after supplementation with thymomodulin, vaccination against *S. agalactiae* at several days post challenge with *S. agalactiae*. Capital letters compare different treatments within the same sampling period. Lowercase letters compared different sampling points within the same treatment. ANOVA and Tukey's test (p<.05).

Serum glucose concentration

No statistical differences on serum glucose levels were observed at Days 7 and 14 post-infection (P > .05) (Fig. 3). In addition, groups L and VL showed significant higher values (p< 0.05) compared to group C and V at Day 21. Glucose kinetics showed that values from group C diminished with time showing statistical differences between consecutive sampling times. Group V showed higher values at 7 DPI, compared to 14 and 21 DPI. Groups L and VL evidenced its highest values at 7 DPI, followed by concentrations at 21 DPI and finally 14 DPI, with significant differences between each one.

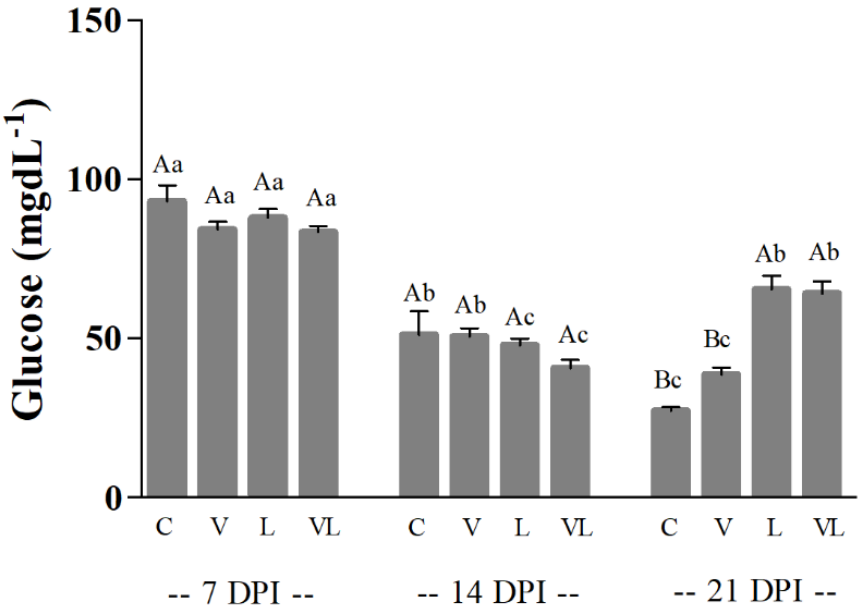

Figure 3. Serum glucose concentration (mean ± SEM) of Nile tilapia after supplementation with thymomodulin and vaccination against *S. agalactiae*, at several days post-infection. Capital letters compare different treatments within the same sampling period. Lowercase letters compared different sampling points within the same treatment. ANOVA and Tukey's test (p<.05).

Biochemical parameters

Total plasma proteins were lower (p<.05) on animals supplemented with thymomodulin at 7 and 21 DPI compared to the remaining groups. In addition, the evolution of each treatment showed no significant differences, except for the group L, which showed significant lower values on Day 21 compared to 7 and 14. No differences were observed for neither albumin nor globulin values (p<.05) (Table 1).

Table 1. Biochemical profile of Nile tilapia supplemented with thymomodulin and vaccinated against *S. agalactiae* at 7, 14 and 21 days after infection.

Days	Treatment	PPT	Albumin	Globulin
7	C	$4.23\pm0.22_{Aa}$	$1.08\pm0.50_{Aa}$	$3.15\pm0.36_{Aa}$
	V	$4.40\pm0.41_{Aa}$	$1.17\pm0.33_{Aa}$	$3.23\pm0.30_{Aa}$
	L	$3.90\pm0.48_{Ba}$	$0.73\pm0.25_{Aa}$	$3.13\pm0.42_{Aa}$
	VL	$4.30\pm0.37_{Aa}$	$1.14\pm0.25_{Aa}$	$3.24\pm0.36_{Aa}$
14	C	$4.60\pm0.22_{Aa}$	$1.32\pm0.15_{Aa}$	$3.27\pm0.19_{Aa}$
	V	$4.30\pm0.58_{Aa}$	$1.02\pm0.33_{Aa}$	$3.32\pm0.38_{Aa}$
	L	$4.60\pm0.14_{Aa}$	$1.03\pm0.28_{Aa}$	$3.57\pm0.27_{Aa}$
	VL	$4.35\pm0.32_{Aa}$	$0.93\pm0.10_{Aa}$	$3.42\pm0.35_{Aa}$
21	C	$4.35\pm0.21_{Aab}$	$1.23\pm0.17_{Aa}$	$3.13\pm0.17_{Aa}$
	V	$4.26\pm0.57_{Aab}$	$0.98\pm0.19_{Aa}$	$3.28\pm0.43_{Aa}$
	L	$3.63\pm0.33_{Bb}$	$0.77\pm0.15_{Aa}$	$2.87\pm0.27_{Aa}$
	VL	$4.45\pm0.29_{Aa}$	$1.12\pm0.15_{Aa}$	$3.33\pm0.25_{Aa}$

Capital letters compare different treatments within the same sampling period. Lowercase letters compared different sampling points within the same treatment. ANOVA and Tukey's test (p<.05).

