Lysozyme activity as an indicator of innate immunity of tilapia (Oreochromis niloticus) when challenged with LPS and Streptococcus agalactiae

Jaqueline Dalbello Biller1*, Gustavo do Valle Polycarpo1, Basia Schlichting Moromizato1, Ana Paula Diogo Sidekerskis1, Thaís Daltoso da Silva2, Ingrid Camargo dos Reis3, Camino Fierro-Castro4

1 Universidade Estadual Paulista “Júlio de Mesquita Filho”, Faculdade de Ciências Agrárias e Tecnológicas, Dracena, SP, Brasil.
2 Universidade Estadual Paulista “Júlio de Mesquita Filho”, Programa de Pós-Graduação em Aquicultura, Jaboticabal, SP, Brasil.
3 Universidade Estadual Paulista “Júlio de Mesquita Filho”, Programa de Pós-Graduação em Ciência e Tecnologia Animal, Dracena, SP, Brasil.
4 Universidad de León, Facultad de Ciencias Biológicas y Ambientales, Departamento de Biología Molecular, Campus de Vegazana, León, España.

ABSTRACT - The objective of this study was to fully describe the protocol with standardized modifications and evaluate the lysozyme activity, an indicator of innate immunity in tilapia, to compare lipopolysaccharide (LPS) with Streptococcus agalactiae injections. Lysozyme was determined in serum using the turbidimetric method, in which lysozyme activity was evaluated by Micrococcus lysodeikticus lysis, with modifications for microplate assay. The experiment was conducted in a completely randomized design. Juvenile tilapia was divided in the following six treatments: challenged with phosphate buffer PBS (control) and 100, 250, 500, and 600 μg kg−1 LPS and S. agalactiae. All treatments were challenged for 72 h and seven days and then sampled to determine lysozyme activity. After 72 h or seven days, concentrations of LPS promoted changes in lysozyme production, either lesser or equal, depending on concentration when compared with fish injected with S. agalactiae. It was possible to standardize the analysis and determine that the treatment with LPS promotes immunomodulation at a concentration of 250 μg kg−1 LPS, this response being similar to challenge with S. agalactiae.

Keywords: aquaculture, cell wall, enzyme, fish, immune response

1. Introduction

The innate humoral system is the first to respond to pathogens through several soluble components in the body fluids. These compounds (C-reactive protein, amyloid A serum, transferrin, α-2 macroglobulin, complement C3, lysozyme, and lectins) are known as acute phase proteins (acute phase response) and are produced in high concentrations after an infection or cell damage. The vast majority of these proteins are often used as diagnostic indicators (Bayne and Gerwick, 2001; Biller-Takahashi et al., 2013; Biller-Takahashi and Urbinati, 2014).

Lysozyme was accidentally discovered by the Scottish bacteriologist, Sir Alexander Fleming (1881-1955), after his nasal secretion (resulting from a cold) fell on microbiological plaques, and he observed bacterial lysis (Fleming, 1922). Since then, lysozyme has been used as a protein model for several areas of knowledge, such as chemistry, enzymology, crystallography, molecular biology, in the pharmaceutical and food industry, and also has a role in immunology (Callewaert and Michiels, 2010).
Lysozyme is one of several anti-microbial proteins associated with the first barrier of innate immunity in fish (Bayne and Gerwick, 2001). Several types of lysozymes have been described such as c-type (conventional or chicken-type), g-type (goose-type), and invertebrate-type lysozyme (i-type), T4 phage lysozyme, bacterial lysozyme, and plant-type lysozyme (Jiménez-Cantizano et al., 2008; Callewaert and Michiels, 2010; Buonocore et al., 2014). Only g-type and c-type lysozymes have been identified in several fish species (Jiménez-Cantizano et al., 2008; Saurabh and Sahoo, 2008; Buonocore et al., 2014; Mohapatra et al., 2019). In fish, lysozyme genes are expressed in cells of myeloid origin (Hall et al., 2007; Saurabh and Sahoo, 2008), and these are present in mucus, eggs, blood, and tissues with leukocytes (neutrophils, monocytes, and a small amount in macrophages) to defend against microorganisms, such as bacteria (Li et al., 2021; Murray and Fletcher, 1976).

*Streptococcus agalactiae* is a bacterium that causes infections and affects many fish species in pisciculture worldwide. It is a major health challenge for fish and is characterized by septicemia, exophthalmos, and meningoencephalitis (Evans et al., 2008; Olivares-Fuster et al., 2008). In 2016, an outbreak of *Streptococcus agalactiae* resulted in high mortality rates in six tilapia farms in northeastern Brazil, causing significant economic losses for the tilapia aquaculture industry (Mian et al., 2009; Chideroli et al., 2017). The Nile tilapia (*Oreochromis niloticus*) has excellent growth and reproductive performance and is classified as one of the most important fish produced in Brazil and worldwide, and has been used in many studies on immunomodulators (Ha et al., 2017; Zaminhan-Hassemer et al., 2020), thus the reason for it being chosen for this study.

