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Original Article

Phagocytotic activity and gene expression of leukocytes isolated from *Astyanax lacustris*

Atividade fagocítica e expressão gênica de leucócitos isolados de *Astyanax lacustris*

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Abstract

The constant intensification of aquaculture has considerable increased the stress levels of farmed fish and, consequently, the number and intensity of diseases outbreaks. Thus, studies on fish immune response, especially regarding the interaction of fish leukocytes with potential pathogens and xenobiotics are of great importance in order to develop new prophylactic and curative strategies. We isolated leukocytes from the head kidney of Astyanax lacustris—an important Neotropical fish species for aquaculture and a potential model for Neotropical aquaculture research—using a Percoll centrifugation protocol. The isolated leukocytes were incubated with lipopolysaccharide (LPS), and the expression of genes IL-1β, IL-8, LysC, and LysG were measured. We assessed the phagocytotic activity of leukocytes using Congo red-dyed yeast, a novel and cost-effective protocol that has been developed in this study. The isolated leukocytes responded to LPS induction, exhibiting strong IL-1β and IL-8 upregulation, two of the most important pro-inflammatory interleukins for vertebrates immune reponse. The optimal concentration of yeast for the phagocytic assay was 106 cells mL-1, resulting in acceptable phagocytic capacity (PC) but without excess of yeasts during the counting process, ensuring a high precision and accuracy of the method. To the best of our knowledge, the present study is the first to investigate the in vitro gene expression and phagocytic activity of leukocytes isolated from A. lacustris. Our findings will serve as a reference for future studies on the immunology and toxicology of Neotropical fish.

Keywords: leukocytes, interleukins, Neotropical aquaculture, phagocytosis, yellow-tailed tetra.

Resumo

A constante intensificação da aquicultura tem aumentado consideravelmente os níveis de estresse dos animais cultivados e, consequentemente, o número e a intensidade dos surtos de doenças. Logo, estudos sobre a resposta imune dos peixes, especialmente relacionados com a interação dos leucócitos de peixes com potenciais patógenos e xenobióticos, são de grande importância para o desenvolvimento de novas estratégias profiláticas e curativas. No presente trabalho, nós obtivemos sucesso ao isolar leucócitos oriundos do rim cranial de Astyanax lacustris – uma importante espécie de peixe Neotropical para a aquicultura e um modelo em potencial para pesquisas em aquicultura Neotropical – usando um protocolo de centrifugação com Percoll. Os leucócitos isolados foram incubados com lipossacarídeo (LPS) e, a expressão dos genes IL-1β, IL-8, LysC, e LysG foi avaliada. Ainda, um novo protocolo para avaliação da atividade fagocítica dos leucócitos utilizando leveduras coradas com Vermelho Congo foi estabelecido. Os leucócitos isolados responderam à indução com LPS, exibindo up regulation dos genes IL-1β e IL-8, duas das interleucinas pró-inflamatórias mais importantes para a resposta imune de vertebrados. Além do mais, a concentração ótima de leveduras para a avaliação da fagocitose foi de 106 células mL-1, resultando em uma capacidade fagocítica (PC) aceitável, mas sem excesso de leveduras durante o processo de contagem, garantindo maior precisão e eficácia do método. Até o presente momento, o presente estudo é o primeiro a investigar a expressão gênica e atividade fagocítica de leucócitos isolados de A. lacustris através da abordagem in vitro. Ainda, nossos resultados servirão de referência para futuros estudos em imunologia e toxicologia de peixes Neotropicais.

Palavras-chave: leucócitos, interleucinas, aquicultura Neotropical, lambari-do-rabo-amarelo.

