Fish and Shellfish Immunology 73 (2018) 133–140

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Glucan-MOS® improved growth and innate immunity in pacu stressed and experimentally infected with *Aeromonas hydrophila*

Michelly Pereira Soares[a], Fulvia Cristina Oliveira[a], Israel Luz Cardoso[a], Elisabeth Criscuolo Urbinati[b], Cristiane Meldau de Campos[a], Hamilton Hisano[c,*]

[a] Programa de Pós-Graduação em Zootecnia, Universidade Estadual de Mato Grosso do Sul, Unidade Universitária de Aquidauana, Rodovia Aquidauana-UEMS, Km 12, CP 25, Aquidauana, 79200-000, Mato Grosso do Sul, Brazil

[b] Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista UNESP, Via de Acesso Prof. Paulo Donato Castelane, Jaboticabal, 14 884-900, São Paulo, Brazil

[c] Empresa Brasileira de Pesquisa Agropecuária, Embrapa Meio Ambiente, Rodovia SP 340, Km 127,5 Caixa Postal 69, Jaguariúna, 13820-000, São Paulo, Brazil

ARTICLE INFO

Keywords:
Bacterial challenge
β-1,3-1,6 glucans
Mannan
Handling
Immunostimulants
Stress

ABSTRACT

We tested the efficacy of a commercial product (Glucan-MOS®) derived from yeast *Saccharomyces cerevisiae*, containing two combined products, β-1,3-1,6 glucans and mannans on the growth, feed efficiency, stress and innate immune responses of juvenile pacu (*Piaractus mesopotamicus*) after a stressful handling and bacterial inoculation. For this, we evaluated the serum cortisol and plasma glucose levels, the respiratory activity of leukocytes, the serum lysozyme levels, as well as the number of circulating erythrocytes and leukocytes of fish fed during 30 days with diets containing increased levels of Glucan-MOS (0.0, 0.1, 0.2, 0.4 and 0.8%). The supplementation of 0.1% improved weight gain, feed conversion and the protein efficiency ratio compared to a control diet. The 0.2 and 0.4% Glucan-MOS® diets were sufficient to increase the respiratory burst of leukocytes and lysozyme activity, the number of thrombocytes, neutrophils and monocytes in the blood after a stressful handling and bacterial challenge, and minimized stress response as shown by decreased cortisol and glucose levels when compared to the control. The results of this work reinforce the benefits of the adoption of feeding strategies including combination of both β-1,3-1,6 glucans and mannans as a dietary supplement in periods prior to intensive management. The 30-day period was sufficient to stimulate growth performance, improve nutrient utilization, minimize stress response and modulate innate immunity responses.

1. Introduction

Intensive aquaculture depends on high stocking densities, large quantities of feed and routine handling, all of which representing stressful conditions for fish, and may trigger diseases and negatively affect their performance and survival [1–3]. Therefore, the rapid and uncontrolled growth of pathogens in the aquatic environment and the indiscriminate use of antibiotics to prevent them have resulted in the emergence of several resistant pathogens, immunosuppression, destabilization of beneficial gastrointestinal bacteria, and accumulation of antibiotics in the fish musculature [4–6] what are important concerns for both researchers and farmers [7].

In aquaculture, alternative strategies such as the use of immunostimulants and products to improve gastrointestinal health are modern tools for enhancing resistance against infectious diseases through naturally occurring compounds that modulate the immune system and balance the gut microflora [7–11]. Polysaccharides derived from the yeast cell wall (*Saccharomyces cerevisiae*), such as glucans [7] and mannan-oligosaccharides (MOS) [11] have been used for these purposes. These products are generally included as dietary supplements during stressful operations or during crucial life stages to help the animal ward off pathogens and maintain good health [10].

In fish, β-glucans are considered an immunostimulant that acts on non-specific defense mechanisms, inducing phagocytic activity of macrophages, as well as the release of lysozymes and leukocyte migration through macrophage activation [12,13]. The dietary MOS has been used to manipulate the intestinal microbial flora and lessening the impact of pathogenic colonization. On the other hand, MOS can activate receptors for pattern recognition and protein, triggering the cascade of the complement system and activating the immune system. In addition to the gut health and immune system stimulation effects, MOS dietary supplementation has an additional dimension by promoting

* Corresponding author.
E-mail addresses: michelly_psoares@hotmail.com (M.P. Soares), fulcris@yahoo.com.br (F.C. Oliveira), israelcardoso2008@hotmail.com (I.L. Cardoso), bethurb@fcav.unesp.br (E.C. Urbinati), cmeldau@uems.br (C. Meldau de Campos), hamilton.hisano@embrapa.br (H. Hisano).

https://doi.org/10.1016/j.fsi.2017.11.046
Received 9 June 2017; Received in revised form 22 November 2017; Accepted 24 November 2017
Available online 27 November 2017

growth and food conversion improvement [11].

However, few studies have evaluated the combination of yeast polysaccharides β-glucans and MOS as a supplement in fish diets [14–16]. Glucan-MOS® is a promising prophylactic product in aquaculture, composed primarily of glucans and mannans. However, for its effective use in aquaculture, the time of administration, dosage, administration methods and the physiological condition of each tested species must be known [17].

The pacu (*Piaractus mesopotamicus*) is one of the most important farmed fish in Brazil [18–20]. Studies focusing on the physiology and immunology of the species are still scarce [21–23]. The test of the efficacy of new products for improving the immune resistance of pacu should be important tools for its farming. Thus, the objective of this study was to evaluate the effect of the dietary supplementation of Glucan-MOS® on growth performance, physiological, immunological and hematological responses and resistance of juvenile pacu submitted to a stressful procedure and challenged with *Aeromonas hydrophila*.

2. Material and methods

2.1. Fish and maintenance

Three hundred and ninety juvenile fish (63.8 ± 3.7 g) were distributed randomly in 30 polyethylene tanks with 80 L of water in a flow-through system, with controlled temperature and continuous aeration. During the experimental period, the temperature (25.3 ± 0.91 °C), dissolved oxygen (7.1 ± 0.89 mg L^{-1}) and pH (6.3 ± 0.04) were measured daily. Total ammonia (0.02 ± 0.01 mg L^{-1}) was measured weekly using a commercial kit (Hach, Loveland, CO, USA). The water quality parameters values were adequate for the species according to [24].

2.2. Experimental diets

The experimental diets were isoproteic and isoenergetic [23% of digestible protein, 3200 kcal of digestible energy kcal g^{-1}] (Table 1) and were formulated according to [18]. The diet ingredients were ground in a mill (0.5 mm), mixed, moistened (20% of water) at 50 °C and processed in a meat grinder (2.5 mm). Diets were dried in a forced ventilation oven at 55 °C for 24 h and stored at -6 °C.

The Glucan-MOS® (Yessinergy do Brasil Agroindustrial LTDA, Campinas, São Paulo, Brazil; http://www.yes.ind.br/prod-glucan-mos.html) was composed of yeast cell walls (*Sacharomices cerevisiae*), containing mannan (14.3%), whole β-glucan (31.1%) and β-1,3-1,6 glucan (24.4%) (Lot: 510880820L102) and was added to the formulated diet according to the treatments.