The presence of lysozyme activity in fish blood can be measured, and it is an important tool for analyzing the innate immune system (Franco Montoya et al., 2017; Amphan et al., 2019). Standardization of this technique would provide a valuable analytical method for studies of resistance to bacterial infections such as those by *S. agalactiae*. Lipopolysaccharide (LPS) is a complex molecule present in the cell wall of Gram-negative bacteria, has been used to promote immunomodulation in fish, and is a good alternative for reproducing immunological challenges (Liu et al., 2016; Ha et al., 2017; Schug et al., 2019).

The objective of this work was to fully describe the protocol with standardized modifications of the lysozyme activity, which is an indicator of innate immunity in tilapia. And our intention was also to compare two immunomodulators, LPS and *S. agalactiae* injections, by their effects on lysozyme activity and their potential as immunological challenge factor in tilapia.

### 2. Material and Methods

#### 2.1. Experimental design and sampling procedure

This study was approved by the Institutional Ethics Committee on the use of Animals (case number 16/2018.R1), in Dracena, São Paulo, Brazil (Latitude: 21°29'0" South, Longitude: 51°32'1" West, altitude: 419 m). A total of 288 juvenile Nile tilapia with an initial weight and total length of 75.7±8.5 g and 16.1±0.7 cm, respectively (mean ± standard deviation), were distributed into 24 polythene tanks, with 12 animals per tank, and four tanks for each treatment. Each tank had a capacity of 130 L and was equipped with an open circulation system with continuous water supply and forced aeration. The water temperature was approximately 26 °C, which was maintained by heating and the use of thermostats, and a natural photoperiod (12 h light: 12 h dark) was established. The water flow in the tanks was approximately 2 to 2.5 L per min. The water temperature and water quality variables of the tanks were assessed and kept within the appropriate ranges, i.e., temperature: 26.1±1.4 °C, dissolved oxygen: 5.8±1.4 mg L⁻¹ (oximeter YSI 55), 0.08±0.02 NH₄ mg L⁻¹ (Nessler method), and pH: 7.54±0.11 (Corning pH meter). When necessary, organic matter in the tanks was removed. The fish were acclimated to laboratory conditions for 30 days in polyethylene tanks and fed a commercial diet four times a day until apparent satiation during the acclimation and experimental periods (28% crude protein and 3,600 kcal digestible energy kg⁻¹) in particles smaller than 8 mm.
For the implantation of the experiment, fish were fasted for 24 h, anesthetized in clove oil dissolved in an alcoholic solution (1 g 10 L⁻¹ of water), individually weighed in a semi-analytical digital scale with an accuracy of 0.01 g, and finally, measured with an ichthyometer. Fish were divided into the following six experimental groups, with four repetitions (each fish collected was considered an experimental unit, n = 12): a control group injected with 0.5 mL of PBS; four groups challenged by an intraperitoneal injection of 0.5 mL of LPS solution at 100, 250, 500, and 600 μg kg⁻¹ extracted from *E. coli* 026: B6, (Sigma, St. Louis, MO, USA) dissolved in PBS buffer (according to Ha et al., 2017); and a challenge group with an injection of 0.5 mL at the concentration of 1.0×10⁸ CFU of *S. agalactiae* per fish, which was diluted in sterile saline solution (0.85%). The MacFarland scale was used for the standardization of the inoculations at 50% lethal dose (LD₅₀), which was previously determined, and verified by reading in spectrophotometry (600 nm). The *S. agalactiae* strains were isolated from tilapia infected with signs of meningoencephalitis and identified according to culture, morphological, staining, and biochemical characteristics. The bacteria were obtained from the collection of the Laboratory of Pathology of Aquatic Organisms (CAUNESP-UNESP).

Fish were sampled twice, at 72 h and seven days after being challenged for the study of acute and long-term immune effects. Three tilapia per tank were sampled and anesthetized, subjected to blood sampling for standardization and evaluation of lysozyme activity. The blood from caudal vessels was collected with a syringe and then allowed to clot for 4 h at 4 °C and subsequently centrifuged (3000 rpm for 10 min). The serum was separated and kept at −80 °C until analyses were performed.