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1. Introduction

In 2020, the world aquaculture production reached the milestone of 57.5 million tons, representing 66% of the global fisheries production and was evaluated at USD 141 billion (FAO, 2022). From this total, 2.3 million tons were produced through Neotropical aquaculture, thus accounting for a share of 1% of the global. After China, which is responsible for 91% of the global aquaculture production, the neotropical region is considered the world's most promising region for aquaculture due to its great farmable area and water (FAO, 2020, 2022). Concomitant with the growth of aquaculture, however, the frequency and severity of disease outbreaks has increased (Jhingan et al., 2003; Pickering and Pottinger, 1989; Bonga, 1997), resulting in annual economic losses of approximately USD 10 billion (Evensen, 2016). Thus, more studies on fish immune responses are warranted, so that new treatments and prophylactic methods can be developed.

The immune system of teleost bears major similarities with the responses observed in mammals (Bjørgen and Koppang, 2022; Rauta et al., 2012; Zapata et al., 2006). However, fish innate immunity plays a more decisive role against pathogens when compared to adaptive immunity. This occurs because their adaptive response lacks of important immunoglobulin (Ig) subtypes, such as IgG and IgA, with IgM representing the vast majority of the produced Igs (Rauta et al., 2012; Sunyer, 2013).

Teleost head kidney is considered the equivalent of mammalian bonne marrow, as it contains a large site of leukopoiesis responsible by the production of granulocytes, lymphocytes and macrophages (Uribe et al., 2011). Macrophages are the most commonly leukocyte in teleost head kidney. In fish, macrophages are the first line of defense against pathogens, and they are involved in several steps of the innate immune response such as pathogen elimination and tissue regeneration (Neumann et al., 2001). Macrophages can rapidly kill pathogens by engulfing them and releasing toxic substances through phagocytosis (Esteban et al., 2015). In addition, macrophages regulate inflammation by producing and releasing signaling substances, such as interleukins (ILs), which induce cell migration, differentiation, and tissue regeneration (Hodgkinson et al., 2015). Neutrophils, the most common granulocyte in fish blood, share similarities with macrophages; they are also known for their killing activity, phagocytizing pathogens, degranulating neutrophilic granules, secreting antimicrobial peptides and forming extracellular traps, but lack the tissue healing activity presented by macrophages (Rieger and Barreda, 2011). Lymphocytes present different activities according to their subtypes; T lymphocytes produce cytokines that regulate the inflammation or present cytotoxic activity, while B lymphocytes are commonly known as responsible for the production of antibodies (Scapigliati, 2013). Interestingly, there are evidences that B lymphocytes can participate of inflammation, also phagocytizing and killing pathogens (Li et al., 2006).

Recently, culture of leukocytes (specifically, monocyte/macrophage lineages), have garnered much attention as a cellular system for *in vitro* studies (Awasthi et al., 2015; Joerink et al., 2006; Mulero et al., 2008). These studies allow for the direct observation and quantification of the response of leukocytes, both morphologically and molecularly, to pathogens or molecules of interest. This provides a better understanding of the host–pathogen interaction as well as the functions of these cells during the development of several pathologies (Cencič and Langerholc, 2010; Quarta et al., 2019; Stansley et al., 2012).

The yellow-tailed tetra *Astyanax lacustris* (former *A. altiparanae*) represents a good model for studies on Neotropical fish because it is a small sized fish (3–14 cm) whose technology for growth and reproduction in captivity are well stablished (Bertolini et al., 2018; Nascimento et al., 2017b; Nascimento et al., 2017a, 2020; Santos et al., 2018; Siqueira-Silva et al., 2021). Moreover, this fish species belongs to the group of Characiform, such as *Piaractus mesopotamicus* and *Colossoma macropomum*, which are important fish species for Neotropical aquaculture (FAO, 2022). Recently, our research group conducted studies on the *in vivo* immune response of diploid and triploid *A. lacustris* (Levy-Pereira et al., 2020; Levy-Pereira et al., 2021) allowing a better understanding of *A. lacustris* innate immune response, the development of a new technique to assay *in vivo* phagocytosis and the evaluation of the expression of immune-related genes.

Thus, in this study we took a next step by successfully isolating leukocytes from *A. lacustris* for the first time and cultivating them *in vitro*. We also evaluated the leukocyte responsiveness to LPS through gene expression and developed a new and cost-effective protocol for *in vitro* phagocytosis assay using the isolated cells.

