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Routes of β-glucan administration affect hematological and immune responses of *Oreochromis niloticus*

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Additional keywords

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Vaccine.
Juveniles.

SUMMARY

Immunostimulants, such as β-glucans are an efficient alternative to the use of antibiotics in aquaculture. However, it is important to determine the ideal route of administration for a better stimulation of fish immune system. Thus, this study evaluated the different routes of β-glucan administration (oral, injection, immersion) on fish hematology and immune response. After 15 days of injection/bath/feeding, blood was sampled to determine fish hematological and immunological parameters. Differential leukocyte count was affected ($p<0.05$) by treatments. Neutrophil number reduced in all treated groups, when compared to control group. β-glucan injected fish presented higher ($p<0.05$) serum lysozyme concentration when compared to others administration routes and leukocyte oxidative burst in comparison to oral administration. In conclusion, this study showed that oral and bath β-glucan administration were not immunostimulant for juvenile Nile tilapia as β-glucan provided by intraperitoneal injection. Further studies must be performed to verify the efficiency of β-glucan as a vaccine adjuvant for Nile tilapia.

Vias de administração de β-glucano afetam as respostas hematológica e imune de *Oreochromis niloticus*

Palavras chave adicionais

Imunoestimulante.
Lisozima.
Tilápia-do-Nilo.
Vacina.
Juvenis

RESUMO

Imunoestimulantes, como o β-glucanos, são eficientes substitutos alternativos ao uso de antibióticos na aquicultura. Entretanto, é importante determinar a via de administração ideal que melhor estimule o sistema imune dos peixes. Neste estudo avaliaram-se as diferentes vias de administração de β-glucanos (oral, injeção, imersão) sobre a hematologia e sistema imune dos peixes. Após 15 dias da inoculação/banho/alimentação, foi realizada coleta de sangue para avaliação dos parâmetros hematológicos e imunológicos dos peixes. A contagem diferencial de leucócitos foi afetado ($p<0,05$) pelos tratamentos. O número de neutrófilos reduziu em todos os grupos tratados, quando comparados com o controle. Peixes tratados com β-glucanos via injeção apresentaram aumento ($p<0,05$) na concentração de lisozima sérica quando comparados com os demais tratamentos e burst oxidativo dos leucócitos quando comparado a administração via oral. Concluindo, este estudo demonstrou que a administração oral e por imersão de β-glucanos não apresentou efeito imunoestimulantes em juvenis de tilápia-do-Nilo como o β-glucano fornecido por meio de injeção intraperitoneal. Futuros estudos podem ser realizados para verificar a eficiência do β-glucano como adjuvante de vacinas para tilápias-do-Nilo.

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INTRODUCTION

Infectious diseases caused by microorganisms are the major constraint in the aquaculture industry for many years and impaired the development and sustainability of aquaculture throughout the world (Bondad-Reantaso *et al*., 2005, Kiron, 2012). Thus, there is a need to develop suitable tools to control the disease outbreaks in fish production (Meena *et al*., 2013).

One alternative strategy to use of antibiotics is the applications of substance with immunostimulatory proprieties. For instance, immunostimulants acts non-specifically and represent the main tools in modern fish farming against disease. The stimulatory action of a row of

structurally non-related substances has been studied for their suitability to prevent disease in aquaculture (Raa *et al*., 1992; Siwicki *et al*., 1998; Meena *et al*., 2013). In this context, the β-glucans appeared to be the most effective in aquaculture (Raa, 1996; Raa, 2000). Therefore, in recent years, attention has focused primarily on possible immune stimulation in farmed fish by the use of β-glucans.

The immunostimulatory abilities of β-glucans in several fish species were made by evaluation of some indicators of innate immune response and survival rate (Welker *et al*., 2007, Sealey *et al.*, 2008) and also specific mechanism of humoral defense (Selvaraj *et al*., 2005). However, the route of administration of immunostimulants is very important and it should be acceptable with regard to labor input, vaccine consumption, and the fish level of protection and stress. The drawback of injection procedures is that it is laborious and stressful to fish. Otherwise, bathing and oral administration are potentially useful alternative methods for mass administration to fish of all sizes (Selvaraj *et al*., 2005).

