

Article - Biological and Applied Sciences

# Immunomodulation of Juvenile Pacu, *Piaractus mesopotamicus*, by Different $\beta(1-3)(1-6)$ -D glucan Products

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Received: 2017.12.23; Accepted: 2019.06.16.

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## HIGHLIGHTS

- Two  $\beta$ -glucan products, with different production process, were tested in pacu feed.
- Bacterial challenge and immunological assays was used to measure  $\beta$ -glucans activity in pacu.
- A similar response was observed comparing a product with less  $\beta$ -glucan with the conventional.

**Abstract:** Stress in intensive fish farming hamper immune function of fish and cause losses by disease outbreaks, a situation that can be minimized, but cannot be completely circumvented, by the use of immunomodulators. Addition of immunomodulators to aquafeeds has thus become a common practice.  $\beta$ -glucan (BG) is one of most studied and effective immunomodulators, aquaculture purposes included. Extracted from cell walls of bacteria, fungi and selected cereals, BG activity depends on the source and extraction methods. This study evaluated effects of two BG products (BG1 and BG2), extracted from *Saccharomyces cerevisiae* under varying extraction methods and with different immune activity, on the feeding of pacu *Piaractus mesopotamicus* juveniles. BG1 provided higher leukocytes respiratory activity when fed at 0.5% inclusion for 10 days and 0.1% inclusion for 15 days. Both products seems to cause negative effect on lysozyme concentration and monocytes profile when fed to pacu for 15 days at 0.5% inclusion. Although the results for

BG2 did not differ from control (diet devoid of BG), the proximity with the BG1 behavior is indicative that a commercial product with smaller BG concentration can be effective when more refined technology is used in extraction process.

**Keywords:** Immunostimulant, Aquaculture, Stress, *Aeromonas hydrophila*, Leukocytes, Lysozyme.

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## INTRODUCTION

Harmful stimuli such as handling, crowding and transport, trigger stress response in intensively farmed fish, hampering immune functions and causing disease outbreaks and economic losses [1, 2]. Stress responses are actually a physiologic state, coordinated by central nervous system, which changing hormonal profile enables organisms to override adverse conditions and survive [3, 4]. Although physiological alterations resulting from stress have homeostatic functions, extreme and or prolonged stress can hamper immune function thus turning fish vulnerable to opportunistic pathogens [5]. Once stressors of aquaculture systems can be minimized, but not totally expunged, the improvement of immune function by dietary supply of vitamins, probiotics, prebiotics and natural and synthetic molecules has drawn the attention of the scientific community [6, 7, 8].

$\beta$ -glucan (BG), a structural polysaccharide of fungi, bacteria and some cereals walls, is one of the most studied immunomodulators, for its molecules can interact directly with receptors in leukocytes membrane, thus turning BG one of most promising, wide-ranging immunostimulant, both for human health and animal production, aquaculture included [9]. The direct interaction of BG-receptors in the leukocytes membranes enables considering these molecules ideal immunostimulants [9]. This mechanism occurs as a result of the pathogen-associated molecular patterns (PAMPs), part of BG structure, which activates leukocytes receptors, promoting signaling and transcription of immune factors setting up an "alert state" in the immune system [10], in such a way that BGs are able to promote better immunological status and survival rate in fish and shellfish [11]. Besides the stimulatory activity promoted by BG, down regulation activity has been reported in long-term and/or high dosage trials with immunostimulants [6].

The yielding process and source of BGs may influence their biological activity: BGs extracted from yeast and fungi have more immunological and antitumoral activities [12], while those extracted from cereals are more effective modulating blood cholesterol and glucose [13]. The most conventional source of BG molecules are cell walls of *Saccharomyces cerevisiae* (bakers' yeast), the most important being  $\beta(1-3)$ - and  $\beta(1-6)$ -glucan. Structural differences among BG molecules from varying sources require the use of different extraction processes resulting in varying quality of molecules and degrees of activity, the most limiting points of the industrial process [14, 15]. Once effectiveness of extraction techniques and processing of BG from different sources is recognized, selecting cost-effective products and extending the use of BG to other purposes in human and animal health come as consequent needs [16].