2. Material and Methods

2.1. Ethics and experimental conditions

All the procedures were approved by the Ethics in Animal Use Committee (CEUA) of the Faculty of Animal Science and Food Engineering (FZEA), University of São Paulo (USP), Pirassununga, SP, Brazil, under the protocol number 2403220719.

In the present experiment, adult *A. lacustris* specimens (6.9 ± 1.6 g, 8.6 ± 0.8 cm) were reared in 310 L tanks with constant aeration and daily water exchange. Only dechlorinated tap water was used during the growth period. Fish were fed a commercial diet with adequate energy and protein levels. For this experiment, eight fish were used.

2.2. Leukocyte isolation and cultivation

Leukocyte isolation and *in vitro* culture were performed according to a previously described method (Joerink et al., 2006), with some modifications. Briefly, eight fish were euthanized in clove oil solution (1 g L^{-1}) and washed with ethanol 70% in order to avoid contamination. Thereafter, blood was collected through puncture of the caudal vein. This procedure aimed at decreasing the number of erythrocytes in the head kidney during the sample collection. An incision was made laterally, and the head kidney was aseptically removed and placed on a 100 µm nylon mesh. The mesh was placed in a Petri dish

containing 1 mL of L-15 cell (Lebovitz) culture medium and 100 IU mL^{-1} of heparin. The head kidney fragments were thoroughly macerated against the mesh using a 1 mL syringe plunger. After maceration, an additional 1 mL of culture medium was added, and the cell suspension was collected, transferred to a 2 mL polystyrene tube, and kept on ice. Then, the cell suspension was gently pipetted on top of the Percoll discontinuous gradient (60/42%) and centrifuged for 25 min at 800 ×*g* and 4°C. After centrifugation, a leukocyte-rich layer was obtained between the Percoll layers. The obtained cell suspension was gently pipetted into a 15 mL polystyrene tube, washed by centrifugation (for 5 min at 300 ×*g* and 4°C), and resuspended in RPMI (Roswell Park Memorial Institute) culture medium. Cell viability was determined using the trypan blue method, and only suspensions with >90% viability were used (Strober, 1997).

Next, the cell suspension was adjusted to a concentration of 1.5×10^5 cells mL^{-1} using RPMI (Sigma) culture medium, enriched with 10% fetal bovine serum, 1% penicillin and streptomycin, and 1% l-glutamine. The cells were seeded into 24-well plates using 500 µL of suspension and incubated overnight at 25°C under 5% CO_2. No pooling method was used and all assays were performed for each fish separately.

2.3. LPS-induced gene expression

To evaluate the response of the isolated leukocytes to inflammatory stimuli, we measured the expression of genes encoding two important pro-inflammatory interleukins, namely IL-1β and IL-8, and two genes encoding lysozymes, namely LysC and LysG.

All procedures described below were performed using three fish and in triplicate, incubated or not with LPS, according to Jørgensen et al. (2000). After overnight incubation, each well of the LPS group was incubated with 100 µL of enriched RPMI medium containing LPS (Sigma), resulting in a final concentration in each well of 50 µg mL^{-1} of LPS. In the control group, each well received 100 µL of LPS-free RPMI medium. The plates were incubated at 25°C under 5% CO_2 for 24 h. Next, the culture medium was gently discarded by pipetting. Thereafter, 500 µL of TRIzol (Invitrogen) was pipetted into each well; the suspension was homogenized, transferred to 2 mL polystyrene tubes, frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

To assess the effects of LPS on gene expression in *A. lacustris* leukocytes, transcript levels of pro-inflammatory interleukin 1 beta (IL-1β), chemokine (IL-8), and lysozyme (LysC and LysG) genes were measured using quantitative real-time polymerase chain reaction (qPCR). Total mRNA was extracted using TRIzol reagent following the manufacturer's instructions. mRNA concentration was spectrophotometrically quantified on NanoDrop 2000 (Thermo Scientific) at 260 nm, and purity was assessed by measuring the 260/280 nm ratio. mRNA integrity was tested using 1.5% agarose gel electrophoresis, and the samples were maintained at -80°C for long-term storage.