2.3. Experimental protocol

The experimental design was completely randomized with five treatments: diets with 0.0 (control diet), 0.1, 0.2, 0.4 and 0.8% of Glucan-MOS® with six repetitions (13 fish per repetition/experimental unit). The fish were fed three times per day (0800 h; 1200 h; 1630 h) to apparent satiety during 30 days. In this period, fish consumed an average of 4% of their body weight. After the experimental feeding period, the fish starved for 24 h were anesthetized with eugenol (100 mg L^{-1}) for weighing and blood collection. Growth and nutrient retention parameters were calculated as follows: weight gain (WG) = (final body weight(g) - initial body weight(g)); specific growth rate (SGR, % day^{-1}) = 100 × (ln final weight(g) - ln initial weight(g)/days of experiment); feed conversion ratio (FCR) = feed intake(g)/weight gain(g); protein efficiency ratio (PER) = weight gain(g)/protein intake (g).

Initially, two fish per repetition were used for blood collection to designate the before stress sampling. After the blood collection, the sampled fish were removed from the experiment. Subsequently, to weaken the fish's immune system, they were stressed by chasing, followed by capture and exposure to air for 5 min. Thirty minutes after the stressful handling, two fish per repetition were bled. Then, the remaining fish were challenged by inoculation of the bacterium *Aeromonas hydrophila* in the mesenteric cavity. After that, two fish per repetition were bled at 3, 6 and 24 h after inoculation.

Table 1
Formulation, proximate and estimated composition of experimental diet.

Ingredient	Levels of Glucan-MOS® (%)				
	0.0	0.1	0.2	0.4	0.8
Soybean meal	44.70	44.80	44.80	44.90	45.00
Corn meal	31.72	31.45	31.29	30.87	30.08
Wheat meal	17.35	17.35	17.35	17.35	17.35
Soybean oil	1.60	1.67	1.73	1.86	2.15
Dicalcium phosphate	3.37	3.37	3.37	3.37	3.37
Calcitic limestone	0.20	0.20	0.20	0.20	0.20
L-Lysine (78%)	0.37	0.37	0.37	0.36	0.36
DL-Methionine (99%)	0.07	0.07	0.07	0.07	0.07
Sodium chloride	0.10	0.10	0.10	0.10	0.10
Glucan-MOS®	0.00	0.10	0.20	0.40	0.80
Vitamin and mineral premix[a]	0.50	0.50	0.50	0.50	0.50
Butyl hydroxy toluene	0.02	0.02	0.02	0.02	0.02
Total	100.00	100.00	100.00	100.00	100.00
	Proximate and estimated composition				
Digestible protein (%)[b]	23.00	23.00	23.00	23.00	23.00
Crude protein[c]	26.10	26.60	26.90	26.50	26.50
Digestible energy (kcal/kg)[b]	3200.00	3200.00	3200.00	3200.00	3200.00
Ethereal extract (%)[c]	2.19	2.75	2.32	2.87	3.15
Crude fiber (%)[c]	3.92	3.86	4.07	3.64	3.52
Lysine (%)[b]	1.64	1.64	1.64	1.64	1.64
Methionine (%)[b]	0.38	0.38	0.38	0.38	0.38
Treonine[b]	0.83	0.83	0.83	0.83	0.83
Calcium (%)[b]	1.05	1.05	1.05	1.05	1.05
Available phosphorus (%)[b]	0.70	0.70	0.70	0.70	0.70
Ash (%)[c]	7.10	7.11	7.31	7.40	7.16

[a] Composition of the vitamin-mineral premix (Vaccinar, Belo Horizonte, MG, Brazil) kg $diet^{-1}$: vitamin A: 500.000 UI, vitamin D3, 250.000 UI, vitamin E 5.000 mg, vitamin K3, 500 mg, vitamin B1 1.000 mg, vitamin B2: 1.000 mg, vitamin B6: 1.000 mg, vitamin B12: 2.000 mg, niacin: 2.500, folic acid: 500 mg, biotin: 10 mg, vitamin C 10.000 mg, choline: 100.000 mg, Inositol: 1.000 mg, selenium: 30 mg, iron: 5.000 mg, copper: 1.000 mg, manganese: 5.000 mg, zinc: 9.000 mg, cobalt: 50 mg, iodine: 200 mg.
[b] Estimated values based on digestible nutrients determined by Ref. [20].
[c] Analyzed values according to AOAC [25].

2.4. Preparation of A. hydrophila for inoculation

The *A. hydrophila* strain was isolated from pintado fish (*Pseudoplatystoma* sp.), strain KJ561021 LAPOA, Jaboticabal, São Paulo, Brazil. The strain was kept in medium BHI (Brain Heart Infusion) with glycerol 30% (sterile), at -80 °C. An aliquot of 20 μL (strain stock) was inoculated in 5 mL of autoclaved BHI medium and incubated in a bacteriological incubator at 28 °C for 24 h. Then, 700 mL of autoclaved BHI medium was added to the former solution and incubated according to the same procedure. The bacterial suspension was centrifuged (12,000 rpm for 20 min) and the supernatant was discarded. A PBS buffer (0.01 M) was used to wash the pellets twice. At the end, the suspension with 1 × 10^6 CFU/mL^{-1} was adjusted according to the McFarland Standard and read in a spectrophotometer (OD600 = 0.845) using the PBS buffer (0.01 M). The bacterial suspension was previously determined to cause only infection and no severe mortality in order to stimulate the fish's immune system.

Table 2
Growth and feed performance of pacu fed with diets containing increasing levels of Glucan-MOS® after 30 days of feeding.

Parameters	Levels of Glucan-MOS® (%)				
	0.0	0.1	0.2	0.4	0.8
WG (g)	15.2 ± 0.8b	21.1 ± 0.9a	16.0 ± 1.1b	17.3 ± 0.5 ab	16.8 ± 1.0b
SGR	4.0 ± 0.0a	4.0 ± 0.0a	4.0 ± 0.0a	4.0 ± 0.0a	4.0 ± 0.0a
DFI (g)	33.2 ± 1.0a	33.3 ± 1.2a	30.8 ± 0.5a	31.4 ± 0.8a	32.9 ± 0.3a
FCR	2.2 ± 0.2a	1.6 ± 0.0b	2.0 ± 0.2 ab	1.8 ± 0.1 ab	2.0 ± 0.1 ab
PER	1.8 ± 0.15b	2.4 ± 0.0a	2.0 ± 0.2 ab	2.1 ± 0.1 ab	2.0 ± 0.1 ab

WG - weight gain, SGR - specific growth rate, DFI - daily feed intake, FCR - feed conversion ratio, PER - protein efficiency ratio. Values are mean ± standard error. Means in the same line with different superscripts are significantly different ($P < 0.05$).

2.5. Physiological, immunological and hematological parameters

Blood was collected by caudal vessels puncture using syringes bathed in anticoagulant EDTA (3%) for the hematological analysis, heparin to determine the respiratory burst activity of leukocytes, GLISTAB (EDTA 6 g/dL and KF 12 g/dL, Labtest, Sao Paulo, Brazil; code 29) to obtain plasma to determine the stress parameters and without anticoagulant to obtain serum to evaluate the lysozyme concentrations.

2.5.1. Stress parameters

The plasma was used to determine the glucose concentrations by an enzymatic method using a commercial kit (Labtest, Sao Paulo, Brazil; code 84), and cortisol concentrations by an immunoenzymatic assay using a commercial kit (DRG International, Inc., USA; Cortisol ELISA - EIA - 1887).

