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Distinct β-glucan molecules modulates differently the circulating cortisol levels and innate immune responses in matrinxã (*Brycon amazonicus*)

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ABSTRACT

This study investigated the effects of two β-glucan molecules with different purities and isolated by different biotechnological processes on the immune response of matrinxã (*Brycon amazonicus*) prior and after challenge with *Aeromonas hydrophila*. In this sense, we evaluated serum cortisol and plasma glucose levels, the number of leukocytes (lymphocytes, neutrophils and monocytes), as well as the respiratory activity of leukocytes prior to, 6 and 24 h post infection (hpi). During 15 days, fish were fed with diets containing 0.1% of two β-glucans (β-G 1 and β-G 2, with 71 and 62% of purity, respectively) and then submitted to challenge. Results were compared with a positive control group fed with a β-glucan-free diet. A negative control group, also fed with β-glucan-free diet but inoculated with PBS, was established to evaluate the effect of handling during injection. Our results showed that different β-glucans affected differently the biological responses of matrinxã. The βG 2 modulated the cortisol profile prior to and after the acute infection with *A. hydrophila*, and increased the mobilization and activity of leukocytes. The infection promoted lymphopenia at 6 hpi and both β-glucans increased the circulating lymphocyte population 24 hpi. Moreover, the β-G 2 prevented the infection-induced neutrophilia at 6 and 24 hpi. Finally, the β-G 2 caused a marked increase in the circulating monocytes prior to infection, and a reduction at 6 hpi that was reversed at 24 hpi. In summary, our study demonstrates that β-G 2 was more efficient on the induction of the cell-mediate immunity in matrinxã.

1. Introduction

Modern intensive aquaculture can adversely affect the health of cultured fish by promoting a potentially stressful environment and the growth of infectious diseases. Unfortunately, the indiscriminate use of antibiotics and other drugs to control diseases has contributed to the emergence of several resistant pathogenic organisms [1]. Therefore, efforts aiming to develop strategies to control the pathogen and immuno-prophylactic measures are needed to support the economic viability of the activity.

Dietary addition of immunostimulants has been shown to enhance fish innate humoral and cellular immune responses against infectious diseases [2–5]. These substances induce the proliferation of leukocytes and their phagocytic activity, as well as secretion of immune mediators such as cytokines [6]. Among these immunostimulants are the β(1–3) (1–6)-D-glucans, hereafter referred to as "β-glucans", which are highly conserved carbohydrates found in the cell walls of plants, fungi, yeast, seaweed, and bacteria [7,8]. They consist of a backbone of β-(1, 3)-linked β-D-glucopyranosyl units with β-(1, 6)-linked side chains of varying distributions and lengths [3,6,9]. The β-glucan from the cell wall of *Saccharomyces cerevisiae* has been shown to have immunostimulatory and beneficial properties, including enhanced protection against infections [10], tumor development [11], and sepsis [12,13]. Its effect has been attributed to the binding to multiple toll-like receptors on leukocytes membrane, resulting in the stimulation of immune responses, such as the increase of bactericidal activity [2] and modulation of cytokine production [14].

Several β-glucans are now commercially available to be

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incorporated in the diets of livestock, including farmed fish [6,15]. The activation of the immune response by the β-glucans depends on their molecular weight and degree of branching and the extraction process has implications for the benefits of these compounds [16,17]. However, few are known about the comparative efficiency of β-glucans extracted by different biotechnological methods, as immunostimulants able to strengthen defense mechanisms [18,19]. Therefore, we compared the effects of two insoluble β-glucan molecules over the immune response in matrinxã, a teleost fish from the Amazon basin, with high economic value for aquaculture in some South American countries [20]. In order to achieve our aim, fish previously fed with β-glucans were experimentally inoculated with *Aeromonas hydrophila*, a gram-negative bacterium that is commonly isolated from freshwater environment and used to stimulate immune responses in fis [21]. The results presented herein offer new knowledge about the immunostimulant effects of β-glucan molecules as well as feed strategies to increase disease resistance and improve fish health.