Prior to cDNA synthesis, mRNA was digested with DNase I (Invitrogen) to eliminate possible contamination with residual gDNA. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. Since no IL-1č , IL-8, LysC, and LysG sequences for *A. lacustris* are available in the literature for use as a reference to design specific primers for qPCR, a partial fragment of cDNA of these genes was sequenced using primers designed based on conserved regions in *A. mexicanus* (GenBank accession numbers: XM_022680751 for IL-1č , XM_022675957 for IL-8, XM_007248970 for LysC, and XM_007233023 for LysG), which is the closest related species to *A. lacustris* with available data. PCR was performed with 10 ng of cDNA, 300 nM of each primer, 22.5 µL of Platinum PCR Supermix (Invitrogen), and ultrapure water in a 25 µL reaction system.

The PCR program involved 35 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min, preceded by initial denaturation at 95°C for 5 min and followed by terminal extension at 72°C for 5 min. The amplicon sizes were compared with the 1 kb Plus DNA Ladder (Invitrogen) using 1.5% agarose gel electrophoresis, purified with the PureLink™ Quick Gel Extraction Kit (Invitrogen), and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on the ABI 3730 DNA Analyzer (Applied Biosystems).

The partial sequences obtained were used to design specific qPCR primers for IL-1β, IL-8, LysC, LysG, and the constitutively expressed gene β-actin (Table 1), which was used as an endogenous reference. All qPCR primers were tested for amplification efficiency using the standard curve method by applying the algorithm $E = (10^{-1/slope} - 1) \times 100$. The amplification efficiency was tested three times for each gene, and values ranging from 90% to 110% were accepted as satisfactory.

All qPCR runs were performed in duplicate with 10 ng of cDNA, 5 µ µL of GoTaq® qPCR Master Mix (Promega), 300 nM of each primer, and ultrapure water in 10 µL reaction system on the 7500 Fast Real-Time PCR System (Applied Biosystems). Immediately after every amplification, melting curve analysis was performed to screen for possible non-specific amplicons or primer dimers. Relative gene expression levels were determined using the $2^{-\Delta Ct}$ method, and data were presented as fold-change relative to control.

2.4. In vitro phagocytosis assay protocol

We developed an *in vitro* protocol to assess the phagocytotic activity of the isolated leukocytes by modifying a method described previously (Levy-Pereira et al., 2020). Briefly, 1.5 g of *Saccharomyces cerevisiae* (Fleishman, Brazil) cells were added to 5 mL of phosphate-buffered saline (PBS; 0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, and 0.49 mM $MgCl_2$ in Milli-Q water, pH 7.4) containing 0.83% of Congo red in a 15 mL plastic tube (Falcon) and left to stain for 15 min. Subsequently, 7 mL of ultrapure water (Milli-Q water) was added, and the solution was autoclaved for 15 min. Next, the yeast cells were washed by centrifugation at 250 ×*g* for 5 min, and the volume was raised to 10 mL using autoclaved PBS. This procedure was repeated until excess dye was removed. Finally, the yeast cells were

Table 1. Primers used for the amplification and expression of IL-1β, IL-8, LysC, and LysG genes in leukocytes isolated from *Astyanax lacustris*.