2.5.2. Immunological parameters

Heparinized blood was immediately processed after collection to determine the respiratory burst activity of leukocytes according to [26]. The method consists of a colorimetric determination of the reactive oxygen species (ROS) produced by the leukocytes respiratory burst, which promotes the reduction of nitrobluetetrazolium (NBT, Sigma, St. Louis, MO, USA) into dark blue precipitate inside the phagocyte called formazan granules. The optical density (OD) of the final solution was measured at 540 nm.

After serum extraction, lysozyme activity was measured by a turbidimetric assay, as described by Ref. [27], with partial modifications [28], by lysing a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, M3770). Results were expressed in a concentration of serum lysozyme (ng/dL). One unit is defined as the amount of sample that triggers a reduction in the absorbance of 0.001 min^{-1} at 450 nm compared to the control (*M. lysodeikticus* suspension without serum).

2.5.3. Hematological parameters

Blood collected with EDTA (3%) was used to determine the hemoglobin concentration (Hb) using a commercial kit (Labtest, Sao Paulo, Brazil; code 43). The mean corpuscular hemoglobin concentration [MCHC = (Hb x 100)/Ht] was also calculated. The erythrocyte count was performed in a Neubauer chamber after blood dilution in a citrate formaldehyde solution (1:200).

Total leukocyte, total thrombocytes and leukocytes counts were performed on blood smears stained with May-Grunwald-Giemsa-Wright according to [29]. Differential and total counts were performed under a microscope using an oil immersion objective (100x).

2.6. Statistical analysis

All data were submitted to normality (Shapiro-Wilk) and the means were submitted to an analysis of variance (ANOVA). Means were compared by Tukey's test, with p-value < 0.05 to estimate the level of significance. To assess the stress and innate immune response parameters, a completely randomized design with a factorial arrangement of 5 × 5, being 5 treatments (control diet, 0.1, 0.2, 0.4 and 0.8% of Glucan-MOS®) x 5 sampling times (prior to stress and 30 min post-stress, 3, 6 and 24 h post-infection) was set up. When there was no interaction between sampling times and treatments, the results are presented considering the means of each treatment at all sampling times and the means of all treatments at each time. When there was interaction, the results were presented considering the means of each treatment within each sampling time.

2.7. Ethical statement

All procedures that involved animal use in this study were performed in accordance with ethical principles in animal experimentation, and approved by the Comissão de Ética no Uso de Animais (CEUA) protocol n° 014/2013. UEMS – Aquidauana, Brazil.

3. Results

We tested the efficacy of a commercial product derived from yeast *S. cerevisiae* containing two combined products, β-1,3-1,6 glucans and mannans, on the growth, feed efficiency, stress and innate immune response of juvenile pacu after a stressful handling and bacterial inoculation.

3.1. Growth and feed performance

Pacu juveniles fed with a 0.1% Glucan-MOS® diet showed higher ($P < 0.05$) weight gain (WG) when compared to fish fed with 0.0%, 0.2% and 0.8% diets. Fish fed with 0.4% Glucan-MOS® did not differ from the other treatments (Table 2).

DFI was not significantly affected by Glucan-MOS® supplementation. Fish fed with the 0.1% diet showed the lowest FCR and differed ($P < 0.05$) from the control (no supplementation). However, in comparison with the other treatments (Table 2), FCR did not show statistical difference. SGR did not differ between treatments. On the other hand, pacu fed with the 0.1% diet showed the highest PER, which was higher ($P < 0.05$) than that of the control, but did not differ from the other treatments.

3.2. Response to chasing and air exposure followed by bacterial infection

3.2.1. Stress parameters

3.2.1.1. Plasma concentrations of glucose and cortisol. There was no interaction between Glucan-MOS® levels and sampling times (before and after stress, and after bacterial inoculation) on the plasma glucose profile (Fig. 1 A). At 0.4 and 0.8% of supplementation the plasma glucose concentrations were lower regardless of the sampling time ($P < 0.05$). Regardless of the Glucan-MOS® level, glucose increased after the handling and again at 3 h after the bacterial inoculation ($P < 0.05$). At 6 h the levels were similar to those of the pre-stress condition and at 24 h they increased again to the levels observed after handling ($P < 0.05$).