### 2.2. Lysozyme activity analysis

The standardization of the lysozyme activity methodology was determined using the turbidimetric assay as described by Ellis (1990), in which the lysozyme activity was evaluated by the lysis of *Micrococcus lysodeikticus*; with modifications for the microplate assay, according to Tort et al. (1998). A lyophilized *M. lysodeikticus* suspension (Sigma-Aldrich, St. Louis, MO, USA) was prepared at the concentration of 2 mg mL⁻¹ to be used as a lysozyme substrate. This was prepared with KH₂PO₄ (0.05M), Na₂HPO₄ (0.02M), and NaCl (0.05M) in Milli-Q water qsp 500 mL, with pH adjusted to 6.3 (with 0.1 mol HCl L⁻¹). A lyophilized chicken egg white lysozyme solution (Sigma-Aldrich, St. Louis, MO, USA) was prepared to construct a standard curve, and a stock solution was prepared with 1 mg mL⁻¹ (40,100 units mL⁻¹) in Milli-Q water; then used to prepare a salt solution at 4,010 units mL⁻¹ in NaCl 0.0154 mol L⁻¹ solution. Subsequently, using the salt lysozyme solution, a serial dilution was prepared with Milli-Q water at 668, 334, 167, 83.5, 42, and 21 unit L⁻¹.

Initially, the tilapia serum was heated in a water bath at 56 °C for 30 min to degrade the hemolytic proteins of the complement system, which act on the same substrate (*M. lysodeikticus*). The assay was started in a flat-bottomed microplate, and the construction of the standard curve was in triplicate (three columns). A negative control column was prepared with the addition of 10 μL distilled water. Then, 10 μL of serum samples were placed in duplicates in the rest of wells. To initiate the reaction, 200 μL of *M. lysodeikticus* was then added to each well. The reaction was performed at 25 °C, and the absorbance was measured at 450 nm using a microplate reader (Multiskan GO, Thermo Scientific) after 0.5 and 10 min. In previous tests, not shown in this paper, the activity of the lysozyme was tested up to 30 min, though it was observed that, at times longer than 10 min, the optical density (OD) values were very low and generated errors. Therefore, it was determined that 10 min was the optimal period for obtaining the best data to determine the enzyme activity.

The results of the difference between the final and initial turbidity (OD reduction) were expressed in units of lysozyme per mL of serum. A linear regression equation representing the lysozyme calibration curve was used to determine the serum lysozyme levels (units mL⁻¹). One unit is defined as the amount of sample required to reduce absorbance by 0.001 min⁻¹ at 450 nm compared to the control (*M. lysodeikticus* suspension without serum).
2.3. Statistical analysis

The study was conducted as a completely randomized design according to the following general model:

\[ Y_{ij} = \mu + T_i + e_{ij} \]

in which \( Y_{ij} \) = response variable in each experimental unit, \( \mu \) = general mean of the response variable, \( T_i \) = effect of the \( i \)-th treatment level, and \( e_{ij} \) = residual error. The experimental unit was a 130-L polythene tank with 12 fish. One-way ANOVA was used to assess any differences among treatment groups. The comparison of any two mean values was performed using Tukey’s multiple range test (\( P<0.05 \)). The results are presented as the mean ± standard deviation, and two comparisons were made, differences between sampling times (A, B) and differences among treatments in each time (a, b, c), and were analyzed using SAS computer software (Statistical Analysis System, version 9.2). Before performing the analysis of variance, normality and variance homogeneity were tested according to Kolmogorov-Smirnov and Bartlett tests, respectively.

3. Results

The initial optical density value is higher, since, over time, there is substrate lysis (\( M. \) lysodeikticus). Thus, after 10 min, as determined in the methodology, the OD reading of the substrate will be lower, due to lysozyme-mediated cell lysis. In the samples with a higher concentration of lysozyme, the OD reading will be lower, since there will be greater lysis of the substrate.

This followed the same previously described procedure, and the results were expressed in units of lysozyme per mL of serum. From the standard curve, the linear regression equation representing the lysozyme calibration curve was used to determine the serum lysozyme levels (units mL\(^{-1}\)) of the samples (Figure 1). The standardized analysis was adequate for measuring the lysozyme concentration of the tilapia challenged with LPS and \( S. \) agalactiae after 72 h and seven days of challenge.

Regarding the sampling times, all treatments showed a decrease in lysozyme activity after seven days. In the comparison between the PBS control group and each of the treatment groups, at 72 h, except for dose of 100 μg kg\(^{-1}\), all the other treatments were significantly lower, especially for 250 μg kg\(^{-1}\) and the \( S. \) agalactiae-challenged samples. After seven days, the higher doses of LPS (500 and 600 μg kg\(^{-1}\)) promoted significantly lower levels of lysozyme activity (Figure 2).

In the comparison between \( S. \) agalactiae and each of the treatment groups, at 72 h only the PBS control group and LPS dose of 100 μg kg\(^{-1}\) showed significantly higher values of lysozyme activity, while the

![Figure 1](image-url) - Graphical demonstration of the standard curve evidencing the optical density reading of samples, according to the concentration of egg lysozyme.
other LPS treatments were similar to the *S. agalactiae*-challenged samples. At seven days, significant differences were found for the higher doses of LPS (500 and 600 μg kg⁻¹) compared with samples challenged with the bacteria (Figure 2).