Primer	Sequence (5'→3')	Product length	Application
IL1βF1	ATCGAGATTTCCCAGCATCCTC	765 bp	Initial amplification
IL1βR1	CCGGTCTCCAGAGTAAGAACTG		
IL8F1	GTCTTCTTCACTCTGGCTGAAGG	154 bp	
IL8R1	ATGATCTCAGTGTCTTTGCAGTG		
LysCF1	GGTCATTGTGCTGTGTGTGATG	353 bp	
LysCR1	TAAGCCCCTCTCTCTTCACGAT		
LysGF1	CACTACTGGAGCGTCTGAGAAA	410 bp	
LysGR1	CGTTGTAGGCTGCTATTCCTCC		
IL1βF2	CCTCACAGCATGAGGAAGGTGG	105 bp	Gene expression
IL1βR2	GTGCTCGGTGAACTCTGTGGAG		
IL8F2	CTTCTTCACTCTGGCTGAAGGTAT	115 bp	
IL8R2	GAGTTCGATGCTCTCGATCAGTTT		
LysCF2	AAGTGGAAGACCCACAAGGTGC	116 bp	
LysCR2	CACAGGTTCTGTCCGTTCCTGG		
LysGF2	GAGATCACGGCTATGCCTTCGG	84 bp	
LysGR2	CATGCTCCTCACTGTTCCAGGC		
βactF1	TGTTATTTTTGGCGCTTGACTCAG	105 bp	
βactR1	CTCAGATGCATTGTAGAACTTCGG		

suspended in autoclaved PBS and stored at 4°C until further use. Before inoculation, four suspensions were prepared by adjusting the yeast concentration to 10^4, 10^5, 10^6, and 10^7 cells μL^{-1} using enriched RPMI culture media.

Subsequently, the isolated leukocytes from five fish were incubated with 100 µL of yeast suspension of each concentration, and microphotographs were obtained after 6 h of incubation at 25°C under 5% CO_2 (400× magnification). The phagocytic capacity (PA) and phagocytic index (PI) were determined from five microphotographs per well using the following Equations 1-2:

$$PA = Number\ of\ phagocytizing\ leukocytes / Number\ of\ leukocytes\ x100 \quad (1)$$

$$PI = Number\ of\ phagocytized\ yeast\ cells / Number\ of\ phagocytizing\ leukocytes \quad (2)$$

For PA analysis, besides the statistical difference among groups, a selection of the most indicated concentrations of yeast was made in order to be used in further assays. PA values too close to 100% would make it difficult to observe increases in PA after incubating the leukocytes with potential immunostimulants substances or composts. In the same way, PA values too close to 0% would make it difficult to observe the effects of toxic or immunosuppressive substances in PA. Thus, a PA of 50% was desirable because it allows a more precise determination of immunostimulant or immunosuppressive effects of substances or composts in further studies.

Since there is no consensus for PI value in literature, the indication for PI for further studies was based on the concentration of yeast that showed the highest value of PI.

2.5. Statistics

Results are expressed as mean ± standard error. Statistical analyses were performed using R V3.4.0. Data were tested for homoscedasticity and normality using Levene's test and the Cramér–von Mises test, respectively. Gene expression data were analyzed using *t*-test and PA and PI using ANOVA. PA and PI means were compared using Tukey's multiple range test (α = 5%).

3. Results

We successfully isolated leukocytes from the head kidney of *A. lacustris*, resulting in cell suspensions with at least 80% viable cells; however, only those cell suspensions with >90% viable cells were used in the experiments (Figure 1).

The isolated leukocytes were successfully cultivated for up to a week under the described conditions without changing the culture medium. The isolated leukocytes presented different degrees of adherence to the plates among individuals. Thus, to avoid losing cells, we did not perform the usual lavage after cell seeding to remove non-adherent cells. However, most leukocytes induced with LPS adhered to the plates after 24 h.

Expression of the tested genes was detected in all groups (Figure 2). In LPS-induced leukocytes, the two pro-

inflammatory interleukins, namely IL-1β (146-fold change, *P = 0.0275, T = 4.8487, n = 3*) and IL-8 (111-fold change, *P = 0.0348, T = 3.8964, n = 3*), were up-regulated. Although marginally not significant, LPS induction increased LysG expression (15-fold change, *P = 0.0668, T = 2.6201, n = 3*).