Nile tilapia is one of the most important cultured fish in several countries around the world. In Brazil, bacterial disease outbreaks cause significant losses to fish farmers (figueiredo *et al*., 2005). Thus, the present study aimed investigate the impact of different routes of β-glucan administration on hematology and immune system of juvenile tilapia.

MATERIAL AND METHODS

Fish and animal conditions

Forty Nile tilapia (225.3 ± 15.9 g) obtained from fish farm were randomly distributed into four polyethylene tanks (250 L, 10 fish per tank) in water recirculation system and kept for one week acclimatization period. During this time, fish were fed until apparent satiety twice a day with commercial diet (320 g kg^{-1} of crude protein). Weekly, the water temperature (26 ± 0.5 ºC) and dissolved oxygen (> 5.0 mg dL^{-1}) were monitored.

Experimental design and sampling

Fish were submitted to three administration routes of β-glucan as follows: immersion in a solution containing β-glucan (100 µg mL^{-1}) in the first day of experiment; β-glucan provided in diet (1% diet inclusion); β-glucan injected intraperitoneally (10 mg kg^{-1} of fish) in first day of experiment; and control group (without β-glucan administration). At the end of 15 day trial, 10 fish per treatment were anaesthetized in benzocaine (0.1 mg mL^{-1}) and blood were drawn from the caudal vessel, dispensed in microtubes with EDTA (whole blood) and microtubes without anticoagulant (serum). Whole blood was immediately used to measure leukocytes respiratory activity, hematologic parameters and plasma total protein. Blood was allowed to clot at room temperature for 3 h and thereafter centrifuged (600 x *g* during 10 minutes). Serum was stored at -20 ºC until analysis of serum lysozyme.

Specific procedures

Treatments

At first day of experiment, fish submitted to immersion treatment were captured and transferred in a 65-L bucket with solution of 100 µg mL^{-1} β-glucan during 30 minutes. Fish submitted to immersion, injection and control were fed with a commercial diet (32% crude protein, Anhambi Alimentos, Ltda. Itapejara do Oeste, Parana, Brazil). In the same way, fish submitted to dietary β-glucan treatment were also fed with the same basal diet with 1% glucan.

Hematological assays

The blood hemoglobin concentration (HGB) was determined by the cyanmethemoglobin method at a wavelength of 540 nm on spectrophotometer; total red blood cell count (RBC) was performed manually in Neubauer chamber using formaldehyde citrate buffer as a diluting fluid; and the hematocrit (HCT) was determined by centrifugation of whole blood for 5 min at 4000 x *g* in microhematocrit capillary tubes. Hematimetric indexes calculated were mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) according to Wintrobe (1934).

Blood smears from individual fish were stained with May-Grünwald-Giemsa (Rosenfeld, 1947) and examined under light microscopy using an oil immersion objective for differential leukocyte count, and white blood cell (WBC) and thrombocyte. White blood cell (WBC) and thrombocyte count were performed by indirect method (Garcia *et al*., 2007).

Total plasmatic protein

Total plasma protein were determined in portable refractometer (WZ-301/Protein 0.0-12 g dL^{-1}) according to Sado *et al.* (2008).

Leukocytes respiratory activity assay

The production of reactive oxygen species (ROS) was measured using NBT (Nitrotetrazolium Blue chloride, Sigma-Aldrich, St Louis, MO, USA), following protocol of Anderson and Swicki (1995) with modifications as suggested by Biller-Takahashi *et al.* (2013).

