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

Jaqueline Dalbello Biller[1]*, Gustavo do Valle Polycarpo[1], Basia Schlichting Moromizato[1], Ana Paula Diogo Sidekerskis[1], Thaís Daltoso da Silva[2], Ingrid Camargo dos Reis[3], Camino Fierro-Castro[4]

[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.

***Corresponding author:**
jaqueline.biller@unesp.br

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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 NH_4 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 1.0×10^8 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 (LD_{50}), 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 KH_2PO_4 (0.05M), Na_2HPO_4 (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, μ = 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

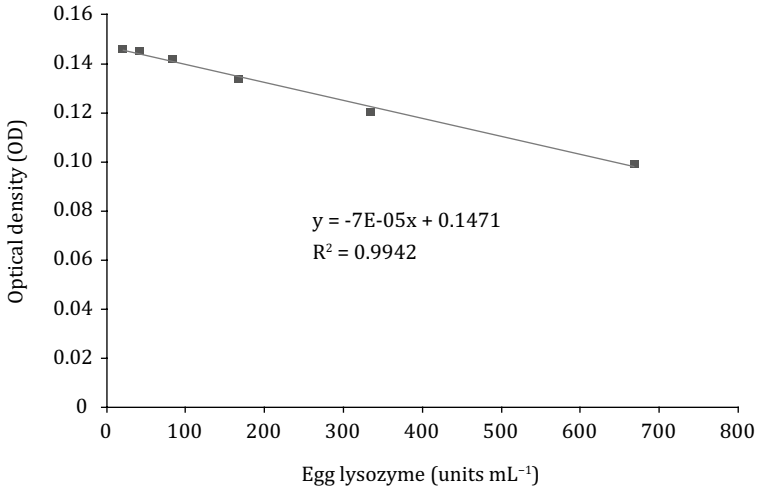
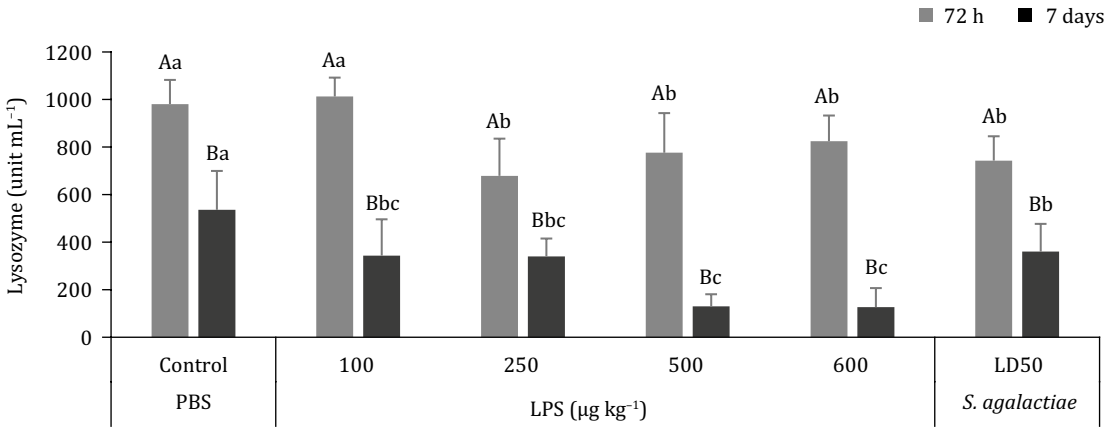


Figure 1 - Graphical demonstration of the standard curve evidencing the optical density reading of samples, according to the concentration of egg lysozyme.

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).



Bars represent the mean±SD (n = 12) of lysozyme activity.
Uppercase letters indicate difference between sampling times and lowercase letters indicate difference among treatments (P<0.05).

Figure 2 - Lysozyme activity after 72 h (gray bars) and seven days (black bars) of challenge with different doses of lipopolysaccharides (LPS) and *Streptococcus agalactiae*.

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 salmoide* 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.

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.

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