Leukocytes isolated from *A. lacustris* phagocyted yeast in our tests (Figure 3). Very few phagocytizing leukocytes were observed in the 10^4 yeasts mL^{-1} group. The number of phagocytizing leukocytes increased with the increasing yeast cell density. The concentration of 10^7 yeasts mL^{-1} resulted in fields that were considerably difficult to count due to the excess of non-phagocytized yeast. Moreover, the same leukocytes phagocytized several yeasts, indicating that both PA and PI could be calculated.

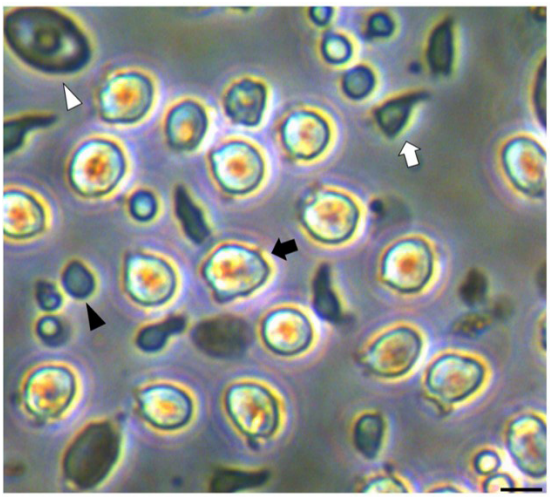

Figure 1. Cell suspension obtained after the isolation protocol was observed under an inverted phase-contrast microscope. Black arrow: possible macrophage/granulocyte. White arrow: moving leukocyte. White arrow head: erythrocyte. Black arrow head: possible lymphocyte/thrombocyte. Bar = 10 µm.

The results of phagocytic assay are presented in Figure 4. Yeast concentrations of 10^6 and 10^7 cells mL^{-1} were considered optimal for phagocytic assay because of their proximity to 50% of PA (*P = 0.0184,* $F_{3,16}$ *= 6.642, n = 5*). However, yeast concentration of 10^5 cells mL^{-1} resulted in a higher PI (*P = 0.0007,* $F_{3,16}$ *= 9.623, n = 5*) than yeast concentration of 10^6 cells mL^{-1}, indicating that 10^5 cells mL^{-1} can be used for phagocytic assays in which PI results are more important than PA.

4. Discussion

To the best of our knowledge, the present study is the first to describe the isolation and cultivation of leukocytes from *A. lacustris*—an important model for Neotropical fish studies.

Here, cells from the head kidney of *A. lacustris* were collected and centrifuged on a discontinuous Percoll gradient column (60/42%). Using a centrifugation protocol, we obtained a suspension rich in leukocytes. Different Percoll gradients have been reported to achieve great efficiency in different species: 51/34% in *Onchorrynchus mykiss* (Stafford et al., 2001) and 41/35 in *Dicenthrachus*