### 4. Discussion

In immunological studies, measurement of lysozyme activity has been performed since the 1960s through three classical methods: turbidimetric assay (Parry et al., 1965), lysoplate assay (Osserman and Lawlor, 1966), and lyso-rocket electrophoresis (Virella, 1977). Currently, advances in molecular biology technologies enable researchers to focus on the molecular characterization of the lysozyme gene through sequencing analysis. Consequently, this permits to estimate lysozyme dosage that is assessed by gene expression, and often the production of the enzyme can be further verified through direct or indirect ELISA assays with the use of a specific monoclonal antibody to identify lysozyme (Marsh and Rice, 2010; Mohapatra et al., 2019).

In this study, the lysozyme activity assay was suitably established for tilapia and proven to be reliable and easy to perform, thus generating a valuable tool for the assessment of the innate immune system of this fish species. In addition, this method has the advantage over current gene expression technique because it represents the dosage of proteins produced by the individual, since the classical technique can quantitatively measure the proteins produced even after the complex biological networks, which can interfere with the result of gene expression. The transcriptome can undergo epigenetic changes that will influence the result of the components produced by the cells. Only the protein dosages and evaluation of its activities prove the animal’s production capacity (Jalili et al., 2018; O’Flaherty et al., 2020).

The standardization of methods is important for comparing results found in the scientific literature (Biller-Takahashi et al., 2012; Biller-Takahashi et al., 2013). The test applied in the present study is widely used in several fish immunology studies (Franco Montoya et al., 2017; Amphan et al., 2019; Li et al., 2021; Mohammadian et al., 2021); however, new methods should be standardized and used for a more complete assessment of the entire network of reactions that occurs in the fish body (Martin and Król, 2017).

Concentrations of LPS promoted changes in lysozyme production, which were either lesser or equal depending on the concentration, after 72 h or seven days, when compared with fish injected with...
S. agalactiae. This indicates that the commercial chemical also promotes immunomodulation as the traditional microbiological challenge and can thus be used for fish immunology studies.

Lysozyme level or activity is an important indicator of innate immunity of fish. The recognition of pathogens mediated by pattern recognition receptors (PRR) is critical to the initiation of innate immune responses and subsequent host immunity through multiple signaling pathways, such as increased lysozyme production, which contribute to the eradication of the pathogen (Janeway Jr. and Medzhitov, 2002).

Lysozyme has the ability to attack the peptidoglycan layer of bacterial cell walls by hydrolysis of beta-1,4-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine acid, and leads to destruction of Gram-positive bacteria. However, it can damage Gram-negative bacteria if their outer cell wall is disrupted due to the previous action of the proteins of the complement system or other enzymes (Paulsen et al., 2003; Marsh and Rice, 2010). When bound to the microorganisms, it acts as an opsonization agent, promoting phagocytosis. Lysozyme may also present a fungicidal action, since it binds to structures with muramic acid and hydrolyses glycol chitin present in fungi and invertebrates (Saurabh and Sahoo, 2008).

The innate immune response is a rapid response to the presence of pathogens and triggers multiple mechanisms and networks of action, such as the production of antimicrobial substances and acute phase proteins, non-classical complement activation, release of cytokines, inflammation and phagocytosis, and finally gives way to the development of an adaptive response. Possibly, for this reason, after contact with the pathogen for a time, the levels of lysozyme activity decrease, giving way to other defense mechanisms, so it is not surprising to find low levels of lysozyme as time passes. Perhaps earlier samplings, such as a few hours after challenge, would detect increased values. Zhang et al. (2018) showed how the mRNA levels of lysozyme in different tissues of Micropterus salmoides at different time points under heat stress decreased after 24 h. Whang et al. (2011) also found this in the head kidney tissue of Oplegnathus fasciatus in response to challenges with LPS.

Lysozyme activity is dependent on the degree of stress, intensity, and its duration and type of stressors (Yildiz, 2006; Mohammadian et al., 2021). Demers and Bayne (1997) showed that, following exposure to a handling stressor, lysozyme activity was significantly increased in rainbow trout, and Caruso and Lazard (1999) reported that plasma lysozyme activity of Nile tilapia stressed by social pressure was lower than in unstressed fish.

The suppression of lysozyme activity was observed especially in higher LPS dose treatments at seven days. The challenge with the immunomodulator is usually applied to compare the effects of treatments. As such, a number of studies have investigated the action of immunostimulants (such as vitamins, β-glucan, and others) through challenges with LPS (Ha et al., 2017; Schug et al., 2019) or bacterial challenges (Biller-Takahashi et al., 2013; Souza et al., 2019). After immunomodulation, the defense system may trigger proinflammatory or anti-inflammatory pathways, depending on many interactions, to modulate the systemic response (Tort, 2011). As a result, it is possible to prove the beneficial action of the immunostimulant even after the challenge, when the animals present improvement in their immunological responses, since the challenge causes stress and immune defense impairment (Rebl and Goldammer, 2018).