Studies comparing different BG molecules have being carried out with humans [17] and pets [18], but are still scarce with fish, a sole example registered is a recent study with Nile tilapia (*Oreochromis niloticus*) [19]. Therefore, this study aims at evaluating the immunomodulatory effect of two BG products obtained from *Saccharomyces cerevisiae* via different extraction process and bearing different immune stimulatory activity on the feeding of juvenile Neotropical species pacu, *Piaractus mesopotamicus*. As a rule, fish species less domesticated, such as pacu, are more susceptible to effects of stress [20], a solid the reason why the species was chosen for this study, which also sets up new research protocol for the study of immunostimulants in Neotropical fish.

## MATERIAL AND METHODS

### Experimental Diets and Design

Graded levels of a novel, insoluble BG (1-3)-, (1-6)-based product (BG2) and conventional BG (BG1) extracted from *Saccharomyces cerevisiae* (Biorigin, Lençóis Paulista, SP, Brazil) using different processes (55,5% and 68,5% activity, respectively) and immune effects (See composition in [19]), were amalgamated to a commercial fish feed (28% crude protein; 4,000 kcal kg<sup>-1</sup> crude energy) at 0.1% or 0.5%. A control diet (C) was obtained by milling and reprocessing the feed. Diets were fed for 15 days to juvenile pacu, *Piaractus mesopotamicus* (75 g ± 10) stocked in 18, 60-L plastic aquaria (n=10), under continuous aeration and open-water flow, natural photoperiod, in a completely randomized experimental design (n=3). A negative control group (NC) feeding on the control diet was not handled before sampling procedures. Fish were fed twice a day at a rate of 2 % of stock biomass, that is, daily dose of 2 and 8 mg BG/g, respectively for 0.1% and 0.5% inclusion.

Blood samplings were drawn at 5, 10 and 15 days of feeding period. Two fish were sampled from each tank after handling stress – four minutes hypoxia – and transferred to an adjacent corresponding tank for intraperitoneal inoculation with 5 µL/g *Aeromonas hydrophila* suspension (1x10<sup>6</sup> CFU) three hour after handling. Sampling, hypoxia and inoculation protocols aimed at mimicking acute stress situation and consequent white blood cell mobilization [21] eliciting evaluation of the response of treated groups to bacterial challenge. Blood samples (1.0 mL) were drawn three hours after inoculation by puncture of caudal vein with heparinized syringe (sodium heparin, 5,000 IU/mL), aliquoted and processed according to analysis requirements. Experimental and sampling procedures were performed in accordance with “Conselho Nacional de Controle de Experimentação Animal” (Brazil’s Council for Control of Experiments with Animals – CONCEA) and approved by the “Comissão de Ética no Uso de Animais” (Committee of Ethics on Use of Animals – CEUA), of “Escola Superior de Agricultura Luiz de Queiroz da Universidade de São Paulo (ESALQ-USP), Protocol CEUA 2016-21.

### Hematological Parameters

Leucocytes profiles were obtained from blood smears stained with Rosenfeld hematologic pigment (May-Grünwald-Giemsa-Wright) according to [22] under optic microscopy using the indirect method [23]. Leukocytes respiratory burst assay was carried out following [24] protocol, modified by [25]. Total blood (50 µL) was incubated with nitroblue-tetrazolium (NBT) (Sigma® ref: N6876) in phosphate buffer (pH 7.4) (50 µL) solution (0.2%), during 30 minutes at 25°C. A 50-µL aliquot of the incubate was added to 1.0 mL of n-n,dimethylformamide (DMF) (Sigma® ref: 227056), centrifuged at 3,000 g for 5 minutes and supernatant read in spectrophotometer at 540 nm.