2. Material and methods

2.1. Experimental animals and lab condition

This study utilized 64 juvenile fish (250.9 ± 45.9 g and 25.7 ± 1.4 cm) that were kept individually in 64 40-liter fiber tanks (1 fish per tank, density near to 6.2 g fish L^{-1}), during 10 days for acclimatization to the laboratorial conditions being fed with a commercial feed (28% crude protein CP). Temperature, oxygen and ammonia levels were 30° ± 0.4 °C, 5.7 ± 0.2 mg L^{-1}, and 71.1 ± 21.2 μg L^{-1}, respectively. Photoperiod was 14 h light: 10 h dark, during summer season.

2.2. Experimental design and diets

The present study evaluated the individual immunostimulant effect of the two β-glucan molecules (β-G 1 and β-G 2) added to a rate of 0.1% in commercial feed (28% CP) and their effects were compared with a β-glucan-free diet as positive control. The matrinxa displays a highly aggressive behavior [22], and fish were allocated in individual tanks in order to avoid agonistic behavior which could drastically affect the stress and immune responses. After acclimatization, fish were fed with 1.5% of their body mass twice a day (11:00–16:00 h) during 15 days. We used 18 fish per treatment (Control, 0.1% β-G 1 and 0.1% β-G 2). Another 10 fish were fed with the control feed to represent the negative control group that would be inoculated with phosphate buffered saline solution (PBS) in instead of bacterial inoculation. On the 16th day, six fish were sampled to determine the prior infection condition. Following that, 12 fish per treatment, starved for 12 h, were anesthetized (benzocaine, 0.05 g L^{-1}) and inoculated in the mesenteric cavity with a sub lethal concentration of *A. hydrophila* (2.5 μL g^{-1}), while the 10 fish from the negative control group were inoculated with PBS. At 6 and 24 h post infection (hpi), anesthetized fish were bled in order to evaluate biochemical and cellular indicators of their immunological condition.

Experimental diets were prepared using an extruded commercial feed that was ground, and to which 0.1% of two different glucans coded as β-G 1 and β-G 2 were incorporated. Then, in order to re-pelletize the feed, the mixture was moistened with 40% water, passed through a food processor, and finally dried in an oven with air extraction at 40 °C for 24 h. The control feed was β-glucan-free. The two β-glucans were isolated from *Saccharomyces cerevisiae*, being β-G1 Macrogard 71% pure (batch number Q513187) and β-G 2 is a research and development substance for now called “R&D β-glucan” 62% pure (batch number T1411201). Both products contain β-glucans plus lipids, protein, ash and moisture, and no nucleotides according to the manufacturer. The batches were kindly provided by Biorigin, Brazil; (http://www.biorigin.net/biorigin/macrogard/macrogard_en/index.html).

2.3. Acute bacterial challenge

The *A. hydrophila* strain was isolated from carp *Cyprinus carpio* (strain A135, LAPOA, UNESP) and identified by sequencing of the 16S rDNA (similarity of 97% with GenBank access: ATCC 7966). The strain was stocked in TSB (Tryptic Soy Broth, Media) medium with 30% glycerol (sterile), at −80 °C. An aliquot of 20 μL (strain stock) was inoculated in 5 mL of autoclaved TSB medium and incubated in bacteriological incubator at 28 °C, for 24 h. Subsequently, 200 mL of autoclaved TSB medium was added and incubated again with the same procedure. The bacterial suspension was centrifuged at 8.000 ×g for 10 min and supernatant was discarded. Then PBS buffer (0.01 M) was used to wash the pellets twice and the suspension was centrifuged again. The bacterial PBS suspension was lower than the lethal concentration CL 50 ($7.6 * 10^8$ CFU mL^{-1}) adjusted by the UFC counting after bacterial culture and determined based on the optical density (OD600 = 1.095), using PBS buffer (0.01 M). For stimulation of the fish immune response, the bacterial suspension was previously determined as a sub lethal dose (pre-experimental tests, data not shown). No mortality was recorded during the experiment period.

2.4. Sampling

At each sampling time, 6 fish per treatment (different fish at each sampling time were used) were anesthetized and blood samples were drawn from the caudal vein using syringes without anticoagulant. Blood was dispensed in 2 mL microtubes with and without anticoagulant. Blood with the anticoagulant Glistab® was maintained under refrigeration for plasma glucose determination and NBT activity measure. The blood smears preparations were made with fresh blood. Blood in microtubes without anticoagulant was maintained at room temperature for 3 h and then centrifuged (3000 rpm for 5 min) for serum separation. Serum samples were stored at −20 °C and further used to measure cortisol levels.