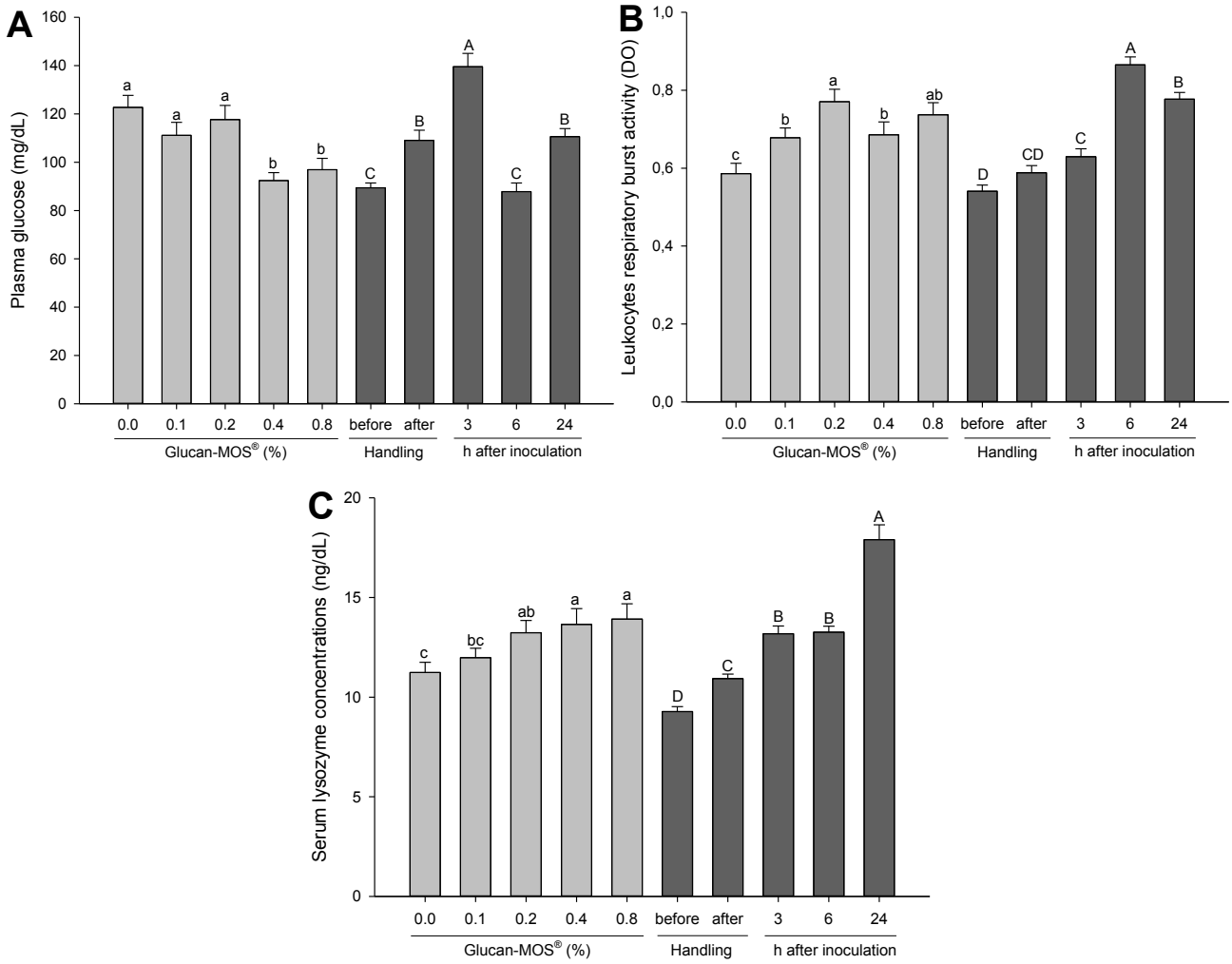

Fig. 1. Plasma glucose concentrations(A), leukocytes respiratory activity (B) and serum lysozyme concentrations (C) in pacu fed Glucan-MOS®, stressed and inoculated with *Aeromonas hydrophila*. Bars represent means ± standard error. Small letters compare grouped sampling times in each treatment and capital letters compare grouped treatments in each sampling time ($P < 0.05$).

Table 3
Plasma cortisol concentrations (ng/mL) in pacu fed Glucan-MOS®, stressed and inoculated with *Aeromonas hydrophila*. Before handling and after handling; 3 h, 6 h and 24 h after bacterial inoculation.

Time	Levels of Glucan-MOS® (%)				
	0.0	0.1	0.2	0.4	0.8
Before	42.6 ± 1.6ABa	37.9 ± 2.0ABa	38.9 ± 1.2ABa	38.2 ± 0.8 Aa	32.6 ± 4.3Cb
After	46.5 ± 0.8Aa	42.4 ± 1.3Aab	40.2 ± 1.9 Ab	39.4 ± 1.5Ab	36.8 ± 5.3Aab
3 h	46.4 ± 1.2Aa	37.4 ± 1.7Bb	36.8 ± 1.4ABb	34.9 ± 0.7Ab	38.8 ± 1.5ABb
6 h	39.9 ± 1.9BCa	34.6 ± 0.1Bb	34.7 ± 0.2Bb	34.8 ± 0.2Ab	35.1 ± 0.2BCb
24 h	37.2 ± 1.6C	34.5 ± 1.3B	34.6 ± 0.2B	34.7 ± 0.2A	34.9 ± 0.2BC

Values are mean ± standard error. Lowercase letters differ in line and uppercase letters differ in column ($P < 0.05$).

There was interaction between dietary Glucan-MOS® supplementation and sampling time (before and after stress, and after bacteria inoculation) on the plasma cortisol profile (Table 3). Glucan-MOS®, at 0.8%, reduced the plasma cortisol concentrations after 30 days of feeding ($P < 0.05$) and also reduced after chasing and air exposure (0.2, 0.4 and 0.8%) and at 3 and 6 h after bacterial inoculation in all concentrations ($P < 0.05$). Comparing the cortisol levels observed before and after chasing and air exposure, in the control group, they reduced after 6 h of the bacterial inoculation, while in the 0.1% group the highest value was observed after the chasing and air exposure and decreased after that. A similar profile was observed in the other Glucan-MOS® groups 6 h after the inoculation ($P < 0.05$).

3.2.2. Immunological parameters

3.2.2.1. Respiratory activity of leukocytes (RAL) and serum lysozyme concentrations (SLC). There was no interaction between dietary Glucan-MOS® supplementation and sampling times (before and after stress and after bacterial inoculation) on the RAL (Fig. 1 B) and on the serum lysozyme concentrations (Fig. 1 C).

The respiratory activity of the leukocytes in all Glucan-MOS® treatments was higher compared to the control group, regardless of the sampling time ($P < 0.05$). Regardless of the Glucan-MOS® level, RAL

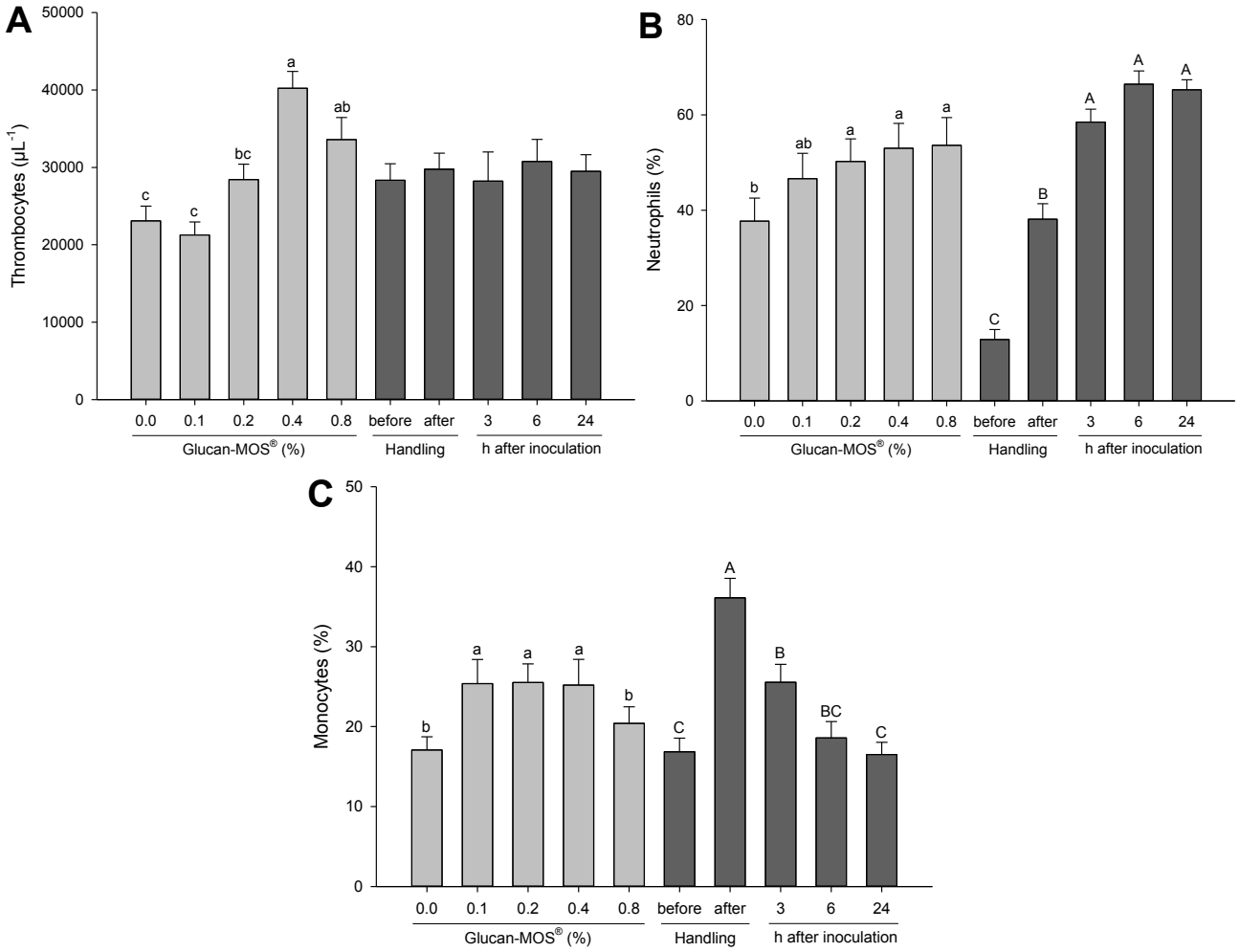

Fig. 2. Thrombocytes (A), neutrophils (B) and monocytes (C) counts in pacu fed Glucan-MOS®, stressed and inoculated with *Aeromonas hydrophila*. Bars represent means ± standard error. Bars represent means ± standard error. Small letters compare grouped sampling times in each treatment and capital letters compare grouped treatments in each sampling time ($P < 0.05$).

increased at 3 and 6 h after bacterial inoculation, reducing at 24 h but still in values higher than those observed at 3 h ($P < 0.05$).