Serum lysozyme concentration assay

Serum lysozyme concentration was determined following the protocol proposed by Ellis (1990) modified for use in continuous reading spectrophotometer (Abreu *et al*., 2009). The assay is based on the lysis of *Micrococcus lysodeikticus* (Sigma-Aldrich, St Louis, MO, USA) suspension using hen egg white lysozyme (Sigma-Aldrich, St Louis, MO, USA) as standard. To ensure the quality of assay, we observed the linearity of decrease rate every 30 seconds during five minutes. Only samples with coefficient of determination up to 0.98 were considered.

Statistical Analysis

The experiment was conducted in an entirely randomized design and results were analyzed by a one-way ANOVA. Tukey's post-hoc test were used to examine the effect of different routes of β-glucan administration

(α=0.05) after being tested for normality (Cramer Von Mises) and homoscedasticity tests (Brown-Forsythe).

RESULTS

To evaluate the efficiency of β-glucan as immune modulator in tilapia, it was used different administration routes of β-glucan and assessed hematologic and immune response after 15 days. Overall results suggest that Nile tilapia juvenile responds differently according administration routs. During all experiment period, there was no observed mortality.

Different administration routes of β-glucan did not affected (p>0.05) hematological parameters such as hematocrit, hemoglobin concentration and RBC as well as for hematimetric indexes **(table I)**. In the same way, total leukocytes and thrombocytes counting were not influenced (p>0.05) by treatments **(table II)**. However, administration routes and/or glucan treatment influenced (p<0.05) differential leukocyte count **(table II)**. Glucan treated fish by feed and injection administration routes presented decreased lymphocytes number when compared to immersion treatment. Moreover, fish treated with glucan, independent to administration route, presented decreased (p<0.05) neutrophils number. Finally, glucan administration by feed decreased monocytes and lymphocytes number as well as in injected fishes regarding lymphocytes number when compared to control untreated fish **(table II)**.

Fish immune response was significantly influenced by glucan administration route, except the total plasmatic protein **(table II)**. Leukocyte respiratory burst was increased (p<0.05) in glucan injected fish in comparison to feed administration route **(figure 1)**. In addition, glucan injection route also improved serum lysozyme concentration when compared to control and others administration routes **(figure 2)**.

Table I. Hematological parameters $(\overline{X} \pm SD)$ of Nile tilapia exposed to different routes of β-glucan administration. Red blood cell (RBC), hematocrit (HTC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), concentration of mean corpuscular hemoglobin (CMCH) (Parâmetros hematológicos $(X \pm SD)$ de tilápias do Nilo submetidos a diferentes vias de administração de β-glucano. Número de eritrócitos (RBC), hematócrito (HTC), hemoglobina (HB), volume corpuscular médio (MCV), hemoglobina corpuscular média (MCH), concentração de hemoglobina corpuscular média (CMCH).

Variables	Treatments			
	Control	Feed	Immersion	Injected
RBC x 10^3 (cells)	902.5 ± 540.0	865.0 ± 330.4	877.7 ± 376.0	1073.0 ± 143.7
HTC (%)	25.0 ± 2.07	25.05 ± 2.70	23.05 ± 1.58	25.6 ± 2.77
HGB (g dL^{-1})	0.301 ± 0.232	0.448 ± 0.109	0.287 ± 0.160	0.368 ± 0.108
MCV x10^{-4} (fL)	2.85 ± 0.57	3.66 ± 0.27	3.17 ± 0.14	2.43 ± 0.24
MCH x 10^{-5} pg $cell^{-1}$	0.33 ± 0.27	0.74 ± 0.82	0.24 ± 0.14	0.34 ± 0.11
CHCM (g dL^{-1})	1.22 ± 0.95	1.80 ± 0.51	1.27 ± 0.74	1.44 ± 0.43

DISCUSSION

Nowadays, commercial and intensive aquaculture systems are not dependent only on good water quality. Fish are continuous exposed to stressful condition that impairs its immune system and disease resistance that can lead to disease outbreaks, and to avoid this scenario, fish farmers can use antibiotics, vaccines and immunostimulants. The last one, non-specific immunostimulants represents the primary tool in modern aquaculture (Vetvicka *et al*., 2013).

