Immunomodulatory effects of dietary β-glucan in silver catfish (*Rhamdia quelen*)[1]

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ABSTRACT.- Di Domenico J., Canova R., Soveral L.F., Nied C.O., Costa M.M., Frandoloso R. & Kreutz L.C. 2017. **Immunomodulatory effects of dietary β-glucan in silver catfish (*Rhamdia quelen*).** *Pesquisa Veterinária Brasileira 37(1):73-78.* Laboratório de Microbiologia e Imunologia Aplicada, Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo, Campus I, Bairro São José, BR-282 Km 171, Passo Fundo, RS 99052-900, Brazil. E-mail:lckreutz@upf.br

The immunomodulatory effects of dietary β-glucan were evaluated in silver catfish. β-glucan was added to the diet (0.01%, and 0.1%) and fed to the fish for 21 days, to evaluate effects on blood and some innate immune parameter, or fed for 42 days, to evaluate growth rate and resistance to challenge with pathogenic *Aeromonas hydrophila*. We found that adding β-glucan to the diet had no effect on fish growth and no effect on blood cells, or serum bacterial agglutination and serum myeloperoxidase activity. However, fish that received β-glucan in the diet had the natural hemolytic activity of complement significantly higher compared to control fish. Furthermore, fish fed with β-glucan and challenged with *A. hydrophila* had fewer bacteria in blood and presented a significantly higher survival rate compared to control fish. Thus, we concluded that β-glucan might be explored as feed additive aiming to improve silver catfish innate immunity and resistance to specific pathogen.

INDEX TERMS: Silver catfish, *Rhamdia quelen*, fish, immunostimulants, β-glucan, *Aeromonas hydrophila.*

RESUMO.- [**Efeito imunomodulador da β- glucana usada como aditivo alimentar em jundiás (*Rhamdia quelen*).**] O uso da β-glucana como suplemento alimentar foi avaliado em jundiás. A β-glucana foi adicionada à ração na proporção de 0.01%, e 0.1% e fornecida aos peixes por 21, para avaliar dados hematológicos e parâmetros do sistema imune natural, ou 42 dias, para avaliar ganho de peso e resistência ao desafio com *Aeromonas hydrophila*. A adição da β-glucana na dieta não afetou o ganho de peso e não induziu alterações hematológicas nem alterações nos níveis de aglutininas e mieloperoxidase sanguínea. No entanto, a atividade hemolítica natural do sistema do complemento foi significativamente maior nos peixes alimentados com β-glucana. Além disso, nos peixes alimentados com β-glucana e desafiados com *A. hydrophila*, o número de bactérias isoladas do sangue foi significativamente menor, e a sobrevivência ao desafio foi significativamente maior do que nos peixes que não receberam β-glucana. Consequentemente, concluímos que a β-glucana tem potencial imunomodulador quando adicionada à dieta, nas condições experimentais aqui indicadas, e contribui para aumentar imunidade natural e a resistência dos jundiás ao desafio com patógenos específicos.

TERMOS DE INDEXAÇÃO: Aditivo alimentar, jundiá, *Rhamdia quelen*, imunoestimulantes, β-glucana, *Aeromonas hydrophila.*

INTRODUCTION

The occurrence of infectious diseases is one of the major causes of losses in modern aquaculture (Sitjà-Bobadilla

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2008). Although antibiotics have been widely used for disease treatment, mainly in fries and fingerlings (Brudeseth et al. 2013), the surge of antibiotics-resistant bacteria, and antibiotics residues in water and meat, raised major concerns towards this management procedure. In this scenario, strengthening the fish defense mechanisms by vaccination to specific pathogens or by adding immune modulating molecules in the diet has been increasingly explored as an economically viable procedure to prevent disease outbreaks (Bricknell & Dalmo 2005, Sommerset et al. 2005, Plant & Laptra 2011, Bairwa et al. 2012).

Immunomodulating molecules interact with immunological cells and are widely used to improve defense mechanisms. Medicinal herbs and plants are major source of such molecules (Galina et al. 2009, Van Hai 2015); pre- and probiotics (Nayak 2010), vitamins (Ortuño et al. 2001, Azad et al. 2007, Kiron 2012) and synthetic molecules (Maqsood et al. 2009) have also been evaluated for their effects on the immune system. Amongst immune modulating molecules, β-glucan, a linear polysaccharide extracted from the cell wall of yeast, algae and fungi (Dalmo & Bøgwald 2008) stands as a model of pathogen associated molecular pattern (PAMP) molecule, and as such has been widely exploited as additive in fish diets aiming to improve resistance to infection. The inclusion of β-glucans in the diet of *Anabas testudineus* (Das et al. 2009), *Cyprinus carpio* (Miest et al. 2012), *Oncorhynchus mykiss* (Ghaedi et al. 2015), *Salmon salar* (Pionnier et al. 2013) amongst other fish species, for instance, improved several innate immune parameters like respiratory burst, macrophage phagocytic index, serum lysozyme and myeloperoxidase, and improved the production of total IgM. However, conflicting results have also been reported, such as in *Salmo salar* and *Scophthalmus rhombus*, and have been attributed to differences in β-glucan concentration on diet, the molecular characteristics of the molecule (1,3-β-glucan or 1,6-β-glucan), time and feeding regimen (Dalmo & Bøgwald 2008).