Glucan-MOS® at 0.2, 0.4 and 0.8% increased SLC after 30 days of feeding ($P < 0.05$). Regardless of the Glucan-MOS® level, SLC increased after chasing and air exposure and at 3, 6 and 24 h after bacterial inoculation ($P < 0.05$).

3.2.2.2. Thrombocytes count and total and differential counts of leukocytes. There was no interaction between Glucan-MOS® supplementation and sampling times (before and after stress and after bacterial inoculation) on the number of thrombocytes (Fig. 2 A). Regardless of the sampling times, Glucan-MOS® supplementation, at 0.4 and 0.8%, increased the number of thrombocytes ($P < 0.05$).

3.2.2.3. Total and differential counts of leukocytes. There was interaction between Glucan-MOS® levels and sampling times on the total count of leukocytes in the blood ($P < 0.05$) (Table 4). Before stress, the 0.4% treatment showed a higher number of leukocytes compared to the control and 0.1% treatments, but Glucan-MOS® had no effect after chasing and air exposure. Three hours after the bacterial challenge, the 0.4 and 0.8% treatments presented higher leukocytes counts compared to the control and 0.1% treatments, at 6 h only the 0.8% treatment differed and at 24 h there were no differences among treatment (Fig. 2 A).

There was interaction between Glucan-MOS® levels and sampling times on the percentage of lymphocytes ($P < 0.05$) (Table 4). Before handling, the 0.4% treatment presented a lower number of lymphocytes, not differing from the 0.2% treatment. After stress and at all samplings after bacterial challenge, all Glucan-MOS® treatments presented a reduced number of lymphocytes.

There was no interaction between Glucan-MOS® levels and sampling times (before and after stress and after bacterial inoculation) on the percentage of neutrophils in the blood ($P < 0.05$) (Fig. 2 B) and on the percentage of monocytes in the blood ($P < 0.05$) (Fig. 2 C). Regardless of the sampling time, the 0.2, 0.4 and 0.8% treatments had a higher percentage of neutrophils in the blood. Regardless of the Glucan-MOS® levels, a higher number of these cells were observed after stress and at 3, 6 and 24 h after the bacterial challenge. Regardless of the sampling time, the 0.1, 0.2, 0.4% treatments showed a higher percentage of monocytes in the blood ($P < 0.05$). Regardless of the Glucan-MOS® level, the percentage of monocytes increased after the stress and decreased gradually along the samplings after bacterial challenge, returning to the before handling condition at 24 h ($P < 0.05$).

3.2.3. Hematological parameters

3.2.3.1. Hemoglobin concentrations, MCHC and erythrocytes number. There was no interaction between dietary Glucan-MOS® supplementation and sampling times (before and after stress and after bacterial inoculation) on erythrocytes number (Fig. 3 A), hemoglobin concentrations (Fig. 3 B) and MCHC (Fig. 3 C). Regardless of the sampling times, the dietary supplementation of Glucan-MOS® for 30 days did not change these variables ($P > 0.05$).

Table 4
Leukocytes and lymphocytes counts in pacu fed Glucan-MOS®, stressed and inoculated with *Aeromonas hydrophila*. Before handling and after handling; 3 h, 6 h and 24 h after bacterial inoculation.

Time	Levels of Glucan-MOS® (%)				
	0.0	0.1	0.2	0.4	0.8
Leukocytes (μL^{-1})					
Before	8.1 ± 1.1b	7.9 ± 1.6b	11.6 ± 1.7 ab	19.0 ± 1.13a	13.6 ± 0.6 ab
After	12.7 ± 1.0	8.7 ± 1.2	17.3 ± 0.7	13.2 ± 0.7	11.5 ± 1.0
3 h	6.8 ± 1.9b	5.4 ± 1.7b	10.8 ± 0.4 ab	16.7 ± 1.7a	16.1 ± 1.7a
6 h	6.5 ± 2.4b	8.9 ± 1.5 ab	9.8 ± 1.5 ab	12.7 ± 0.4 ab	15.3 ± 1.5a
24 h	7.1 ± 1.3	10.5 ± 1.1	10.3 ± 2.4	12.8 ± 0.7	10.1 ± 1.3
Lymphocytes (%)					
Before	69.2 ± 1.4Aa	70.5 ± 2.0Aa	66.0 ± 2.0Aab	54.1 ± 0.7Ab	78.4 ± 2.6Aa
After	41.1 ± 7.5Ba	9.4 ± 1.5BCb	8.1 ± 1.5Bb	7.5 ± .8Bb	7.6 ± 0.9Bb
3 h	30.4 ± 6.1BCa	4.0 ± 1.0Cb	3.1 ± 1.0Bb	0.9 ± 1.4Bb	1.9 ± 0.6Bb
6 h	20.4 ± 3.5BCa	7.7 ± 1.9BCab	5.4 ± 0.6Bb	3.7 ± 0.7Bb	6.5 ± 0.8Bab
24 h	30.2 ± 3.9Ca	19.5 ± 1.5Bab	6.1 ± 1.3Bb	5.6 ± 1.3Bb	6.4 ± 1.3Bb

Values are mean ± standard error. Leukocyte mean values x10^3. Lowercase letters differ in line and uppercase letters differ in column ($P < 0.05$).