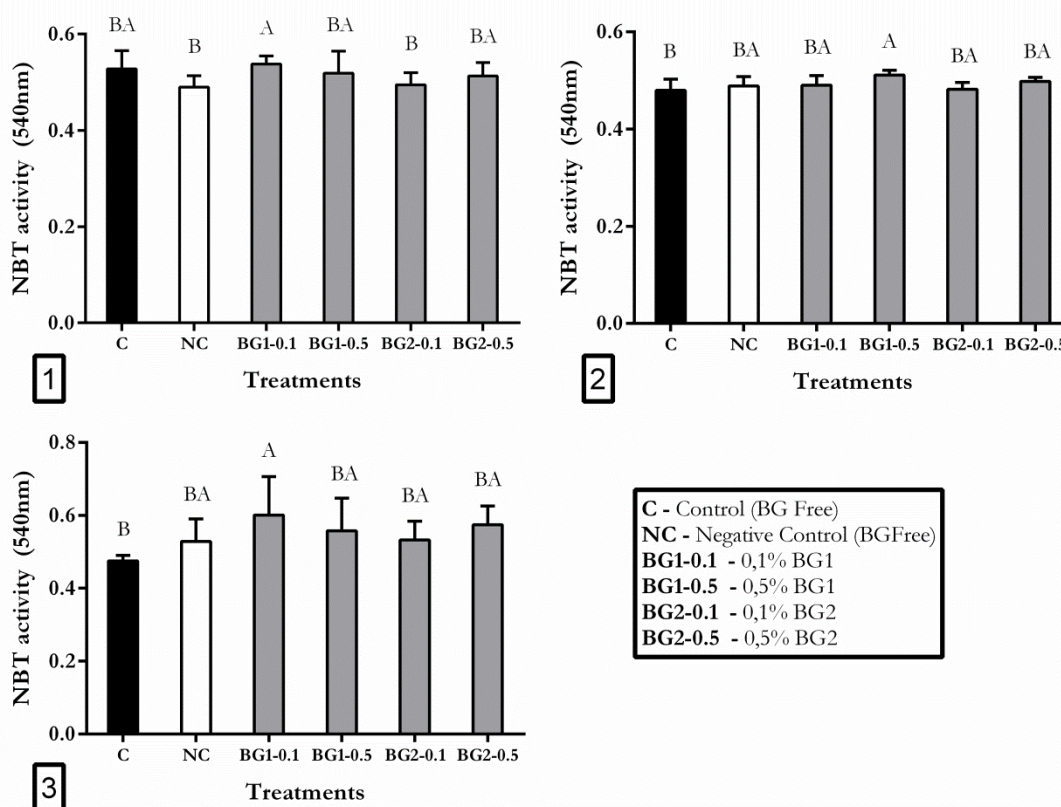
The lysozyme assay was carried out based in [26] method, which is the lysis of a *Micrococcus lysodeikticus* (Sigma® ref: M3770) suspension by the lysozyme present in sample. The procedure followed the turbidimetric protocol described by [27] and adapted to ELISA plates by [28]. Samples kept at -20°C were heat-treated in water bath at 56°C for inactivation of complement system proteins. A curve with 10 crescent standard concentrations of lysozyme (Sigma® ref: L6876) (0.5 to 5.0 ng/µL) in sodium phosphate buffer (SPB) (NaH<sub>2</sub>PO<sub>4</sub>; 0.05 M; pH 7.4), was used to set a linear regression and the equation was used to calculate the sample concentration. Diluted samples (25 µL plasma + 75 µL SPB) and standards (n = 3) were mixed with 100 µL of *Micrococcus lysodeikticus* suspension in SPB (0.075%) in a 96-well plate, and immediately read at 450 nm optical density (OD), using SPB as blank. Readings were taken at time zero (IOD) and at five minutes (5OD) incubation. The difference between the readings (ΔOD = IOD - 5OD) was used to set the standard curve and estimate the samples concentration, multiplied by four (1:4 dilution of sample) and expressed in ng/dL.

### Statistical Analysis

Recorded data were tested for normality (Cramer-Von Mises) and homoscedasticity tests (Brown-Forsythe), and analyzed by one way ANOVA. Duncan's multiple range test ( $\alpha=0.05$ ) was used to compare the means of variables sets.