2.5. Serum cortisol and plasma glucose concentrations

Blood cortisol concentration was measured by enzyme-linked immunosorbent assay (ELISA) with a commercial kit (DRG® Cortisol ELISA, EIA-1887; DRG International, Inc., USA; http://www.drg-international.com). The plasma glucose concentrations were measured by enzymatic method (Labtest kit, Sao Paulo, Brazil, code 84) following the instructions of the manufacturer.

2.6. Leukocyte respiratory burst – NBT activity

The production of reactive oxygen species (ROS) was measured using NBT (Nitro tetrazolium blue chloride – Sigma Aldrich - N6876), following protocol by Anderson & Siwicki (1995) [23], modified by Biller-Takahashi et al. (2013) [24]. Immediately after fish bleeding, 50 μL of heparinized blood was incubated with an equal volume of NBT buffer (0.2%) at room temperature for 30 min. Subsequently, 1 mL of DMF (Dimethylformamide, Sigma Aldrich – 227056) was added to the samples, and they were read using a spectrophotometer (Thermo Scientific; Genesys 10S), at room temperature, at 540 nm.

2.7. Cellular counts

The total count of red cells was performed in a Neubauer chamber, using whole blood diluted in formaldehyde citrate buffer 1:200. The total and differential count of leukocyte was performed using optical microscopy on blood smears stained with methanol blue eosin solution May-Grünwald-Giemsa-Wrigth (MGGW), according to [25]. The leukocytes were measured by the indirect method, which considers the number of leukocytes for 2000 erythrocytes counted. To the leukocytes differentiation, 200 cells were counted and the amount of each cell type

was expressed as cells μl^{-1}.

2.8. Data analysis

To evaluate the immunostimulant effect of both β-glucans and to compare the control group prior to and after bacterial challenge all data was submitted to normality (Shapiro-Wilk) and homoscedasticity (Levene). A two-way-ANOVA was used with a factorial of 3 × 3, being 3 treatments (positive control, β-G 1° and β-G 2°) × 3 sampling times (prior to infection, 6 hpi, and 24 hpi). Duncan's post-hoc tests were made for comparison of means. Finally, a *t*-test was used to compare the means of positive and negative control groups 6 and 24 h after the respective inoculations. Values in the text and figures are represented by means ± standard error (SE) of the mean. P value < 0.05 was used to estimate the level of significance for statistical differences.

2.9. Ethical statemen

All procedures that involved animal use were performed in accordance with ethical principles in animal experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA), Brasilia, Brazil, and approved by the Comissão de Ética no Uso de Animais (CEUA) protocol n° 014679/14 UNESP - Jaboticabal campus.

3. Results

To evaluate the immunostimulant effect of two β-glucan molecules (β-G 1 and β-G 2) on the induction of fish immune response, matrinxã juveniles were fed for 15 days with or without the β-glucans before being inoculated with *A. hydrophila.* Biochemical and cellular indicators were evaluated just before inoculation and again 6 and 24 h after inoculation.

3.1. Blood cortisol and glucose concentrations

Prior to infection, fish fed with β-G 2 showed the highest levels of serum cortisol, followed by animals fed with β-G 1 and lastly by the positive control group. Fish from the positive control group presented similar serum cortisol levels 6 h post infection (hpi). However, cortisol levels were higher in fish fed with β-G 2 compared to fish fed with β-G 1 ($P < 0.05$), or the positive control diet ($P < 0.001$). Only fish fed with the β-glucans reduced cortisol levels at 24 hpi, when compared to the levels measured prior to infection and at 6 hpi. Serum cortisol levels in fish of the negative control group were always lower than those of the positive control (Fig. 1A).

Prior to infection, plasma glucose concentrations did not differ among treatments. However, at 6 hpi all the challenged fish had reduced plasma glucose concentrations ($P < 0.05$). At 24 hpi, the glucose levels of fish fed with β-G 1 were higher than those observed in the positive control group; fish fed with β-G 2 revealed intermediate values. Negative control glucose values did not differ between 6 and 24 h after PBS inoculation, being higher at 6 hpi compared to the positive control group (Fig. 1B).