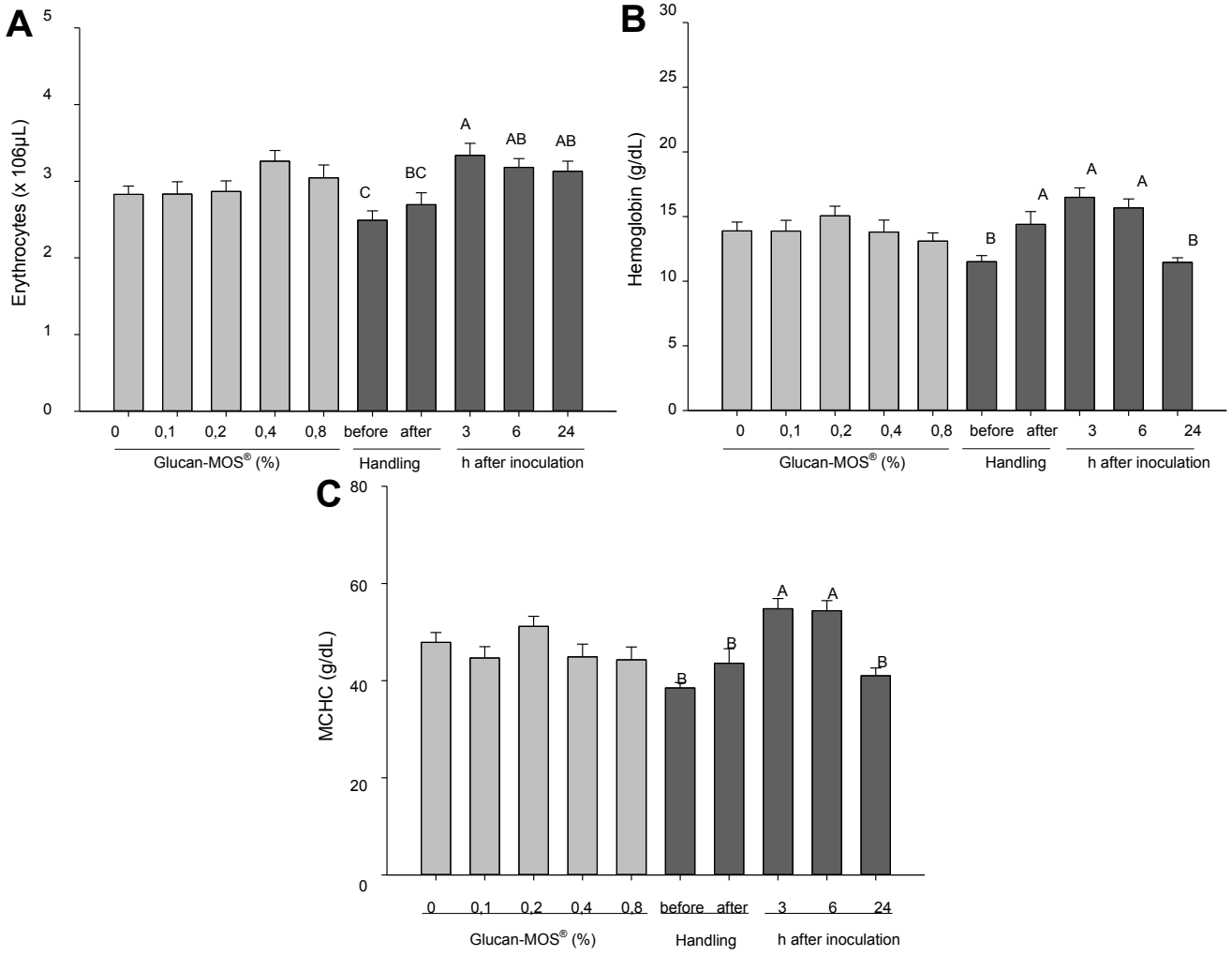

Fig. 3. Erythrocyte number (A), hemoglobin concentrations (B) and MCHC (C) in pacu fed Glucan-MOS®, stressed and inoculated with *Aeromonas hydrophila*. Bars represent means ± standard error. Small letters compare grouped sampling times in each treatment and capital letters compare grouped treatments in each sampling time ($P < 0.05$).

Regardless of the Glucan-MOS® level, hemoglobin concentrations increased after stress and at 3 and 6 h after bacterial inoculation, MCHC increased at 3 and 6 h after bacterial challenge, and the number of erythrocytes increased 3 h after bacterial challenge ($P < 0.05$).

4. Discussion

In this study, we tested a commercial preparation containing two combined components, β 1,3-1,6 glucan and mannan-oligosaccharide, in stressed juvenile pacu infected with *A. hydrophila* and previously fed

with Glucan-MOS® during 30 days. Feeding the fish with the lowest level of the product (0.1%) increased the body weight and protein efficiency ratio, and decreased the feed conversion ratio. Therefore, Glucan-MOS® decreased the response of the stress indicators (plasma glucose and cortisol levels), and increased the innate immune indicators (respiratory activity of leukocytes, serum concentrations of lysozyme, and the thrombocytes, neutrophils and monocytes number).

Regardless of the Glucan-MOS®, the exposure of fish to a stressor and immediate bacterial infection increased the plasma glucose, respiratory activity of leukocytes, serum lysozyme concentrations and neutrophils number and decreased the monocytes number. On the other hand, after stress and after bacterial inoculation, the Glucan-MOS® prevented the elevation of plasma cortisol, increased the total count of leukocytes in addition to decreasing the number of lymphocytes.

In our study, the 30-day supplementation of Glucan-MOS® (0.1%) improved the growth of fish and their feed efficiency (FCR and PER). Most previous studies that evaluated growth performance in fish used one yeast subcomponent to supplement the diets [30–34] or both alternately [35,36]. Few studies have tested the combined effect of both β glucan and mannan-oligosaccharide. Consistent with the results we found in pacu, dietary supplementation with β-Glucan and MOS in combination during 60 days improved the growth and nutrient utilization in Nile tilapia (*Oreochromis niloticus*) [14]. In another study, a prebiotic composed of mannan-oligosaccharide and β-glucans improved the growth and the feed conversion rate in juvenile kutum (*Rutilus kutum*) after 8 weeks of supplementation [16].

The β-Glucan and MOS have two different modes of action. The mannan acts indirectly and can improve growth through the modulation of intestinal microbiota, increasing villi integrity and resistance to pathogenic bacteria, with the consequent increase of digestion and absorption efficiency [37]. Glucan supplementation, on the other hand, may benefit fish performance by improving the digestibility coefficient of the feed, which provides improvements in weight gain and feed conversion [38].

In aquaculture, fish are routinely affected by stressful agents. To establish adequate management practices, the quantification of the stress imposed on fish is fundamental [39]. The most commonly used indicators in stress assessment are plasma cortisol and glucose levels [40–42]. Erythrogram indicators, especially the number of erythrocytes and the hemoglobin concentration, can be also considered stress indicators because they reflect the capacity of blood to transport oxygen to tissues, demonstrating the energy requirement of fish during the stress [43].

As observed in this study, plasma glucose and cortisol concentrations, and erythrocytes and hemoglobin concentrations indicate the stress condition triggered by the chasing and air exposure followed by bacterial challenge. Interestingly, the Glucan-MOS® reduced the glucose and cortisol levels before the stressful handling and prevented the elevation of plasma cortisol after stress and bacterial inoculation. Likewise, Welker et al. (2007) [35] observed lower levels of cortisol in channel catfish (*Ictalurus punctatus*) supplemented with glucans and mannans and exposed to low-water stress (6 wk). The reduction of cortisol levels in fish supplemented with β-glucan or mannans individually was not significant, although the reduction of lactate concentration was. However, no other studies using a combination of glucans and mannans in fish are known. The stress-reducing effect of β-glucan was related by Cain [44] who observed a reduction in circulating cortisol but not in glucose concentration after handling in Nile tilapia at week 3 of feeding. Jeney et al. (1997) [45] showed that feeding rainbow trout with glucan in low doses (0.1%) several weeks before transportation can help to prevent negative effects of stress, as they found reduced levels of glucose and cortisol. Another study showed that dietary supplementation of β-glucan improved stress resistance of red sea bream (*Pagrus major*), assessed by fish survival in a low salinity stress test, but it did not measure cortisol or glucose levels [13].