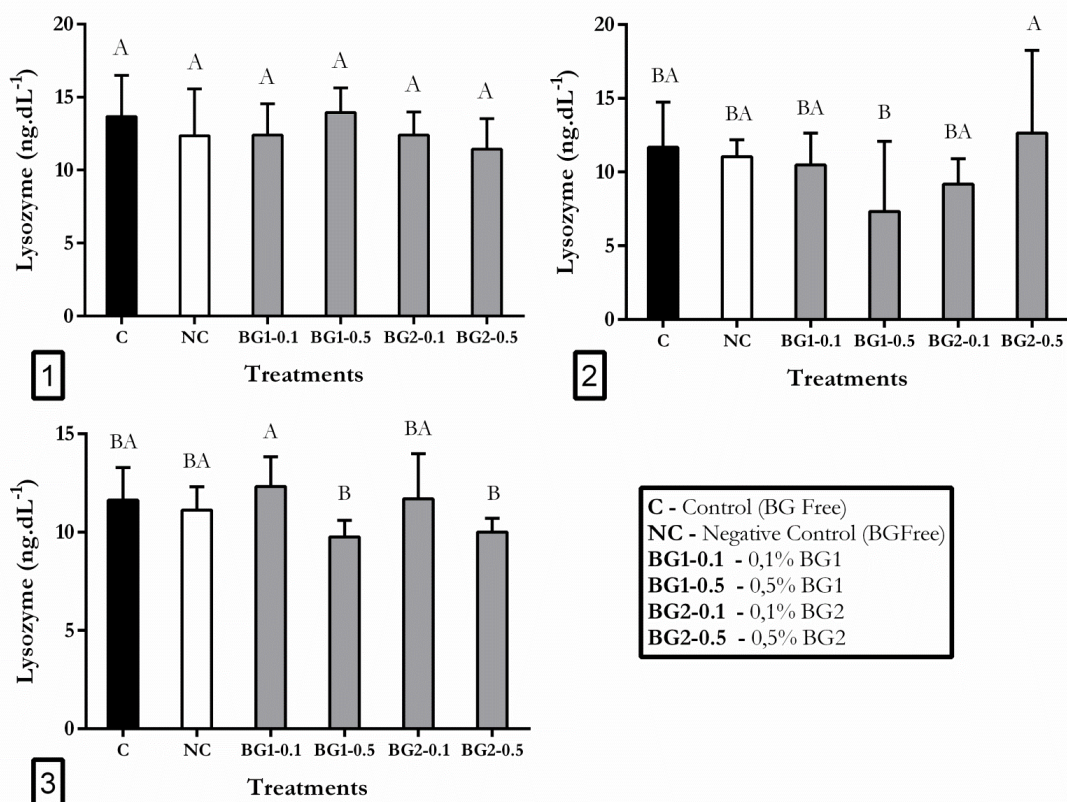
## RESULTS

The leukocytes respiratory activity was comparatively higher in BG1-0.1 group than BG2-0.1 and NC at day five (Fig.1-1); no differences were recorded regarding the other treatments. On days 10 (Fig. 1-2) and 15 (Fig. 1-3), higher leukocytes respiratory activity was recorded in treatments BG1-0.5 and BG1-0.1, respectively, but only in comparison to control group.



**Figure 1.** Mean and standard deviation of leukocytes respiratory activity of pacu juveniles fed 5, 10 and 15 days ([1], [2] and [3], respectively) with experimental diets, inoculated intraperitoneally with suspension of *Aeromonas hydrophila* ( $1 \times 10^6$  CFU) in 0.9% saline three hours after stress induction (air exposure, 4min) and sampled three hours after inoculation. The negative control group (CN) was not stressed or inoculated. Capital letters indicate statistical difference (Duncan 5%) between treatments.

Concentrations of lysozyme did not differ among treatments at day five (Fig.2-1), but at day 10 (Fig.2-2), lysozyme concentration was higher in BG2-0.5 group as compared to BG1-0.5, but did not differ from other treatments. At day 15 day (Fig.2-3), higher concentration of plasma lysozyme was recorded in treatment BG1-0.1 in comparison to BG1-0.5 and BG2-0.5.