Often the humoral and cell-mediated responses may differ upon contact with some immunomodulators, besides demonstrating early or short-term effectiveness depending on the studied compound (Biller et al., 2019). As an example, Ha et al. (2017) demonstrated the efficacy of mannan-oligosaccharide immunostimulant in tilapias that promoted increased lysozyme activity even after being challenged with LPS.

Often the concentration of this enzyme may vary due to season, sex, and sexual maturity, as well as stress conditions (Saurabh and Sahoo, 2008). Hernández and Tort (2003) determined annual variations of lysozyme activity in Sparus aurata, evidencing how the defense can be modulated by several factors. Immunomodulators may also alter lysozyme activity. Reda et al. (2018) observed increased enzyme...
activity in tilapia supplemented with dietary yeast nucleotides. Supplementation with probiotic *Bacillus subtilis* endospores also promoted increased lysozyme activity in tilapias (Galagarza et al., 2018). Administration of β-glucan for two to four weeks promoted increased lysozyme activity in tilapia challenged with *Aeromonas hydrophila* and *Flavobacterium columnare* (Amphan et al., 2019).

Regarding the LPS doses, different doses were tested to find the most suitable, since it is known that very high doses can produce less stimulation, as Tahir and Secombes (1996) observed in the culture of *Limanda limanda* macrophages or as in this study. Similarly, very low doses in fish do not generate an immune response. In our experiment, it was possible to observe the same suppression response for both immunomodulating agents, 250 μg kg⁻¹ of LPS and *S. agalactiae*, indicating that the microbiological challenge may be replaced by challenge with this dose of LPS.

5. Conclusions

The standardized turbidimetric method for determination of lysozyme activity in tilapia proved to be reliable and easy to perform, and has provided a valuable tool for the assessment of the innate immune system of this fish species. Additionally, the lipopolysaccharide challenge is an effective alternative for activating innate immunity and can be used, mostly with 250 μg kg⁻¹ of lipopolysaccharides for samplings, both at 72 h and seven days. Therefore, it is possible to affirm that the injection of lipopolysaccharides modulates the immune system.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: J.D. Biller. Data curation: J.D. Biller, A.P.D. Sidekerskis, T.D. Silva and C. Fierro-Castro. Formal analysis: J.D. Biller, A.P.D. Sidekerskis, T.D. Silva and C. Fierro-Castro. Funding acquisition: J.D. Biller. Investigation: J.D. Biller, B.S. Moromizato, A.P.D. Sidekerskis, T.D. Silva and I.C. Reis. Methodology: J.D. Biller, B.S. Moromizato, A.P.D. Sidekerskis, T.D. Silva and I.C. Reis. Project administration: B.S. Moromizato, A.P.D. Sidekerskis and I.C. Reis. Software: G.V. Polycarpo. Supervision: J.D. Biller and G.V. Polycarpo. Validation: G.V. Polycarpo. Visualization: G.V. Polycarpo and I.C. Reis. Writing-original draft: J.D. Biller, G.V. Polycarpo, B.S. Moromizato, A.P.D. Sidekerskis, T.D. Silva, I.C. Reis and C. Fierro-Castro. Writing-review & editing: J.D. Biller and C. Fierro-Castro.

Acknowledgments

The authors would like to thank the Faculdade de Ciências Agrárias e Tecnológicas, Dracena, Universidade Estadual Paulista (UNESP), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This study was funded through a grant from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; process number 2012/22016-3).

References

Amphan, S.; Unajak, S.; Printrakoon, C. and Areechon, N. 2019. Feeding-regimen of β-glucan to enhance innate immunity and disease resistance of Nile tilapia, *Oreochromis niloticus* Linn., against *Aeromonas hydrophila* and *Flavobacterium columnare*. Fish and Shellfish Immunology 87:120-128. https://doi.org/10.1016/j.fsi.2018.12.062

Bayne, C. J. and Gerwick, L. 2001. The acute phase response and innate immunity of fish. Developmental and Comparative Immunology 25:725-743. https://doi.org/10.1016/S0145-305X(01)00033-7

Biller, J. D.; Takahashi, L. S.; Moromizato, B. S.; Miasaki, C. T. and Urbinati, E. C. 2019. Levamisole modulates the cell-mediated immunity of matrixá, *Brycon amazonicus*. Boletim do Instituto de Pesca 45:e.445.
Lysozyme activity as an indicator of innate immunity of tilapia (*Oreochromis niloticus*) when challenged...