3.1.1. Respiratory activity of leukocytes (RAL)

Prior to infection, RAL did not differ among treatments. However, at 6 hpi, we observed increase in the reactive oxygen species (ROS) in all challenged fish. Furthermore, this increase was higher in fish fed with β-G 2 followed by fish from the positive control group and lastly by fish fed with β-G 1. At this sampling time, RAL did not differ between fish from positive and negative control groups. Twenty-four hpi, RAL returned to the initial values in all treated fish, but remained higher in fish from the negative control group compared to the positive control group (Fig. 2).

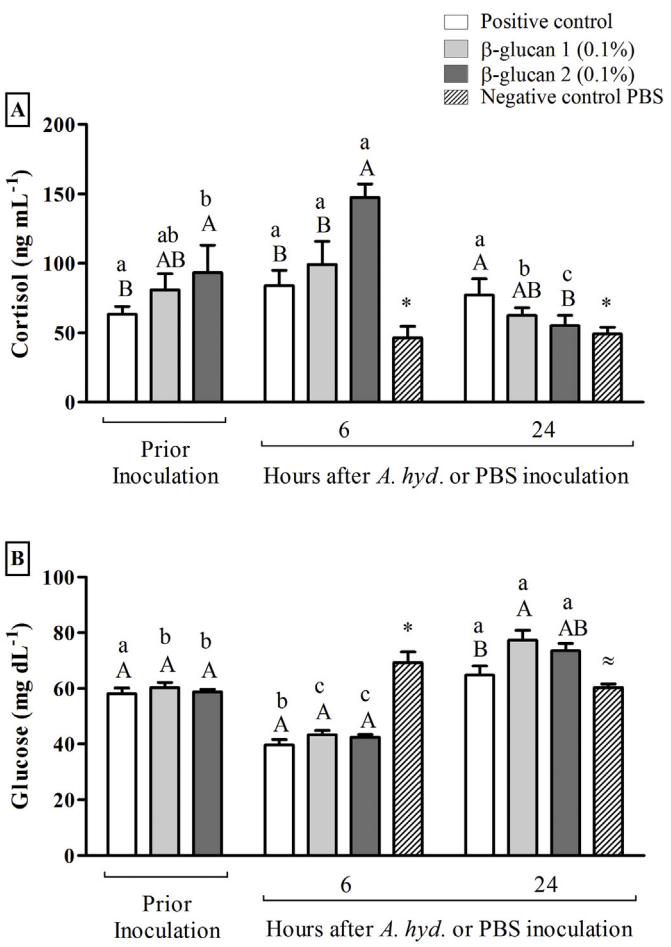

Fig. 1. *Serum cortisol (A) and plasma glucose concentrations (B) in matrinxã.* Fish fed with β-G 1 and β-G 2. The results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control groups, prior to and after A. hydrophila inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ indicates no difference and * indicates difference between positive and negative control groups 6 and 24 hpi. Columns represent the means ± SEM ($n = 6$, $P < 0.05$).

3.2. Number of circulating leukocytes, lymphocytes, neutrophils and monocytes

Prior to infection, the number of leukocytes did not differ among treatments. However, at 6 hpi, there was a drop of approximately 42% in the number of these circulating cells, being lower in fish fed with β-G 2 ($P < 0.05$). At 24 hpi, the number of leukocytes was around 38% lower than the initial values. At 6 and 24 hpi, fish from the negative control group showed lower numbers of leukocytes than the positive control group (Fig. 3A).

Prior to infection, the number of lymphocytes did not differ among treatments. However, at 6 hpi, all fish from challenged groups showed a drop of approximately 83% in the number of circulating lymphocytes ($P < 0.0001$). Fish from the negative control group presented higher number of lymphocytes than those from the positive control group. At 24 hpi, all challenged fish revealed an increase in the number of lymphocytes. However, fish fed with both β-glucans (1 and 2) showed higher circulating lymphocytes values compared to the positive and negative controls ($P < 0.05$), indicating an immune modulation by β-glucan (Fig. 3B).