In addition to the promising effect of Glucan-MOS® on growth performance and feed utilization and the stress-reducing response of pacu, our results confirm the efficiency of the product as an immunostimulant. Before stress and bacterial challenge, the dietary Glucan-MOS® activated the immune system of fish and after fish were exposed to the stressor and bacterial challenge, the dietary supplement reinforced the immune response.

Because β-Glucan and MOS have different modes of action, it is not possible to ensure which is contributing to stimulate the immune system of fish or whether they are acting synergistically. Although the immunostimulant effect of β-glucans in fish is well known [12], there is increasing evidence that prebiotics can also induce immune responses [11,46]. A previous study found an increase in nonspecific immunity after β-Glucan and MOS supplementation in Nile tilapia, as evidenced by increased serum globulin, serum killing activity, phagocytic activity, and lysozyme activity after 15 d of feeding and by improved nitric oxide activity after 30 d [15]. Additionally, a mixture of β-1,3/1,6-glucans and mannan-oligosaccharide as a dietary supplement for channel catfish (*Ictalurus punctatus*) resulted in higher monocyte number, higher phagocytic rate and enhanced phagocytic index [14]. These findings are consistent with our study, which showed that dietary Glucan-MOS® increased the respiratory activity of leukocytes, the serum concentrations of lysozyme, as well as the count of thrombocytes, neutrophils and monocytes.

In addition to the immunostimulant effect of the dietary supplement, we observed activation of the immune responses by the stressor imposed to fish and additionally by the bacterial inoculation. According to Tort (2011) [47], in acute stress episodes immune activation occurs through enhanced innate response and leukocyte mobilization. On the other hand, fish in aquatic environments protect themselves from pathogens mostly with the help of innate or non-specific immunity [48]. Through pattern recognition receptors (PRRs), fish recognize the pathogen-associated molecular patterns of pathogens (PAMPs), and trigger the signaling pathways that activate immune cells in response to pathogen infection [49].

In conclusion, our results reinforce the benefits of the adoption of feeding strategies including combination of both β-1,3-1,6 glucans and mannans as a dietary supplement in periods prior to intensive management. The 30-day period was sufficient to stimulate growth performance, improve nutrient utilization, minimize stress response and modulate innate immunity responses.

Acknowledgements

We would like to thank Dr. Fabiana Pilarski for supplying the strains of *A. hydrophila*. This study was financially supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/CAPES, Brazil, YesSinergy do Brasil Agroindustrial LTDA (Campinas, SP, Brazil) and Proc. n. 552395/2011-0 CNPq.

References

[1] B.A. Barton, G.K. Iwama, Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids, Annu. Rev. Fish Dis. 1 (1991) 3–26.
[2] G.K. Iwama, A.D. Pickering, J.P. Sumpter, C.B. Schreck (Eds.), Fish Stress and Health in Aquaculture, Cambridge University Press, 201162.
[3] B.A. Barton, Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids, Integr. Comp. Biol. 42 (3) (2002) 517–525.
[4] D.J. Alderman, T.S. Hastings, Antibiotic use in aquaculture: development of antibiotic resistance-potential for consumer health risks, Int. J. Food Sci. Technol. 33 (1998) 139–155.
[5] M. Teuber, Veterinary use and antibiotic resistance, Curr. Opin. Microbiol. 4 (2001) 493–499.
[6] J. Romero, C.G. Feijoó, P. Navarrete, Antibiotics in aquaculture–use, abuse and alternatives, in: E.D. Carvalho, G.S. David, R.J. Silva (Eds.), Health and Environment in Aquaculture. In Tech, Rijeka, Croatia, 2012, pp. 159–198.
[7] D.K. Meena, P. Das, S. Kumar, S.C. Mandal, A.K. Prusty, S.K. Singh, M.S. Akhtar, B.K. Behera, K. Kumar, A.K. Pal, S.C. Mukherjee, Beta-glucan: an ideal immunostimulant in aquaculture (a review), Fish Physiology Biochem. 39 (3) (2013)