Several studies have proven β-glucans as potent and promising immunostimulant for improving healthy and disease control in fish culture as summarized by Meena *et al*. (2013). However, the dosage as well as administration routes of glucans for aquatic animals play an important role in the immune system stimulation and in this study, β-glucan administration routes significantly affected fish hematological and immunological responses.

Immunostimulants such as glucans have been given to aquatic animals by feeding, immersion or injection to improve health and disease resistance (Dalmo and Bøgwald, 2008). In this study, the immersion route of administration of glucan resulted higher lymphocytes numbers than feed and injection routes. In contrast, common carp immersed in glucan solution (100 and 1000 μg ml^{-1}) at day 1, 7 and 14 and did not influenced lymphocyte numbers (Selvaraj *et al*., 2006). Moreover, common carp injected with 100, 500 and 1000 μg intraperitoneally also showed no effects on lymphocytes number besides increased total leukocyte numbers (Selvaraj *et al*., 2005). Increased number of lymphocytes can represent a better immunological status, since fish lymphocytes are considered immunocompetent and multipotential hematopoietic cells that can present immunoglobulin-like effects eliciting protection against parasites (Yamamoto *et al.*, 2001).

Independent of the route of β-glucan administration, in comparison to control treatment it was observed reduced neutrophils numbers as well as for monocytes. The reduction of neutrophil number can be an important indicator on stress attenuation (Urbinati and Carneiro, 2004), in this way, glucan dietary supplementation could attenuated the negative effects of stress, as observed in rainbow trout *Oncorhynchus mykiss* (Jeney *et al*., 1997). In accordance to our results, decreased

monocyte numbers in fish supplemented with dietary β-glucan was observed in *Pangasianodon hypophthalmus* (Sirimanapong *et al*., 2015) however, the increasing of monocytes with dietary β-glucan also were observed in mirror carp *Cyprinus carpio* (Kühlwein *et al*., 2013) and common carp glucan intraperitoneally injected (Selvaraj *et al*., 2005). Monocytes are phagocytic cells that plays an important role in fish defense mechanisms due to its capacity to migrate from blood vessels to the inflammatory site differentiating in macrophages (Sado and Matushima, 2007).

In fact, hematological parameters vary to great extent depend on fish species as well as by prebiotic supplementation and unclear and contradictory results can be found (Sado *et al*., 2010, Vetvicka *et al*., 2013) as observed in common carp *C. carpio* fed 0.3% dietary glucan (Sahan and Duman, 2010) or intraperitoneally injected (Selvaraj *et al*., 2005), that showed increased neutrophils and monocytes number and decreased lymphocytes.

Glucan administrations improve immune responses and disease resistance (Das *et al*., 2009, Mastan, 2015, Soltanian *et al*., 2007, Vetvicka *et al*., 2013). However, glucan route of administration plays an important role on the effectiveness as well as negative/toxic effects on fish. In accordance to our results, several fish species presented increase in serum lysozyme concentration and/or activity after glucan injection, such as yellow tail *Seriola quinqueradiata* (Matsuyama *et al*., 1992), rohu *Labeo rohita* (Misra *et al*., 2006a) and common carp (Selvaraj *et al*,. 2005). In the same way, leukocyte oxidative burst also increased in glucan injected fish when compared to dietary glucan administration, but not different from control treatment. Regarding the lack of response in leukocytes respiratory burst activity in all routes of glucan administration in comparison to control treatment, previous reports demonstrated that β-glucan administration enhanced this parameter in *Pseudosciaena crocea* (Ai *et al*., 2007), *Pangasianodon hypophthalmus* (Sirimanapong *et al*., 2015) and *Laboe rohita* (Misra *et al*., 2006a, Misra *et al*., 2006b). Moreover, this absence of effect in respiratory burst activity can be due to the anti-coagulant used, since EDTA (Ethylenediamine Tetraacetic Acid) is an organic compound that chelates metallic ions such as calcium (Holleamn and Wiberg, 2011) which has an important function in mobility of leukocytes cytoskeleton during the phagocytosis process.