Several putative β-glucan receptors have been found in mammal cells (Kerrigan & Brown 2009, Van Bruggen et al. 2009) but not yet in fish. The ligation of β-glucan to its cognate molecule on immune cells such as macrophage and dendritic cells triggers the expression of proinflammatory (IL-2 and IFNγ) and anti-inflammatory (IL-10) cytokines (Aoki et al. 2008, Dalmo & Bøgwald 2008) and potentiates phagocytosis and the removal of iC3b and C3b opsonized antigens (Hawlisch & Köhl 2006). As a consequence of macrophage and dendritic cell activation, and the resulting cytokine cascade, both innate and acquired immune response meliorates. This makes of β-glucan an ideal immune stimulating molecule for aquaculture.

Silver catfish (*Rhamdia quelen*) is endemic in South American rivers and lakes and has been widely used for aquaculture alone or comingled with other fish species (B (Gomes et al. 2000, Barcellos et al. 2004b). However, bacteria and parasitic infections (Barcellos et al. 2008, Martins et al. 2011) remains a major challenge to improve productivity and triggers the need to improve fish health by means of vaccination and PAMPs- enriched diets. Thus, in this work, we aimed to evaluate the effects of β-glucan-enriched diet on innate immunity and resistance to challenge by *Aeromonas hydrophila* infection.

MATERIALS AND METHODS

Fishes. All fishes used in these experiment were produced and obtained from our experimental unit (centro de pesquisa agropecuária – Cepagro) and were free of specific infections. During the acclimatization period (7 days) and up to the end of the experiments, fish were kept in self-cleaning tanks containing 1000L of continuously running water, protected from direct sun light, and fed a commercial pelleted feed containing 42% protein. Water conditions were within the expected values, as previously reported (Kreutz et al. 2014). For inoculations and blood sampling, fish were anesthetized with clove oil (50mg/L - Sigma, Brazil). The experiments were approved by the Ethics Committee for the Care and Use of Experimental Animals (CEUA, protocol 011/2012) of the Universidade de Passo Fundo. Prior to and after the experiments all fish were weighted and measured to evaluate relative weight gain (WG) and specific growth rate (SGR): WG = 100 x (final weight - initial weight)/initial weight) and SGR = 100 x [ln final weight - ln initial weight]/days of the experiment (Lugert et al. 2014).

Evaluation of β-glucan as feed additive aiming to modulate the immune system. Two experiments were carried out to evaluate the effect of mixing β-glucan (MacroGard®, Biorigin, Brazil) on blood cells and innate immune parameters, WG, SGR and survival to challenge with *A. hydrophila*. In the first trial, silver catfish juveniles (70-90 g) were allocated into three groups: one group received pelleted food added with 0.01% of β-glucan; a second group had 0.1% of β-glucan added to the food, and a third group had no β-glucan on the food (control group). All fish were fed *at libitum* twice a day. β-glucan was mixed to the food pellets as recommended by the manufacturer and fed to fish for 28 days. Each group consisted of 15-17 fishes and the experiment was carried out in duplicates. At the end of the feeding trial, all fish were captured and anesthetized for blood sampling at the caudal vein. One blood aliquot was dropped in EDTA-containing tubes aiming hematological analysis. A second aliquot was allowed to clot at 4º and centrifuged at 1500 x g to separate the serum, which was further aliquoted and stored at -20º C and used to evaluate innate immune parameter. One week later, all fish were captured again, anesthetized and immunized with BSA (200 µg/fish) mixed to montanide (20% v/v) aiming to evaluate the effect of the feeding trial in the production of antibodies to BSA. During this time β-glucan was removed from the diet and all fish received the same pelleted food. Then, after 28 days of vaccination with BSA (at day 63 of the experimental trial) all fish were captured and blood samples were collected without anticoagulant, and processed as described above to evaluate anti-BSA antibodies by ELISA.

In the second feeding trial, 150 fish were allocated equally to three feeding groups in duplicates: no β-glucan, 0.01% and 0.1% of β-glucan in the food, and *fed at libitum* twice a day. All fish were weighted and measured prior to the feeding trial and at 42 days to evaluate weight gain and SGR. Then, at the fortieth second day, immediately after measurements, all fish were intraperitoneally challenged with *Aeromonas hydrophila* ($2x10^8$ Colony Forming Units – CFU /fish) as previously described (Kreutz et al. 2010). After 24h, 10 fish from each group were captured for blood sampling aiming to detect bacteremia.