431–457.
[8] E. Ringo, R.E. Olsen, J.L.G. Vecino, S. Wadsworth, S.K. Song, Use of immunostimulants and nucleotides in aquaculture: a review, J. Mar. Sci. Res. Dev. 1 (2012) 104.
[9] S.K. Song, B.R. Beck, D. Kim, J. Park, J. Kim, H.D. Kim, E. Ringo, Prebiotics as immunostimulants in aquaculture: a review, Fish Shellfish Immunol. 40 (1) (2014) 40–48.
[10] V. Kiron, Fish immune system and its nutritional modulation for preventive health care, Animal Feed Sci. Technol. 173 (1) (2012) 111–133.
[11] S. Torrecillas, D. Montero, M. Izquierdo, Improved health and growth of fish fed mannan oligosaccharides: potential mode of action, Fish Shellfish Immunol. 36 (2) (2014) 525–544.
[12] E. Vallejos-Vidal, F. Reyes-López, M. Teles, S. MacKenzie, The response of fish to immunostimulant diets, Fish Shellfish Immunol. 56 (2016) 34–69.
[13] M.A.O. Dawood, S. Koshio, S. Ishikawa, M. F. El Basuini, M.S. Hossain, T.H. Nhu, A.S. Moss, S. Dossou, H. Wei, Dietary supplementation of β-glucan improves growth performance, the innate immune response and stress resistance of red sea bream, *Pagrus major*, Aquac. Nutr. 23 (2017) 148–159.
[14] H. Zhu, H. Liu, J. Yan, R. Wang, L. Liu, Effect of yeast polysaccharide on some hematologic parameter and gut morphology in channel catfish (*Ictalurus punctatus*), Fish Physiology Biochem. 38 (5) (2012) 1441–1447.
[15] K.M. Selim, R.M. Reda, Beta-glucans and mannan oligosaccharides enhance growth and immunity in Nile tilapia, North Am. J. Aquac. 77 (1) (2015) 22–30.
[16] A.K. Amirkolaie, S. Karimzadeh, S.P. Miandehy, The effects of dietary Betaplus® and TechnoMos® on growth performance, blood parameters, and intestinal microflora in juvenile kutum, Rutilus kutum (Kamensky, 1901), in press, J. Appl. Ichthyology (2017), http://dx.doi.org/10.1111/jai.13322.
[17] M. Sakai, Current research status of fish immunostimulants, Aquaculture 172 (1) (1999) 63–92.
[18] IBGE-Instituto Brasileiro de Geografia e Estatística- IBGE, Produção da Pecuária Municipal, Rio de Janeiro vol. 43, (2015), pp. 1–49.
[19] R.K. Jomori, D.J. Carneiro, M.I.E.G. Martins, M.C. Portella, Economic evaluation of *Piaractus mesopotamicus* juvenile production in different rearing systems, Aquaculture 243 (1) (2005) 175–183.
[20] E.G. Abimorad, G.H. Squassoni, J.D. Carneiro, Apparent digestibility of protein, energy, and amino acids in some selected feed ingredients for pacu *Piaractus mesopotamicus*, Aquac. Nutr. 14 (2008) 374–380.
[21] J.D. Biller-Takahashi, L.S. Takahashi, M.V. Saita, R.Y. Gimbo, E.C. Urbinati, Leukocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus*, Braz. J. Biol. 73 (2) (2013) 425–429.
[22] R.Y. Gimbo, G.C. Fávero, L.N.F. Montoya, E.C. Urbinati, Energy deficit does not affect immune responses of experimentally infected pacu (*Piaractus mesopotamicus*), Fish Shellfish Immunol. 43 (2) (2015) 295–300.
[23] J.D. Biller-Takahashi, H.J. Montassier, L.S. Takahashi, E.C. Urbinati, Levamisole promotes an adjuvant effect on the immunity of pacu (*Piaractus mesopotamicus*) when immunized with *Aeromonas hydrophila*, even when provided in the diet, Animal Feed Sci. Technol. 211 (2016) 164–173.
[24] E.C. Urbinati, F.D. Gonçalves, L.S. Takahashi, Pacu (*Piaractus mesopotamicus*), in: B. Baldisseroto, L.C. Gomes (Eds.), Espécies nativas para piscicultura no Brasil, Editora UFSM, Santa Maria, RS, 2013, p. 470.
[25] AOAC, Official Methods of Analysis, 17 ed., Association of Official Analytical Chemists, Washington, DC, 2000, p. 937.
[26] J.D. Biller-Takahashi, L.S. Takahashi, M.V. Saita, R.Y. Gimbo, E.C. Urbinati, Leukocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus*, Braz. J. Biol. 73 (2) (2013) 425–429.
[27] A.E. Ellis, Lysozyme assays, in: J.S. Stolen, et al. (Ed.), Techniques in Fish Immunology, SOS Publications, Fair Haven, NJ, 1990, pp. 101–104.
[28] J.S. Abreu, C.M. Marzocchi-Machado, A.C. Uubaczek, L.M. Fonseca, E.C. Urbinati, Leukocytes respiratory burst and lysozyme level in pacu (*Piaractus mesopotamicus*), Braz. J. Biol. 69 (4) (2009) 1133–1139.
[29] M.J.T.R. De Paiva, S.B. De Pádua, M. Tavares-Dias, M.I. Egami, Métodos para análise hematológica em peixes, Editora da Universidade Estadual de Maringá - EDUEM, 2013 140 pp.
[30] R. Whittington, C. Lim, P.H. Klesius, Effect of dietary β-glucan levels on the growth response and efficacy of *Streptococcus iniae* vaccine in Nile tilapia, *Oreochromis niloticus*, Aquaculture 248 (1) (2005) 217–225.
[31] R.A. Shelby, C. Lim, M. Yildirim-Aksoy, T.L. Welker, P.H. Klesius, Effects of yeast oligosaccharide diet supplements on growth and disease resistance in juvenile Nile tilapia, *Oreochromis niloticus*, J. Appl. Aquac. 21 (1) (2009) 61–71.
[32] S. Refstie, G. Baeverfjord, R.R. Seim, O. Elveb, Effects of dietary yeast cell wall β-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (*Salmo salar*) fed sunflower and soybean meal, Aquaculture 305 (1) (2010) 109–116.
[33] R.Y. Sado, A.J.A. Bicudo, J.E.P. Cyrino, Growth and intestinal morphology of juvenile pacu *Piaractus mesopotamicus* (Holmberg 1887) fed dietary prebiotics (mannanoligosaccharides-MOS), Anais da Academia Brasileira de Ciências 86 (3) (2014) 1517–1524.
[34] L. Ji, G. Sun, J. Li, Y. Wang, Y. Du, X. Li, Y. Liu, Effect of dietary β-glucan on growth, survival and regulation of immune processes in rainbow trout (*Oncorhynchus mykiss*) infected by *Aeromonas salmonicida*, Fish Shellfish Immunol. 64 (2017) 56–67.
[35] T.L. Welker, C. Lim, M. Yildirim-Aksoy, R. Shelby, P.H. Klesius, Immune response and resistance to stress and *Edwardsiella ictaluri* challenge in channel catfish, *Ictalurus punctatus*, fed diets containing commercial whole-cell yeast or yeast subcomponents, J. World Aquac. Soc. 38 (1) (2007) 24–35.
[36] T.L. Welker, C. Lim, M. Yildirim-Aksoy, P.H. Klesius, Use of diet crossover to determine the effects of β-glucan supplementation on immunity and growth of Nile Tilapia, *Oreochromis niloticus*, J. World Aquac. Soc. 43 (3) (2012) 335–348.
[37] A. Dimitroglou, D.L. Merrifield, R. Moate, S.J. Davies, P. Spring, J. Sweetman, G. Bradley, Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Animal Sci. 87 (10) (2009) 3226–3234.
[38] G. Burr, M. Hume, W.H. Neill, D.M. Gatlin III, Effects of prebiotics on nutrient digestibility of a soybean-meal-based diet by red drum *Sciaenops ocellatus* (Linnaeus), Aquac. Res. 39 (15) (2008) 1680–1686.
[39] G.A. Wedemeyer, Effect of rearing conditions on the health and physiological quality of fish in intensive culture, in: G.K. Iwama, A.D. Pickering, J.P. Sumpter, C.B. Schreck (Eds.), Fish Stress and Health in Aquaculture, Cambridge University Press, Cambridge, England, 1997, pp. 35–71 (Society for Experimental Biology Seminar Series, 62).
[40] L. Robertson, P. Thomas, C.R. Arnold, J.M. Trant, Plasma cortisol and secondary stress responses of red drum to handling, transport, rearing density, and a disease outbreak, Progressive Fish-Culturist 49 (1) (1987) 1–12.
[41] B.A. Barton, Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress, North Am. J. Aquac. 62 (1) (2000) 12–18.
[42] N.M. Sopinka, M.R. Donaldson, C.M. O'Connor, C.D. Suski, S.J. Cooke, Stress indicators in fish, in: Fish Physiology, C.B. Schreck, L. Tort, A.P. Farrell, C.J. Brauner (Eds.), Biology of Stress in Fish, vol. 11, Academic Press, New York, 2016, pp. 407–460.
[43] A.H. Houston, Review: are the classical hematological variables acceptable indicators of fish health? Trans. Am. Fish. Soc. 126 (6) (1997) 879–894.
[44] K.D. Cain, L. Grabowski, J. Reilly, M. Lytwyn, Immunomodulatory effects of a bacterial-derivedβ-1,3 glucan administered to tilapia (*Oreochromis niloticus* L.) in a Spiruluna-based diet, Aquac. Res. 34 (2003) 1241–1244.
[45] G. Jeney, M. Galeotti, D. Volpatti, Z. Jeney, D.P. Anderson, Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan, Aquaculture 154 (1) (1997) 1–15.
[46] I. Guerreiro, A. Couto, M. Machado, C. Castro, P. Pousão-Ferreira, A. Oliva-Teles, P. Enes, Prebiotics effect on immune and hepatic oxidative status and gut morphology of white sea bream (*Diplodus sargus*), Fish Shellfish Immunol. 50 (2016) 168–174.
[47] L. Tort, Stress and immune modulation in fish, Dev. Comp. Immunol. 35 (12) (2011) 1366–1375.
[48] P.R. Rauta, M. Samanta, H.R. Dash, B. Nayak, S. Das, Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses, Immunol. Lett. 158 (1) (2014) 14–24.
[49] Y. Palti, Toll-like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (12) (2011) 1263–1272.