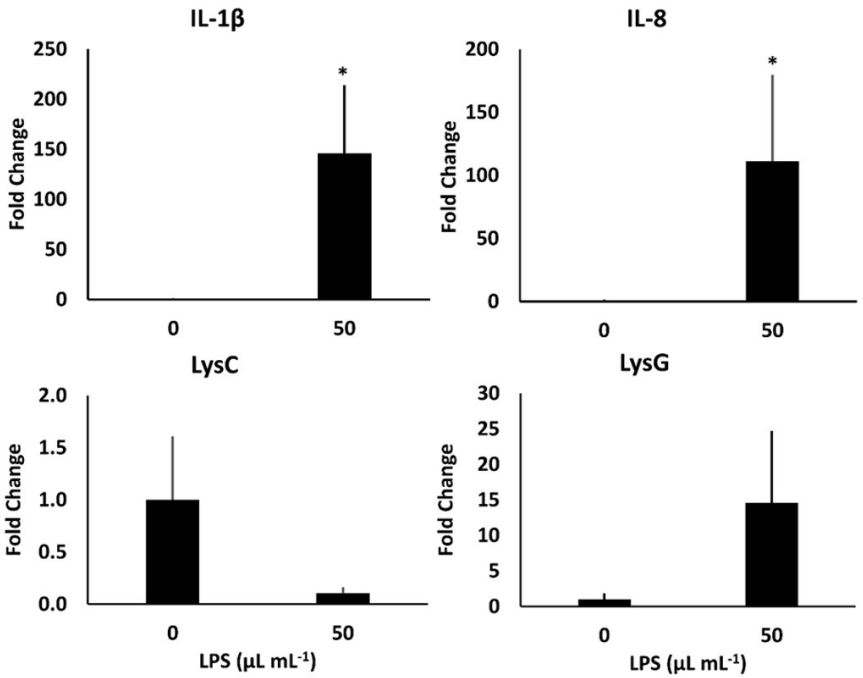

Figure 2. IL-1β, IL-8, LysC, and LysG expression in *Astyanax lacustris* leukocytes incubated with lpopolysaccharide for 24 h. *Significant differences according to *t*-test.

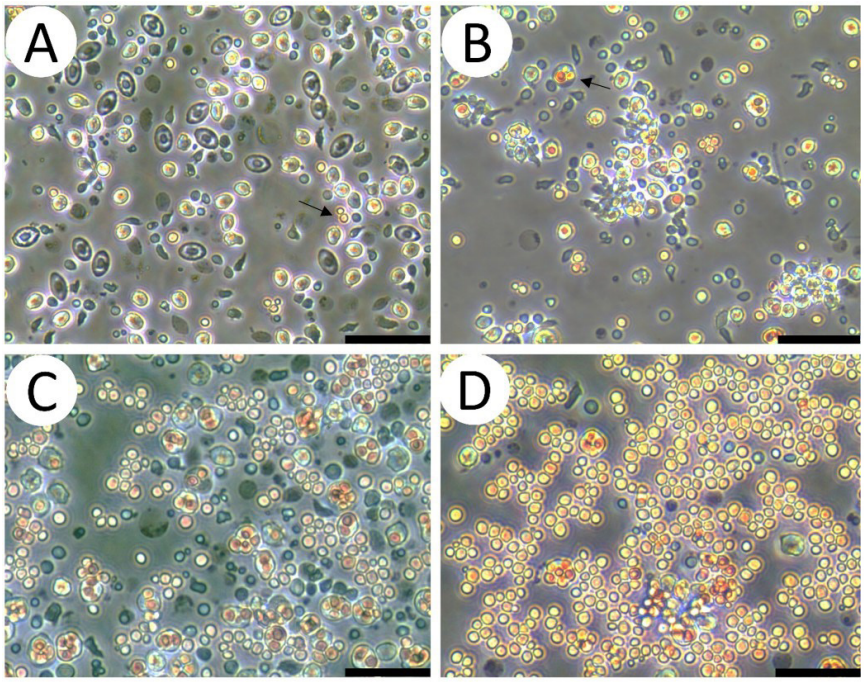

Figure 3. *Astyanax lacustris* leukocytes incubated with different concentrations of yeast. (A) 10^4 cells mL^{-1}; arrow: non-phagocytized yeast. (B) 10^5 cells mL^{-1}; arrow: leukpcyte phagocytizing three yeast cells. (C) 10^6 cells mL^{-1}. (D) 10^7 cells mL^{-1}. Bar = 50 µm.

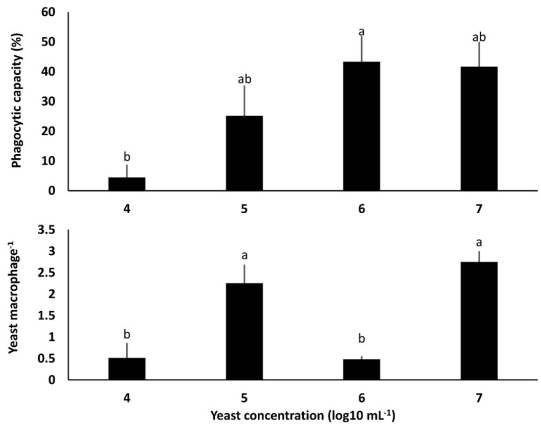

Figure 4. Phagocytic capacity and the phagocytic index of leukpcytes isolated from *Astyanax lacustris* and incubated with yeast. Different letters indicate different means according to Tukey's multiple range test ($P < 0.05$).

labrax (Sarmento et al., 2004). In a previous study (Ribas et al., 2014) on the head kidney cells of *Hoplias malabaricus*, another a Neotropical species, 60/40% Percoll gradient was found to be optimal, which is very close to that noted in the present experiment, suggesting a pattern for Neotropical Characiforms.