**Figure 2.** Mean and standard deviation of plasmatic lysozyme concentration of pacu juveniles fed 5, 10 and 15 days ([1], [2] and [3], respectively) with experimental diets, inoculated intraperitoneally with suspension of *Aeromonas hydrophila* ( $1 \times 10^6$  CFU) in 0.9% saline three hours after stress induction (air exposure, 4min) and sampled three hours after inoculation. The negative control group (CN) was not stressed or inoculated. Capital letters indicate statistical difference (Duncan 5%) between treatments.

Leukocytes profile at day five (Table 1) revealed occurrence of neutrophilia, monocytosis and lymphopenia on handled groups comparatively to NC, but no effect linked to dietary BG was detected. A similar trend was recorded at days 10 and 15, but only lower monocytes percentage was recorded in BG2-0.1 at day 10, and lower neutrophils and higher lymphocytes percentage was recorded in BG2-0.1 at day 15, comparatively to control.

**Table 1.** Leukocytes profile of juveniles pacu fed experimental diets for 5, 10 and 15 days. After handling stress and bacteria inoculation.

Treatment	5 days feeding			10 days feeding			15 days feeding		
	Neutr.	Lymph.	Mon.	Neutr.	Lymph.	Mon.	Neutr.	Lymph.	Mon.
C	62,50(±16,71)A	13,66(±10,54)B	21,75(±10,55)A	62,10(±3,94)A	10,60(±2,38)B	26,00(±3,40)BA	50,08(±18,04)A	10,66(±7,43)C	37,16(±17,37)BA
NC	16,20(±5,63)B	64,80(±10,54)A	12,40(±3,847)B	46,87(±13,30)A	49,65(±24,80)A	16,53(±5,20)B	14,60(±16,37)C	60,00(±26,74)A	22,60(±12,83)B
BG1-0.1	61,41(±4,78)A	12,75(±3,29)B	21,33(±3,53)A	45,83(±11,71)A	26,66(±13,89)B	25,58(±10,24)BA	44,39(±8,53)BA	16,06(±9,79)BC	38,34(±7,75)A
BG1-0.5	54,50(±10,80)A	16,66(±8,47)B	26,83(±6,57)A	58,08(±13,42)A	12,75(±4,28)B	28,58(±10,28)A	40,58(±16,53)BA	20,83(±18,19)BC	37,08(±8,41)BA
BG2-0.1	60,41(±8,31)A	13,83(±4,10)B	23,58(±5,05)A	60,91(±14,85)A	20,83(±17,05)B	16,50(±4,03)B	29,08(±13,24)BC	22,6(±10,15)B	45,58(±12,06)A
BG2-0.5	54,16(±11,26)A	15,75(±7,05)B	24,00(±4,88)A	51,36(±13,08)A	17,15 (±14,77)B	30,23(±7,84)A	48,50(±12,34)A	15,41(±10,67)BC	34,83 (±10,11)BA

C= Control; NC = Negative control; BG1-0.1 = 0,1% BG1; BG1-0.5 = 0,5% BG1; BG2-0.1 = 0,1% BG2; BG2-0.5 = 0,5% BG2. Different capital letters indicate differences (Duncan 5%) in Neutrophils (Neutr.), Lymphocytes (Lymph.) and Monocytes (Mon.) in the comparison between treatments (columns), in each sampling (five, 10 and 15 days).

## DISCUSSION

The activity of BG depends on the source and isolation process [9]. It seems thus possible to boost activity of the immune system using a product that contains less BG, but is processed under a specific technology which provides a more active molecule. Previous studies with mice and dogs [18] and tilapia [19], comparing the same products used in this trial (BG1 and BG2), registered significant effects in phagocytosis assay and leukocytes respiratory activity, respectively, by both tested products. [19] reported enhanced leukocytes respiratory activity in tilapia feed both BG1 and BG2 (0.1%) during 30 days, but BG1 had greater activity when compared to BG2. Besides the similarly higher leukocytes activity in BG1 group herein recorded at days 10 (BG1-0.5) and 15 (BG1-0.1) of the feeding period after bacterial inoculation, the concentration and time-administration protocol used did not elicit equal immune responses driven by BG1 and BG2.