Surprisingly, it was expected effect of treatments on total plasmatic protein as glucan injected fishes presented expressive increase in serum lysozyme. Total plasmatic protein represents several blood peptides such as lysozyme, immunoglobulins and albumin as well as complement factors (Misra *et al*., 2006b) and this result remains unclear. In the same way, dietary glucan did not improve immune status as glucan particles are supposed to be absorbed by enterocytes when administered orally. However, just particles less than one micron could be capable of passing through intestinal cells and blood capillaries and reach the target cells (Selvaraj *et al*., 2005).

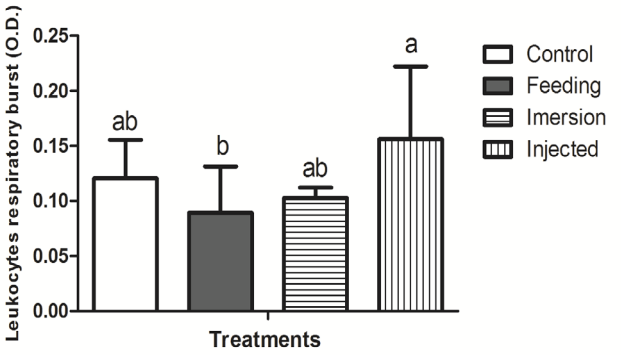

Figure 1. Leukocytes respiratory burst ($\overline{X} \pm SD$) of Nile tilapia exposed to different route of β-glucan administration. Different letter above each column indicates statistical significance by Tukey test ($p<0.05$) (Burst oxidativo ($\overline{X} \pm SD$) de tilápias do Nilo submetidos a diferentes vias de administração de β-glucanas. Letras diferentes acima de cada coluna indicam diferença estatística pelo teste de Tukey ($p<0,05$).

Fish immune system can recognizes non-self-molecules (*i.e.*, glucan prebiotic) through receptors that identify molecular patterns, which are characteristic of microbes (MAMPs-Microbe Associated Molecular Patterns) that stimulates fish leukocytes to produce lysozyme and others antimicrobial peptides (Song *et al.,* 2014). In this context, immunostmulant effects observed in injected fishes, reflect the method, since glucan can reach the target organs and bind to specific receptors on monocyte/macrophages, neutrophils, and natural killer cells (Muller *et al*., 2000), which are the mean cell types involved in natural immunity. After β-glucan receptors engages the β 1,3/1,6 glucans, all immune functions improves, including phagocytosis (Meena *et al*., 2012) as observed in *Labeo rohita* injected intraperitoneally with β-glucan that presented increased both phagocytic ration and superoxide anion production (Misra *et al*., 2006a).

In this experiment, fish immersed in glucan solution probably did not absorb the immunostimulant, as they are insoluble in nature (Selvaraj *et al*., 2005).

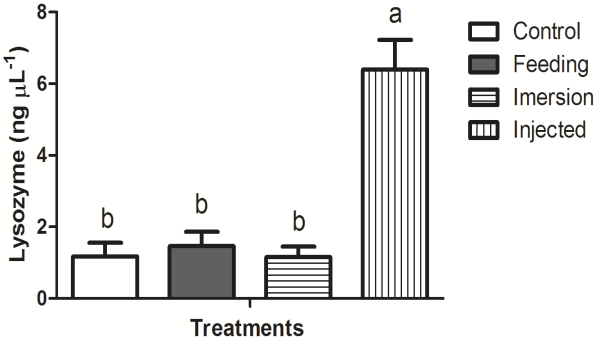

Figure 2. Serum lysozyme concentration ($\overline{X} \pm SD$) of Nile tilapia exposed to different route of β-glucan administration. Different letter above each column indicates statistical significance by Tukey test ($p<0.05$) (Concentração de lisozima sérica ($\overline{X} \pm SD$) de tilápias do Nilo submetidos a diferentes vias de administração de β-glucanos. Letras diferentes acima de cada coluna indicam diferença estatística pelo teste de Tukey ($p<0,05$).