Prior to infection, the number of circulating neutrophils did not differ among treatments. However, at 6 hpi, these levels rose markedly

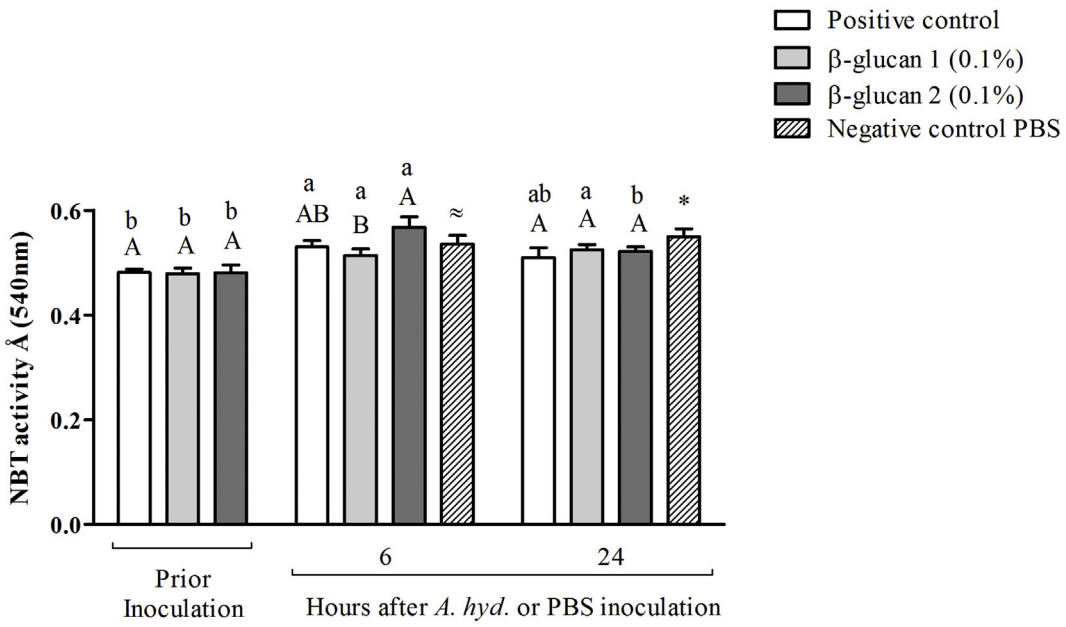

Fig. 2. *Respiratory activity of leukocytes in matrinxã.* Fish fed with β-G 1 and β-G 2. The production of reactive oxygen species was measured using nitro blue tetrazolium (NBT) (see Methods). The results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control, prior to and after A. hydrophila inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ indicates no difference and * indicates difference between positive and negative control groups 6 and 24 h post infection. Columns represented the means ± SEM ($n = 6$, $P < 0.05$).

in all fish groups, reaching a fivefold increase in fish from the positive control and the β-G 1 group ($P < 0.05$). Fish fed with β-G 2 also showed increased number of these cells, albeit lower than fish from the two groups aforementioned. At 24 hpi, the number of circulating neutrophils reduced in all challenged groups; the reduction was more evident in fish fed with β-G 2 than in those fed β-G 1. Fish from the negative control group showed lower numbers of these cells than the positive control group in both samplings (Fig. 3C).

Prior to infection, fish fed with β-G 2 showed the highest number of circulating monocytes, followed by fish fed with β-G 1 and lastly by fish from the positive control group. At 6 and 24 hpi, only fish fed with β-G 2 showed a drop of 83% ($P < 0.0001$), and 60%, respectively. Fish from the negative control group showed lower number of these cells than the positive control group in both samplings (Fig. 3D).

4. Discussion

Herein we tested the immunostimulant effect of two different β-glucan molecules on the immune response of matrinxã. The results showed that the molecules differed in their biological potency and β-G 2 was more efficient in elevating the cortisol serum levels and stimulating the innate immune response, prior to and after an acute experimental infection.

The description of β-glucan effects over cortisol serum levels in baseline conditions in fish is still minimal. In rainbow trout, physiological cortisol serum levels did not change in fish fed with 0.1% of β-glucan during four weeks [26]. Similarly, *Pangasianodon hypophthalmus* fed with different concentrations of β-glucan during 9 weeks also did not show changes in the levels of circulating cortisol [27]. We observed that in matrinxã fed with β-G 2 high levels of cortisol were maintained at 6 hpi. This profile may represent a mechanism to elicit an immune response in order to prepare fish to fight against immune challenges. At 24 hpi, serum cortisol levels decreased in fish fed with both β-glucans, especially in the β-G 2 group reinforcing the modulating effect of β-G 2 on cortisol serum levels. The separate/individual effects of cortisol and β-glucans on the modulation of immune response are well characterized in fish [2,6,28,29].