Biller et al.

Biller-Takahashi, J. D. and Urbinati, E. C. 2014. Fish immunology: The modification and manipulation of the innate immune system: Brazilian studies. Anais da Academia Brasileira de Ciências 86:1483-1495. https://doi.org/10.1590/0001-3765201420130159

Biller-Takahashi, J. D.; Takahashi, L. S.; Pilarski, F.; Sebastian, F. A. and Urbinati, E. C. 2013. Serum bactericidal activity as indicator of innate immunity in pacu (*Piaractus mesopotamicus*) (Holmberg, 1887). Arquivo Brasileiro de Medicina Veterinária e Zootecnia 65:1745-1751. https://doi.org/10.1590/S0102-0935201300600023

Biller-Takahashi, J. D.; Takahashi, L. S.; Marzocchi-Machado, C. M.; Zanuzzo, F. Z.; Sabioni, R. E. and Urbinati, E. C. 2012. Hemolytic activity of alternative complement pathway as an indicator of innate immunity in pacu (*Piaractus mesopotamicus*). Revista Brasileira de Zootecnia 41:237-241. https://doi.org/10.1590/S1516-35992012000200001

Buonocore, F.; Randelli, E.; Trisolino, P.; Facchiano, A.; Pascale, D. and Scapigliati, G. 2014. Molecular characterization, gene structure and antibacterial activity of a g-type lysozyme from the European sea bass (*Dicentrarchus labrax*). Molecular Immunology 62:10-18. https://doi.org/10.1016/j.molimm.2014.05.009

Callewaert, L. and Michiels, C. W. 2010. Lysozymes in the animal kingdom. Journal of Biosciences 35:127-160. https://doi.org/10.1007/s12038-010-0015-5

Caruso, D. and Lazard, J. 1999. Subordination stress in Nile tilapia and its effect on plasma lysozyme activity. Journal of Fish Biology 55:451-454. https://doi.org/10.1111/j.1095-8649.1999.tb00690.x

Chideroli, R. T.; Amoroso, N.; Mainardi, R. M.; Suphoronski, A. S.; Padua, S. B.; Alfieri, A. F.; Alfieri, A. A.; Mosela, M.; Moralez, A. T. P.; Oliveira, A. G.; Zanol, R.; Di Santis, G. W. and Pereira, U. P. 2017. Emergence of a new multidrug-resistant and highly virulent serotype of *Streptococcus agalactiae* in fish farms from Brazil. Aquaculture 479:45-51. https://doi.org/10.1016/j.aquaculture.2017.05.013

Demers, N. E. and Bayme, C. J. 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Developmental and Comparative Immunology 21:363-373. https://doi.org/10.1016/S0145-305X(97)00009-8

Ellis, A. E. 1990. Lysozyme assays. p.101-103. In: Techniques in fish immunology. Fish immunology technical communications, no. 1. Stolen, J. S.; Fletcher, T. C.; Anderson, D. P.; Roberson, B. S. and van Muiswinkel, W. B., eds. SOS Publications, Fair Haven, NJ.

Evans, J. J.; Bohnsack, J. F.; Klesius, P. H.; Whiting, A. A.; Garcia, J. C.; Shoemaker, C. A. and Takahashi, S. 2008. Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan. Journal of Medical Microbiology 57:1369-1376. https://doi.org/10.1099/jmm.0.47815-0

Fleming, A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character 93:306-317. https://doi.org/10.1098/rspb.1922.0023

Franco Montoya, L. N.; Martins, T. P.; Gimbo, R. Y.; Zanuzzo, F. S. and Urbinati, E. C. 2017. β-Glucan-induced cortisol levels improve the early immune response in *Brycon amazonicus*. Fish and Shellfish Immunology 60:197-204. https://doi.org/10.1016/j.fsi.2016.11.055

Galagarza, O. A.; Smith, S. A.; Drahos, D. J.; Eifert, J. D.; Williams, R. C. and Kuhn, D. D. 2018. Modulation of innate immunity in Nile tilapia (*Oreochromis niloticus*). Fish and Shellfish Immunology 83:171-179. Fish and Shellfish Immunology 83:171-179. https://doi.org/10.1016/j.fsi.2018.08.062

Ha, N.; Gonçalves, A. F. N.; Sousa, L. C.; Biller-Takahashi, J. D. and Takahashi, L. S. 2017. Dietary carbohydrates and protein of yeast modulate the early stages of innate immune response in tilapia (*Oreochromis niloticus*) primarily after LPS inoculation. Aquaculture International 25:755-776. https://doi.org/10.1007/s10499-016-0073-2

Hall, C.; Flores, M. V.; Storm, T.; Crosier, K. and Crosier, P. 2007. The zebrafish lysozyme *C* promoter drives myeloid-specific expression in transgenic fish. BMC Developmental Biology 7:42. https://doi.org/10.1186/1471-213X-7-42