The interaction of BG with leukocytes activity is well documented [6, 9,11], as well as the dependence of this activity on administration time and dose [6]. The leukocytes activity registered for BG1-0.5 at day 10 and BG1-0.1 at day 15 was driven possibly by the dose-time protocol, in which the higher inclusion level elicited early activation of the immune system whereas it took an extended amount of time for the lower concentration to yield a similar response. As no differences were recorded between BG2 treatments and control regarding leukocytes activity, and values recorded for BG1 treatments were statistically very close, it is fair to infer that the activity of molecules present in the alternative BG product was smaller than that of the conventional BG. However, it is also fair to infer that the use of a product with smaller BG contents, but resulting from a refined extraction technology and with improved bioactivity, will be every bit as effective.

The higher lysozyme concentration in treatment BG1-0.1 at day 15 matches the registered leukocytes activity at the same stand in time. Lysozymes are conspicuous to neutrophils, monocytes and macrophages [29], and may be associated to cellular defense against pathogens [30], in this work simulated by the bacterial challenge. The same trend was not registered at day 10 probably because of individual variation, higher in the case. Lower lysozyme concentrations were recorded at day 15 in fish of groups BG1-0.5 and BG2-0.5. Actually, effects of dietary  $\beta$ -glucan are time and dose dependent, that is, higher levels of inclusion associated to long periods of administration can actually result in reduction of immune response or even inactivation of immune system [31]. Although mechanism leading to this phenomenon are not yet clearly understood, studies have registered *in vitro* and *in vivo* leukocytes apoptosis [32] and induction of apoptosis-related genes [33] on glucan trials with fish. Therefore, once both treatments with higher BG inclusion resulted in lower lysozyme levels, and this enzyme is closely related to cellular immune response, it is possible that both BG1 and BG2 products may not be effective at 0.5% inclusion level and 15 days of feeding treatment.

One of the physiological responses during acute stress and infection is demand-driven white blood cells mobilization [34]. The leukocytes profile, especially at day 5, followed a pattern registered for fish undergoing acute stress, namely lymphopenia and neutrophilia [35, 36, 37], and can be observed when NC was compared to other treatments, in which fish were submitted to stress protocol. In vertebrates, in addition to the neutrophilia and lymphopenia that may be observed in stress and infection situations, a general increase in monocytes may be registered, mainly because of its function in phagocytosis of invasive particles and pathogens [37]. This phenomenon was actually registered in this trial, mainly at day 5, and with some variations in the subsequent samplings. Treatment BG2-0.1, for instance, was not effective raising monocytes profile at day 10, but an opposite reaction was recorded at day 15. Additionally, the profile of monocytes match the concentration of lysozyme at day 15, lower values registered for BG1-05 and BG2-05. This observation reinforces the hypothesis that usage of BG products may not be feasible at these levels of inclusion and time of administration.

In conclusion, the stress protocol, followed by bacterial challenge, was effective triggering the immune response of fish, and dietary BG1 at 0.5% inclusion for 10 days and 0.1% inclusion for 15 days improved leukocytes respiratory activity of juvenile pacu, comparatively to fish fed diets devoid of glucan products. Both BG1 and BG2 yielded similar responses in comparison with the conventional BG product. The trial also enabled registering a similar response comparing the conventional BG product with a new product formulated with lower BG concentration, an indicative of the potential of producing new BG products with more refined technology and new application.

**Funding:** This work was funded by "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (Brazil's National Council for Technological and Scientific Development - CNPQ), grant # 449499-2014-6.

**Acknowledgments:** Authors are indebted to Biorigin - Zilor for providing the  $\beta$ -glucan sources used in the study.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## REFERENCES