Our results are the first evidence that β-glucan can modulate blood cortisol level in resting condition. This can be explained because β-glucan has been recognized as a major fungal pathogen-associated molecular pattern (PAMP) [2,30]. For this reason, when β-glucan binds to receptors it can be similar to pathogen stimuli that lead to activation of monocyte/macrophages system which increases IL-6 expression and activates the hypothalamus/hypophysis/interrenal (HHI) axis and the cortisol secretion. In fish, a direct association between bacterial infections or treatment with lipopolysaccharide (LPS; bacterial cell walls components of Gram-negative bacteria) with induction of cortisol levels has been described. For instance, *Oreochromis mossambicus* injected with LPS from *Escherichia coli* showed modulation of the HPI axis at the level of the cortisol producing tissue, as well as the corticotropic release hormone CRH production on the ventral telencephalon tissue [31].

Hypoglycemia was detected after challenge with *A. hydrophila* (6 hpi) in all infected fish, suggesting that there was an energetic cost to elicit the immunological response in matrinxã. Recently, a previous study showed that immune responses in pacu (*Piaractus mesopotamicus*) has a high energetic cost reflected by decrease of the plasma glucose levels [32]. However, at 24 hpi, the glucose levels increased in all challenged fish, indicating a recovery of the energetic condition of fish after infection, regardless of dietary treatment.

β-glucans as a PAMP have strongly influenced the natural and adaptive host immune responses [2,30]. On the other hand, β-glucans are also known as leukocyte activators that promote immune protection in several animal disease models [15]. Herein we found that dietary β-G 2 modulated leukocyte response in all infected fish by increasing the number of the circulating leukocytes and by mobilizating them to the infection site.

The immune modulator effect of glucans occurs following their binding to specific receptors in monocytes/macrophages, neutrophils and natural killer cells [33]. Indeed, β-glucan binds to different types of receptors (βGR) in leukocytes, thereby triggering both innate and adaptive immune response [2]. Among these βGR is the scavenger receptor that binds to anionic β-glucans (sulphated β-glucans) (for a review see Meena et al., 2013). The complement receptor 3 (CR3) is more expressed in neutrophils, monocytes, and NK cells compared to