Hernández, A. and Tort, L. 2003. Annual variation of complement, lysozyme and haemagglutinin levels in serum of the gilthead sea bream (*Sparus aurata*). Fish and Shellfish Immunology 15:479-481. https://doi.org/10.1016/S1050-4648(03)00024-X

Jalili, M.; Gebhardt, T.; Wolkenhauer, O. and Salehzadeh-Yazdi, A. 2018. Unveiling network-based functional features through integration of gene expression into protein networks. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1864:2349-2359. https://doi.org/10.1016/j.bbadis.2018.02.010

Janeway Jr., C. A. and Medzhitov, R. 2002. Innate immune recognition. Annual Review of Immunology 20:197-216. https://doi.org/10.1146/annurev.immunol.20.030801.084359

Jiménez-Cantizano, R. M.; Infante, C.; Martín-Antonio, B.; Ponce, M.; Hachero, I.; Navas, J. I. and Manchado, M. 2008. Molecular characterization, phylogeny, and expression of *c-type* and *g-type* lysozymes in *brill* (*Scophthalmus hubbsi*). Fish and Shellfish Immunology 25:57-65. https://doi.org/10.1016/j.fsi.2007.12.009

Li, L.; Cardoso, J. C. R.; Félix, R. C.; Mateus, A. P.; Canário, A. V. M. and Power, D. M. 2021. Fish lysozyme gene family evolution and divergent function in early development. Developmental and Comparative Immunology 114:103772. https://doi.org/10.1016/j.dci.2020.103772
Lysozyme activity as an indicator of innate immunity of tilapia (*Oreochromis niloticus*) when challenged.

Biller et al.

Liu, Q. N.; Xin, Z. Z.; Zhang, D. Z.; Jiang, S. H.; Chai, X. Y.; Li, C. F.; Zhou, C. L. and Tang, B. P. 2016. Molecular identification and expression analysis of a goose-type lysozyme (LysG) gene in yellow catfish *Peleobagrus fulvidraco*. Fish and Shellfish Immunology 58:423-428. https://doi.org/10.1016/j.fsi.2016.09.034

Marsh, M. B. and Rice, C. D. 2010. Development, characterization, and technical applications of a fish lysozyme-specific monoclonal antibody (mAb M24-2). Comparative Immunology, Microbiology and Infectious Diseases 33:e15-e23. https://doi.org/10.1016/j.cimid.2009.10.002

Martin, S. A. M. and Król, E. 2017. Nutrigenomics and immune function in fish: new insights from omics technologies. Developmental and Comparative Immunology 75:86-98. https://doi.org/10.1016/j.dci.2017.02.024

Mian, G. F.; Godøy, D. T.; Leal, C. A. G.; Yuhara, T. Y.; Costa, G. M. and Figueiredo, H. C. P. 2009. Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. Veterinary Microbiology 136:180-183. https://doi.org/10.1016/j.vetmic.2008.10.016

Mohammadian, T.; Ghanem-Motlagh, R.; Molayemraftar, T; Mesbah, M.; Zarea, M.; Mohtashamipour, H. and Nejad, A. J. 2021. Modulation of growth performance, gut microflora, non-specific immunity and gene expression of proinflammatory cytokines in shabout (*Tor grypus*) upon dietary prebiotic supplementation. Fish and Shellfish Immunology 112:38-45. https://doi.org/10.1016/j.fsi.2021.02.012

Mohapatra, A.; Parida, S.; Mohanty, J. and Sahoo, P. K. 2019. Identification and functional characterization of a g-type lysozyme gene of *Labeo rohita*, an Indian major carp species. Developmental and Comparative Immunology 92:87-98. https://doi.org/10.1016/j.dci.2018.11.004

Murray, C. K. and Fletcher, T. C. 1976. The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa*) tissues. Journal of Fish Biology 9:329-334. https://doi.org/10.1111/j.1095-8649.1976.tb04681.x

Oliveares-Fuster, O.; Kleisius, P. H.; Evans, J. and Arias, C. R. 2008. Molecular typing of *Streptococcus agalactiae* isolates from fish. Journal of Fish Diseases 31:277-283. https://doi.org/10.1111/j.1365-2761.2007.00900.x

Osserman, E. F. and Lawlor, D. P. 1966. Serum and urinary lysozyme (*muramidase*) in monocytic and monomyelocytic leukemia. Journal of Experimental Medicine 124:921-952. https://doi.org/10.1084/jem.124.5.921

O'Flaherty, R.; Bergin, A.; Flampouri, E.; Mota, L. M.; Obaidi, I.; Quigley, A.; Xie, Y. and Butler, M. 2020. Mammalian cell culture for production of recombinant proteins: A review of the critical steps in their biomanufacturing. Biotechnology Advances 43:107552. https://doi.org/10.1016/j.biotechadv.2020.107552