1. Mazeaud, M.M.; Mazeaud, F.; Donaldson, E.M. Primary and Secondary Effects of Stress in Fish: Some New Data with a General Review. *Trans Am Fish Soc* 1977, 106(3), 201-12.
2. Wedemeyer, G.A. *Physiology of fish in intensive culture systems*; Chapman & Hall: New York, 1996.
3. Barton, B.A.; Iwama, G.K. Physiological changes in fish from stress in aquaculture with emphasis on the responses and effects of corticosteroids. *Annual Reviews of Fish Disease* 1991, 13-26.
4. Tort, L. Stress and immune modulation in fish. *Dev Comp Immunol* 2011, 35(12), 1366-75.
5. Wendelaar Bonga, S.E. The stress response in fish. *Physiol Rev* 1997, 77(3), 591-625.
6. Sakai, M. Current research status of fish immunostimulants. *Aquaculture* 1999, 172(1-2), 63-92.
7. Sohn, K.S.; Kim, M.K.; Kim, J.D.; Han, I.K. The role of immunostimulants in monogastric animal and fish - Review. *Asian Australas J Anim Sci* 2000, 13(8), 1178-87.
8. Ganguly, S.; Paul, I.; Mukhopadhyay, S.K. Application and Effectiveness of Immunostimulants, Probiotics, and Prebiotics in Aquaculture: A Review. *Isr J Aquacult/Bamidgeh* 2010, 62(3), 130-8.
9. Meena, D.K.; Das, P.; Kumar, S.; Mandal, S.C.; Prusty, A.K.; Singh, S.K.; Akhtar, M.S.; Behera, B.K.; Kumar, K.; Pal, A.K.; Mukherjee, S.C. Beta-glucan: an ideal immunostimulant in aquaculture (a review). *Fish Physiol Biochem* 2013, 39(3), 431-57.
10. Vera-Jimenez, N.J.; Nielsen, M.E. Carp head kidney leukocytes display different patterns of oxygen radical production after stimulation with PAMPs and DAMPs. *Mol Immunol* 2013, 55(3-4), 231-6.
11. Dalmo, R.A.; Børgwald, J.  $\beta$ -glucans as conductors of immune symphonies. *Fish Shellfish Immunol* 2008, 25(4), 384-96.
12. Du, B.; Lin, C.; Bian, Z.; Xu, B. An insight into anti-inflammatory effects of fungal beta-glucans. *Trends Food Sci Technol* 2015, 41(1), 49-59.
13. Zhu, F.; Du, B.; Bian, Z.; Xu, B. Beta-glucans from edible and medicinal mushrooms: Characteristics, physicochemical and biological activities. *J Food Compost Anal* 2015, 41165-73.
14. Akramiene, D.; Kondrotas, A.; Didziapetriene, J.; Kevelaitis, E. Effects of beta-glucans on the immune system. *Medicina (Kaunas)* 2007, 43(8), 597-606.