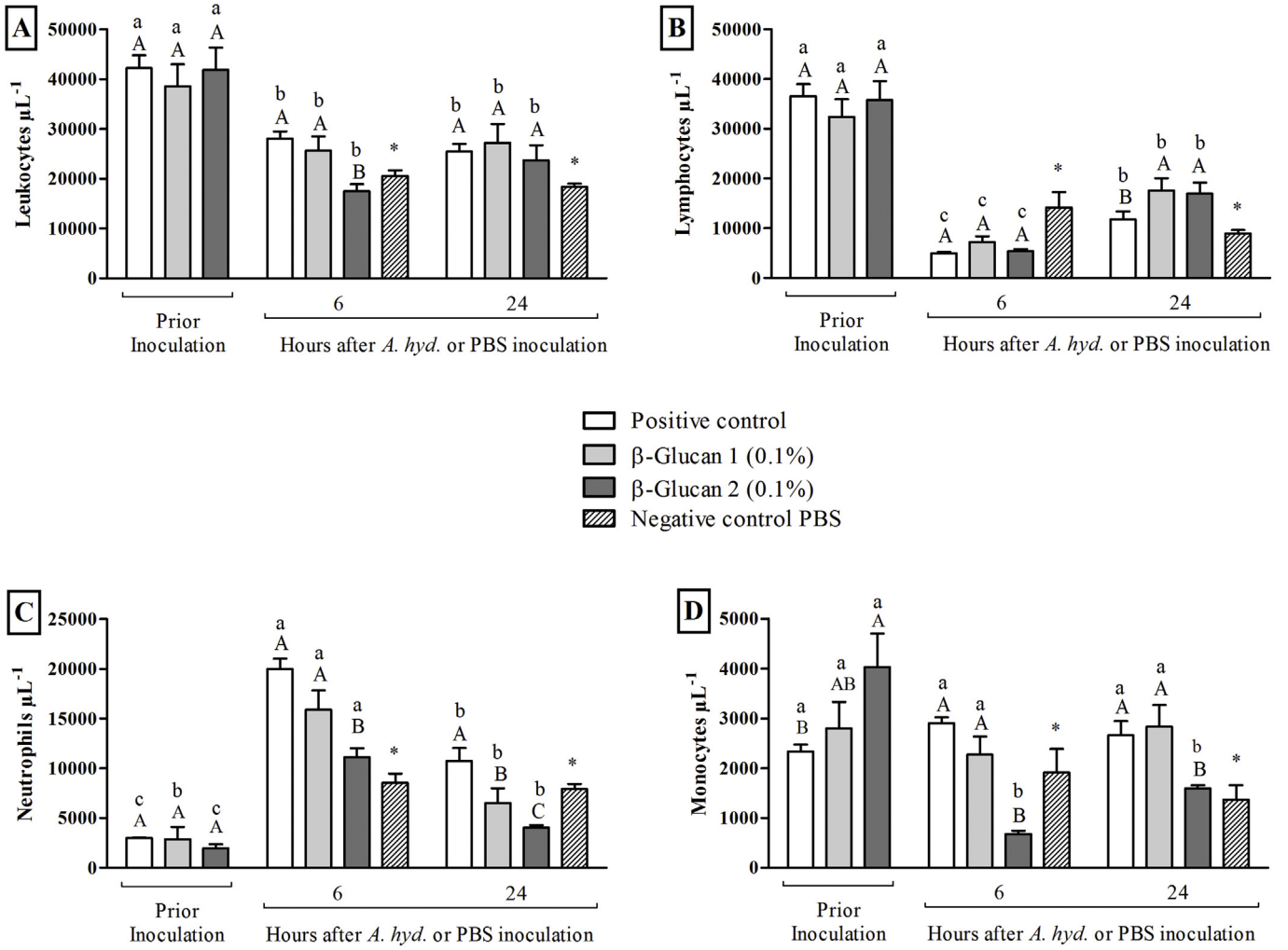

Fig. 3. *Number of circulating leukocytes (A), lymphocytes (B), neutrophils (C) and monocytes (D) in matrinxã.* Fish fed with β-G 1 and β-G 2. The results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control groups, prior to and after A. hydrophila inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, * indicates difference between positive and negative control groups at 6 and 24 h post infection. Columns represent the means ± SEM (n = 6, P < 0.05).

macrophages. Binding of β-glucan to CR3 increases leukocytes' phagocytosis and degranulation as well as their tumoricidal activity [34]. Lactosylceramide is a glycosphingolipid found on leucocytes and endothelial cells which binds to β-glucan and is associated with production of reactive oxygen species (ROS) [4]. In addition, there are various types of Toll-like receptors (TLR2-6). These receptors are used by fungal, glucans and zymosan, all of which induce cytokines after binding to TLR2 and TLR4, thereby favoring Th1 cell differentiation [2]. Lastly, the dectin-1 βGR (dentritic cell-associated C-type lectin-1) is considered the main β-glucan receptor and it recognizes carbohydrates containing β-1,3 and/or β-1,6 glucan linkages, being expressed by monocyte/macrophages, neutrophils, dendrite cells, and T cells [30]. In carp, macrophages recognized β-glucans by multiple pattern recognition receptors that could include TLR but also non-TLR receptors and they were less, but not unresponsive, to selective dectin-1 agonists [35].

In our study, RAL increased in all challenged fish, particularly in those fed with β-G 2 at 6 hpi, which coincided with the reduction in plasma glucose levels, indicating that the fish mobilized energetic blood substrate to support higher immune cell activity. RAL increases were also observed in carp intraperitoneally injected with β-glucan [10] and *in vitro*, in *Salmon salar* macrophages [36]. The phagocytic activity of fish leukocytes is one of the most important immune mechanisms for surviving to pathogen infection [29]. The RAL is an important indicator of the phagocytic activity. The increase in oxygen uptake at the initiation of the respiratory burst is followed by the production of reactive oxygen species which plays a role in the destruction of pathogens [24].

Total leukocyte counting reflected the counting of specific white cell types. It is well known that, during the acute stage of an infection or in conditions of immune exhaustion, there is a suppression of circulating lymphocytes [37]. Moreover, in teleost fish it was demonstrated that B cells have potent *in vitro* and in vivo phagocytic activities [38,39]. Accordingly, after bacterial inoculation, there was a strong lymphopenia caused by the acute infection that was reversed at 24 hpi, especially in fish fed with both β-glucans. Therefore, it is plausible to suggest that there was a migration process stimulated by the immune activation and the reversal was due to immunostimulation by β-glucans that increased these cells' population. In contrast with our results, feeding sea bass *Dicentrarchus labrax* with Macrogard 0.1% during 60 days did not stimulate the lymphocytes populations [40].