Parry, R. M.; Chandan, R. C. and Shahani, K. M. 1965. A rapid and sensitive assay of muramidase. Proceedings of the Society for Experimental Biology and Medicine 119:384-386. https://doi.org/10.3181/00379727-119-30188

Paulsen, S. M.; Lunde, H.; Engstad, R. E. and Robertsen, B. 2003. *In vivo* effects of β-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar*). Fish and Shellfish Immunology 14:39-54. https://doi.org/10.1016/j.fsi.2002.04.016

Rehl, A. and Goldammer, T. 2018. Under control: The innate immunity of fish from the inhibitors’ perspective. Fish and Shellfish Immunology 77:328-349. https://doi.org/10.1016/j.fsi.2018.04.016

Reda, R. M.; Selim, K. M.; Mahmoud, R. and El-Araby, I. E. 2018. Effect of dietary yeast nucleotide on antioxidant activity, non-specific immunity, intestinal cytokines, and disease resistance in Nile Tilapia. Fish and Shellfish Immunology 80:281-290. https://doi.org/10.1016/j.fsi.2018.06.016

Saurabh, S. and Sahoo, P. K. 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquaculture Research 39:223-239. https://doi.org/10.1111/j.1365-2109.2007.01883.x

Schug, H.; Yue, Y.; Krese, R.; Fischer, S.; Kortner, T. M. and Schirmer, K. 2019. Time- and concentration-dependent expression of immune and barrier genes in the RTgutGC fish intestinal model following immune stimulation. Fish and Shellfish Immunology 80:308-317. https://doi.org/10.1016/j.fsi.2019.02.036

Souza, E. M.; Souza, R. C.; Melo, J. F. B.; Costa, M. M.; Souza, A. M. and Copatti, C. E. 2019. Evaluation of the effects of *Ocimum basilicum* essential oil in Nile tilapia diet: growth, biochemical, intestinal enzymes, haematology, lysozyme and antimicrobial challenges. Aquaculture 504:7-12. https://doi.org/10.1016/j.aquaculture.2019.01.052

Tahir, A. and Secombes, C. J. 1996. Modulation of growth performance, gut microflora, non-specific immunity and gene expression of proinflammatory cytokines in shabout (*Tor grypus*) upon dietary prebiotic supplementation. Fish and Shellfish Immunology 112:38-45. https://doi.org/10.1016/j.fsi.2021.02.012

Tort, L. 2011. Stress and immune modulation in fish. Developmental and Comparative Immunology 35:1366-1375. https://doi.org/10.1016/j.dci.2011.07.002

Tort, L.; Rotlant, J. and Rivira, L. 1998. Immunological suppression in gilthead sea bream *Sparus aurata* of the North-West Mediterranean at low temperatures. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 120:175-179. https://doi.org/10.1016/S1095-6433(98)10027-2

Virella, G. 1977. Electrophoresis of lysozyme into *Micrococcus*-containing agarose gel: Quantitative and analytical applications. Clinica Chimica Acta 75:107-115. https://doi.org/10.1016/0009-8981(77)90505-8

Whang, J.; Lee, Y.; Lee, S.; Oh, M. J.; Jung, S. J.; Choi, C. Y.; Lee, W. S.; Kim, H. S.; Kim, S. J. and Lee, J. 2011. Characterization and expression analysis of a goose-type lysozyme from the rock bream *Oplegnathus fasciatus*, and antimicrobial activity of its recombinant protein. Fish and Shellfish Immunology 30:532-542. https://doi.org/10.1016/j.fsi.2010.11.025
Yildiz, H. Y. 2006. Plasma lysozyme levels and secondary stress response in rainbow trout, *Oncorhynchus mykiss* (Walbaum) after exposure to Leteux-Meyer mixture. Turkish Journal of Veterinary and Animal Sciences 30:265-269.

Zaminhan-Hassemer, M.; Michelato, M.; Boscolo, W. R.; Urbich, A. V.; Cruz, T. P.; Almeida, F. L. A.; Furuya, V. R. B. and Furuya, W. M. 2020. Dietary histidine requirement of grow-out Nile tilapia (*Oreochromis niloticus*), based on growth performance, muscle development, expression of muscle-growth-related genes, and blood parameters. Revista Brasileira de Zootecnia 49:e20180210. https://doi.org/10.37496/rbz4920180210

Zhang, C.; Zhang, J.; Liu, M. and Huang, M. 2018. Molecular cloning, expression and antibacterial activity of goose-type lysozyme gene in *Microptenus salmoides*. Fish and Shellfish Immunology 82:9-16. https://doi.org/10.1016/j.fsi.2018.07.058