DISCUSSION

The aim of the present study was not to test the efficacy of inactivated vaccines against *S. agalactiae*, since this has been tested extensively in other studies (MUNANG'ANDU et al. 2016, MIYABE et al. 2017, RAMOS-ESPINOZA et al. 2020a, b), but to test whether a novel immunostimulant for fish, thymomodulin, could improve immune responses in Nile tilapia vaccinated against *S. agalactiae*. Thus, this is the first report on the use of thymomodulin as immunostimulant for fish.

Previous studies stated that supplemented feed could be administered with other treatments such as vaccination in order to boost the immune response in vaccinated fish (RODRÍGUEZ et al. 2016). In the present research, Nile tilapias supplemented with thymomodulin (both vaccinated or not) showed survival rate above 80% after 21 days of intraperitoneal challenge with a homologous *S. agalactiae* strain. On the other hand, non-supplemented and vaccinated fish evidenced lower survival rate (60%). Despite the fact that there were no statistical differences between groups, the data proves that fish supplemented with thymomodulin, with or without vaccination, showed higher protection against *S. agalactiae* infection than fish vaccinated alone.

Research regarding immunostimulant associated to vaccination are scarce and there is no consensus on whether immunostimulants improve vaccination responses in fish. Similar studies using β-glucan-supplemented diets and vaccines for Atlantic salmon (*Salmo salar*) showed that there is a synergy between immunostimulants and vaccines, with clear effects on the expression of immune genes (RODRÍGUEZ et al. 2016). In contrast, WHITTINGTON et al. (2005) found that dietary β-glucan administration (50-200 $mgkg^{-1}$) had no significant effects on humoral responses (agglutinating antibodies, IgM) and survival after challenge in Nile tilapia vaccinated against *S. iniae*.

Immunostimulants, such as thymomodulin, are substances that can increase the magnitude of an adaptive response to vaccines (TAFALLA et al. 2013). Thymomodulin can induce the maturation of T-lymphocytes and enhance the functions of mature T-lymphocytes with cascading effects on B-cell functions (KOUTTAB et al. 1989). Thymus pectid such as thymopeptin and thymosin can induce T-lymphocyte proliferation and IL production (HADDEN 1993). The present results suggests that higher survival rate on animals supplemented with thymomodulin could be due to the enhanced function of both T and B-lymphocytes.

Antibodies are produced by B-lymphocytes and recognize specific microbial antigens, binding to their surface antigens and preventing them from attaching to their target cells (SONG et al. 2014). Antibodies are essential for the protection of fish against *Streptococcus* infections (PASNIK et al. 2005). In our research,

vaccinated groups (with or without thymomodulin) presented higher antibody titers than non-vaccinated groups at 7, 14 and 21 days post-infection, however, there were no differences between groups, thus is not possible to attribute such improvement to either vaccination or thymomodulin supplementation alone. In addition, fish supplemented with thymomodulin but non-vaccinated, did not showed high antibody levels, suggesting that thymomodulin may not induce its immunostimulant actions by itself and requires of stimulus such as vaccines or pathogenic challenges.

Glucose levels can increase in order to supply the energetic demand in stress situations (MOMMSEN et al. 1999). The results of this study showed higher blood glucose levels in all groups at day 7 post-challenge. This could be related to bacteria inoculation that likely produced release of cortisol as a way to redirect energy to vital organs (CLAUDIANO et al. 2019). Higher plasma glucose levels in stressed fish can be result of increased glycogenolysis and gluconeogenesis (VIJAYAN et al. 1997, MOMMSEN et al. 1999). EVANS et al. (2005) reported that after *S. agalactiae* challenge, significantly higher blood glucose levels are seen at 2, 24, 48, and 72 h. After the initial increase, values decreased significantly at day 14 post-challenge in all groups. PASNIK et al. (2008) observed that in Nile tilapia vaccinated against *S. agalactiae*, blood glucose levels decreased at 336 h (14 days) post-challenge with *S. agalactiae* to levels similar to those immediately prior to the challenge.

Increased levels of PPT, albumin and globulins could indicate that there is an increase of protein synthesis to produce more molecules involved in the organism immunity such as immunoglobulins, complement, cytokines, lysozyme, transferrin, acute phase proteins and anti-proteases (VASUDEVA et al. 2006, BILLER-TAKAHASHI et al. 2013). However, this was not observed in the current study, despite the fact that serum antibody titers were higher in vaccinated groups. MARCUSSO et al. (2015) also observed no difference in these parameters after vaccination with *S. agalactiae* and associated to high variation on the concentration of total proteins in teleost.

CONCLUSION

This preliminary study suggests that dietary thymomodulin can promote Nile tilapia adaptive immune response under laboratory conditions. However, further research is necessary to determine the optimum dosage and duration of thymomodulin feeding for tilapias in order to enhance both adaptive and innate immune responses.

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