Assays using cultured cells are useful tools for investigating the cellular mechanisms of responses to important stimuli, such as immunostimulants, xenobiotics, and drugs, and for understanding pathogen–host interactions *in vivo* (Awasthi et al., 2015; Dove, 2014; Ossum et al., 2004). In the present study, we incubated leukocytes with LPS extracted from *Escherichia coli*. LPS induced the expression of macrophage-related genes, specifically the pro-inflammatory cytokines IL-1β and IL-8. This response is usually observed in cultured macrophages, which are known to overexpress both these interleukins under physiological or inflammatory conditions compared with other leukocytes in mammals (Kono et al., 2002; Peddie et al., 2003). As expected, LPS exerted a weak effect on the expression of LysC and LysG, which encode lysozymes—proteins responsible for the lysis of gram-positive bacteria (Saurabh and Sahoo, 2008).

We observed weak adherence of leukocytes to the well bottoms after isolation but strong adherence after LPS incubation in our experiment, which may suggest that: I) the leukocytes presented a low concentration of macrophages, which are known to rapidly adhere to plastic and glass surfaces; II) the isolated leukocytes presented a considerable amount of macrophages but originally in a non-active state, which were activated in the M1 subtype (pro-inflammatory) following LPS exposure, expressing strong adherence to plates and high expression of pro-inflammatory cytokine genes (Ganassin and Bols, 1998; Selvarajan et al., 2011). This second possibility is corroborated by previous observations (Ribas et al., 2014) in which over 71.5% of the cells resulting from Percoll centrifugation were stem cells, only 19.5% were macrophages and 9% were monocytes.

In the present study, we determined the optimal yeast concentration for phagocytic assay. The isolated leukocytes phagocytized stained yeast cells even at lower yeast concentrations, and the results of PA showed a

dose–response trend. Moreover, yeast concentration of 10^6 yeast mL^{-1} was the optimal for *in vitro* phagocytic assay because of its proximity to the threshold of 50% PA, indicating that the proposed protocol is suitable for a wide spectrum of assays. Regarding PI, yeast concentrations of 10^5 and 10^7 cells mL^{-1} appear to be optimal for phagocytic assessment because PI was higher at these concentrations. However, yeast concentration of 10^5 cells mL^{-1} resulted in a considerably lower PA than yeast concentration of 10^6 cells mL^{-1}, while the yeast concentration of 10^7 cells mL^{-1} resulted in poor visualization of microscopic fields, making it difficult to count phagocytizing and non-phagocytizing leukocytes and phagocytized yeast cells. Our results corroborate previous findings in *Cyprino carpio* (Joerink et al., 2006) and *Labeo rohita* (Awasthi et al., 2015; Rebello et al., 2014), from which macrophages were successfully isolated and cultivated and their phagocytizing activity against yeast or fluorescent beads was demonstrated.

In conclusion, we successfully isolated and cultured leukocytes from *A. lacustris* for the first time. The present study showed that leukocytes from *A. lacustris* could be stimulated with LPS, resulting in marked up-regulation of two genes encoding important pro-inflammatory interleukins. Moreover, we developed an efficient and inexpensive protocol for *in vitro* phagocytic assay. Finally, our work can give support for further studies investigating the host-pathogen interaction, the immunostimulant effects of products such as pre and probiotics and the effects of xenobiotics on the innate immune response of *A. lacustris* and other related species from the Characiform group.

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