15. Crognale, S.; Bruno, M.; Fidaleo, M.; Moresi, M.; Petruccioli, M. Production of beta-glucan and related glucan-hydrolases by *Botryosphaeria rhodina*. *J Appl Microbiol* 2007, 102(3), 860-71.
16. Zhu, F.; Du, B.; Xu, B. A critical review on production and industrial applications of beta-glucans. *Food Hydrocolloids* 2016, 52275-88.
17. Noss, I.; Doekes, G.; Thorne, P.S.; Heederik, D.J.J.; Wouters, I.M. Comparison of the potency of a variety of  $\beta$ -glucans to induce cytokine production in human whole blood. *Innate Immunity* 2012, 19(1), 10-9.
18. Vetvicka, V.; Oliveira, C.  $\beta(1-3)(1-6)$ -D-glucans Modulate Immune Status and Blood Glucose Levels in Dogs. *British Journal of Pharmaceutical Research* 2014, 4(8), 981-91.
19. Pilarski, F.; Ferreira de Oliveira, C.A.; Darpossolo de Souza, F.P.B.; Zanuzzo, F.S. Different  $\beta$ -glucans improve the growth performance and bacterial resistance in Nile tilapia. *Fish Shellfish Immunol* 2017, 7025-9.
20. Urbinati, E.C.; Zanuzzo, F.S.; Serra, M.; Bejo Wolkers, C.P.; Sabioni, R.E. Avanço da fisiologia do estresse e suas implicações em espécies nativas. In *Aquicultura no Brasil: Novas Perspectivas: Aspectos biológicos, fisiológicos e sanitários de organismos aquáticos*; Tavares-Dias, M., dos Santos Mariano, W., Eds., Pedro & João Editores: São Carlos, São Paulo, 2015; Volume 1, pp. 381-416.
21. Dhabhar, F.S. Enhancing versus Suppressive Effects of Stress on Immune Function: Implications for Immunoprotection and Immunopathology. *Neuroimmunomodulation* 2009, 16(5), 300-17.
22. Feldman, B.F.; Zinkl, J.G.; Jain, N.C. *Schalm's veterinary hematology*, 5th ed.; Lippincott Williams & Wilkins: Philadelphia, 2000.
23. Mukherjee, K.L.; Ghosh, S. *Medical Laboratory Technology (Volume 1): Procedure Manual for Routine Diagnostic Tests*, 2nd ed.; McGraw Hill Education Private Limited: New Delhi, India, 2010.
24. Anderson, D.P.; Siwicki, A.K. Basic haematology and serology for fish health programs. In: *Diseases in Asian aquaculture*; Shariff, M., Arthur, J.R., Subasinghe, R.P., Eds.; Asian Fisheries Society, Manila, Philippines, 1995; Volume 2, pp. 185-202.
25. Biller-Takahashi, J.D.; Takahashi, L.S.; Saita, M.V.; Gimbo, R.Y.; Urbinati, E.C. Leukocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus*. *Braz J Biol* 2013, 73(2), 425-9.
26. Smolelis, A.N.; Hartsell, S.E. The Determination of Lysozyme. *J Bacteriol* 1949, 58(6), 731-6.
27. Ellis, A.E. Lysozyme assays. In: *Techniques in Fish Immunology*; Stolen, J.S., Fletcher, T.C., Anderson, D.P., Van Muiswinkel, W.D., Eds.; SOS Publications: Fair Haven, NJ; 1990; p. 101-4.
28. Zanuzzo, F.S.; Urbinati, E.C.; Rise, M.L.; Hall, J.R.; Nash, G.W.; Gamperl, A.K. *Aeromonas salmonicida* induced immune gene expression in Aloe vera fed steelhead trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture* 2015, 4351-9.
29. Lie, O.; Evensen, O.; Sorensen, A.; Froysadal, E. Study on lysozyme activity in some fish species. *Dis Aquat Org* 1989, 61-5.
30. Saurabh, S.; Sahoo, P.K. Lysozyme: an important defence molecule of fish innate immune system. *Aquacult Res* 2008, 39(3), 223-39.
31. Miest, J.J.; Hoole, D. Time and concentration dependency of MacroGard (R) induced apoptosis. *Fish Shellfish Immunol* 2015, 42(2), 363-6.

32. Kepka, M.; Verburg-van Kemenade, B.M.L.; Homa, J.; Chadzinska, M. Mechanisms involved in apoptosis of carp leukocytes upon in vitro and in vivo immunostimulation. *Fish Shellfish Immunol* 2014, 39(2), 386-95.
33. Miest, J.J.; Falco, A.; Pionnier, N.P.M.; Frost, P.; Irnazarow, I.; Williams, G.T.; Hoole, D. The influence of dietary beta-glucan, PAMP exposure and *Aeromonas salmonicida* on apoptosis modulation in common carp (*Cyprinus carpio*). *Fish Shellfish Immunol* 2012, 33(4), 846-56.
34. Dhabhar, F.S. Stress-induced augmentation of immune function—The role of stress hormones, leukocyte trafficking, and cytokines. *Brain, Behav, Immun* 2002, 16(6), 785-98.
35. Brenden, R.A.; Huizinga, H.W. Pathophysiology of experimental *Aeromonas hydrophila* in goldfish, *Carassius auratus* (L). *J Fish Dis* 1986, 9(2), 163-7.
36. Plytycz, B.; Flory, C.M.; Galvan, I.; Bayne, C.J. Leukocytes of rainbow-trout (*Oncorhynchus-mykiss*) pronephros: Cell types producing superoxide anion. *Dev Comp Immunol* 1989, 13(3), 217-24.
37. Davis, A.K.; Maney, D.L.; Maerz, J.C. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct Ecol* 2008, 22(5), 760-72.



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