Leukocyte profile was inversely associated to levels of serum cortisol, except in the case of lymphocytes. In fish, cortisol has been shown to affect the number of circulating leukocytes and their activity [29,41–43]. However, different cells of the immune systems show varied responses to cortisol [41]. Previous studies have described cortisol induced lymphopenia and reduced lymphocyte proliferation as well as other effects, such as an increase of the number of circulating

neutrophilic granulocytes [44,45]. In carp, the cortisol induced apoptosis in activated B lymphocytes [46] whereas it caused an inhibition of neutrophil apoptosis [47].

In fish, as in mammals, neutrophils play a primary role in the inflammatory process and are the first phagocytic cells to reach a site of tissue injury or infection [38]. The neutrophils are the most abundant circulating leukocyte in humans and zebrafish and are typically the first responders [48,49]. Moreover, neutrophils can increase rapidly in circulation during acute stress when cortisol levels are elevated [50]. We observed that in matrinxã, under physiological conditions, lymphocytes are the most abundant leukocytes. However, neutrophils observed in low number before bacterial inoculation become highly sensitive after acute infection, and increased significantly in the blood. In addition, at 6 hpi we observed an inverse association between the circulating neutrophils and the cortisol levels indicating the suppressor effect of glucocorticoids over the neutrophils populations. Fish of the β-G 2 group, with the highest cortisol levels, also had the lowest number of neutrophils. However, this association was not observed at 24 hpi. Cortisol-induced neutrophilia was observed also in channel catfish stressed by handling and transport [51].

Lastly, dietary β-G 2 induced a marked increase in the circulating monocyte population before bacterial challenge. Beta-glucans are considered as strong mitogens inducing proliferation of peripheral blood mononuclear cells [15]. However, the monocytes decreased after inoculation with *A. hydrophyla*, indicating that β-G 2 induced cellular migration to the site of infection. A similar finding was described in *Pangasiodon hypophthalmus* supplemented with β-glucan during 4 weeks [13]. Moreover, the profile of cortisol before the inoculation and at 6 hpi is associated with the profile of the circulating monocytes. In the physiological conditions, cortisol would be stimulating proliferation of these cells and the lower number of monocytes at 6 hpi, coincident with the highest cortisol levels observed, suggests the involvement of cortisol in the cell migration process as suggested by Tort (2011). Monocytes/macrophages are part of the first line of defense cells from the innate immune system against infection and are also responsible for the regulation of inflammatory response [52,53]. Our results are in accordance to other studies describing the stimulator effect of β-glucan over monocyte functions [33].

The two different batches of Macrogard® we used promoted different results, the β-G 2 being the most efficient to stimulate cell-mediated immunity of matrinxã. This has occurred with the use of different β-glucans which vary in purity, solubility, primary structure, molecular weight, branching and polymer charge, which have been shown to influence their activity [54,55]. The binding to different cellular receptors has been implicated in these activities [16]. Although not yet fully understood, these attributes influence the way the carbohydrates interact with their receptors. Similarly to our experiment, a study tested the effects of adding two different glucans (β-G 1 68.5% pure; β-G 2 55.5% pure, both from *Saccharomyces cerevisiae*) into commercial feed of dogs to measure immune indicators. The study found that the two glucans had significant immunomodulating effects, but suggested that β-G 1 activity was superior to that of β-G 2 [18]. According to the authors, the samples of glucans were developed using two different biotechnological processes. Moreover, a recent study with Nile tilapia (*Oreochromis niloticus*) fed with diets containing the same two β-glucan molecules (β-G 1 and β-G 2) of the anterior work cited showed that the β-glucans have different magnitudes of effects on growth performance and the immune response. Specifically, even with a similar survival rate, the β-G 1 showed higher immunostimulation than β-G 2, although β-G 2 had improved the fish growth [19].

In summary, in matrinxã, β-G 2 was more efficient to stimulate the immune response cellular indicators and cortisol serum levels prior to and during an experimental acute infection. In addition, we observed an association of cortisol levels and immune response that indicate glucan-induced cortisol improves the start of the early immune response in matrinxã. Our results allow us to suggest that inclusion of β-G 2 in fish diet may help to prepare them to face stressful practices in fish farming.

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