Table II. Mean values for total leukocytes and thrombocytes and differential leukocytes count and total plasmatic protein ($\overline{X} \pm SD$) of Nile tilapia exposed to different route of β-glucan administration (Valores médios para número total de leucócitos, trombócitos, diferencial de leucócitos e proteína total plasmática ($\overline{X} \pm SD$) de tilápias do Nilo submetidos a diferentes vias de administração de β-glucanas).

Variables	Treatments			
	Control	Feed	Immersion	Injected
Leukocytes x 10^3	167.27 ± 49.05	147.55 ± 58.92	139.69 ± 56.16	157.80 ± 34.66
Trombocytes x 10^3	23.51 ± 8.51	31.49 ± 18.07	28.03 ± 12.92	25.01 ± 7.14
Limphocytes x 10^3	72.55 ± 12.26^{ab}	33.44 ± 13.21^{c}	87.16 ± 30.26^{a}	42.15 ± 18.48^{bc}
Neutrophil x 10^3	12.04 ± 4.42^{a}	2.27 ± 1.12^{b}	4.97 ± 2.07^{b}	4.25 ± 2.42^{b}
Monocytes x 10^3	21.07 ± 11.84^{a}	6.45 ± 2.39^{b}	12.13 ± 6.62^{ab}	9.79 ± 3.34^{ab}
Eosinophil x 10^3	9.16 ± 4.97	8.71 ± 4.35	7.83 ± 3.96	9.76 ± 4.14
SGC	973.86 ± 498.95	908.17 ± 846.66	669.53 ± 359.06	791.26 ± 149.09
TPP (g dL^{-1})	4.79 ± 0.47	4.75 ± 0.35	4.69 ± 0.53	4.75 ± 0.66

Different letter in the same line indicates statistical significance by Tukey test ($p<0.05$).
SCG= Special granulocytic cell; TPP= Total plasmatic protein.

Although glucan bath treatment increased immunological parameters and disease resistance in crustacean (*Macrobrachium rosenbergii*) and fish (*Anabas testudineus*) species (Das *et al*., 2009, Misra *et al*., 2004), the glucan source, extraction methods and molecule structure (ramification degree) can influence the solubility of this prebiotic (Przybylska-Diaz *et al*., 2013) and consequently, its biological effects.

As discussed previous, among the three routes of administration tested, the advantage in injection method is that glucan can reach the target organs and induce the macrophages to enhance the non-specific cellular immune response. Fish probably cannot absorb glucan particles through the bathing route, as they are insoluble in water and depending on glucan source and particle size, it cannot pass through intestinal cells wall. In this context, the application procedure of immunostimulants is very important and it should be acceptable with regard to labor input (Selvaraj *et al*., 2005). Otherwise, promising results has been obtained with use of β-glucan as vaccine adjuvant. Early studies showed that the addition of glucan to a vaccine resulted in higher non-specific resistance against vibriosis and yersiniosis in salmon (Robertsen *et al*., 1990) and enhanced protection against infection, glucan also increased production of cytokines, complement and lysozyme production and antibody formation (Raa *et al*., 1992).

Finally, it is an indication that β-glucan can be used as immune synergists of vaccine adjuvant, which would enhance immune response indicated by increase of lymphocytes proliferation, specific antibody titer and the selected genes expression involved in innate and acquired immune responses (Diao *et al*., 2013). Thus, it was demonstrated that dietary and bath routes of administration were not as effective in stimulate immune response in Nile tilapia juveniles as β-glucan provided by intraperitoneal injection. Further studies must be performed to justify the utilization of β-glucan as vaccine adjuvant for Nile tilapias.

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