Hematological, innate immune parameters analysis and bacteremia detection. For hematological evaluation, blood smear were made with EDTA-containing blood aliquots, air-dried and stained with Wrigth-giemsa. Hematocrit, hemoglobin and erythrocyte counts were determined on whole blood within 2h after sampling, as previously described (Barcellos et al. 2004a). Innate immune

parameters (total serum myeloperoxidase, serum bacteria agglutination activity and complement natural hemolytic activity) were performed as previously described (Kreutz et al. 2011).

The presence of bacteremia in fish was assessed by seeding 100 µl of whole blood on Brain Heart Infusion (BHI) plates. After seeding, plates were incubated at 37°C for 24h and the number of CFU was annotated. The remaining fish were observed daily for seven days to evaluate clinical signs, skin lesions and mortality aiming to determine the survival rate following challenge.

Enzyme-linked immunosorbent assay to measure anti--BSA antibodies in fish serum. The ELISA assay was performed as recently described (Kreutz et al. 2016). Briefly, 96-well ELISA plates were coated overnight (4°C) with BSA (5µg/well) diluted in carbonate-bicarbonate buffer (pH 9.6) and then blocked with PBS containing 0.05% Tween 20 (PBST) and 3% skin milk (Sigma) (PBST-Sk3%). Fish serum samples diluted 1:100 in PBST-SK1% were added in duplicates to the wells. After 1h incubation at 23°C and washing with PBST, rabbit anti-silver catfish IgM antibodies diluted 1:400 in PBST-SK1% was added to the wells. The plates were incubated and washed as described above. Horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma) was added to the plates (diluted 1:20.000 in PBST-SK1%) and incubated 1h at 23°C. After washing, color development was performed using O--phenyldiamine (OPD - 0.067%; Sigma). Plates were read at 492 nm with an Anthos 2010 ELISA plate reader.

Statistical analysis. The results obtained were analyzed by the Shapiro-Wilk's test and were found to have normal distribution. Differences amongst treatments were analyzed by t-test or ANOVA followed by Bonferroni's multiple comparisons test, and plotted using GraphPad Prism Software v. 5 (GraphPad Software, Inc., USA). *P*-values of 0.05 or smaller were considered significant. Results are expressed as the mean ± standard error of the mean (SEM).

RESULTS

The effect of feeding β-glucan enriched diet on blood cell and innate immune parameters

Blood cell parameters observed in the current study (Table 1) were within the range reported previously for silver catfish (Barcellos et al. 2004a, Kreutz et al. 2011). In all groups, at the end of the experiment, erythrocyte counts were lower ($p<0.05$) compared to the counts obtained prior to the feeding trial. Monocytes (control and 0.01% β-glucan groups) and thrombocytes (0.01% β-glucan group) were also reduced by the end of the experiment.

The inclusion of β-glucan in the diet had no effect on total serum myeloperoxidase activity or in the capacity of serum to agglutinate inactivated *A. hydrophila* (data not shown). In addition, the β-glucan enriched diet had no effect on the fish capacity to produce antibodies to BSA (data not shown). However, the addition of β-glucan to feed had a significant ($p<0.05$) effect on the complement natural hemolytic activity upon sheep red blood cells (Fig.1).

The effect of feeding β-glucan enriched diet on the resistance of fish to challenge with *Aeromonas hydrophila*

The inclusion of β-glucan on the diet had no effect on weight gain and SGR (Table 2). Resistance to *A. hydrophila* infection was evaluated by measuring bacteremia, at 24 h p.i., and survival rate up to 7 days p.i. The number of CFU in the blood of fish fed diets containing β-glucan was significantly lower ($p<0.05$) compared to fish from the control group (Fig.2). In addition, the survival rate of fish fed β-glucan was significantly higher ($p<0.05$) than the survival rate of the fish from the control (Fig.3). In the control group, mortality was observed 24h p.i. and continued up to 96h p.i. In the group of fish fed a diet containing 0.01% β-glucan, fish mortality was observed at the third and fourth day. No mortality was observed in the group of fish fed with 0.1% β-glucan.

DISCUSSION

Outbreaks of infectious diseases on cultivated fish species are difficult to control and represent a major cause of reduced productivity. In addition, the occurrence of specific infections might raise sanitary barrier to fish products. In this scenario, controlling disease outbreaks by vaccination and the use of immune modulator enriched diets represents a major achievement for aquaculture species. Although fish vaccination is still crawling, it has been successfully used to control important fish diseases (Sommerset et al. 2005, Secombes 2008, Van Muiswinkel 2008, Plant & Lapatra 2011). And, more recently, vaccination commingled with the inclusion of immune-modulator molecules in the diet offered and additional strategy to overcome major pathogen (Newaj-Fyzul & Austin 2015). However, because an efficient immune response to inoculated antigen still relies on intraperitoneal injections, the hurdles and cost of individual vaccination hinders application to low commercial value species. Thus, for this species, feed additives with immune modulating capability might become widely used.

The mechanisms of β-glucan effect on fish innate immune system are not clear but might involve improved phagocytic activity and increased expression of cytokines in macrophage, neutrophils and dendritic cell. Indeed, *in vitro*

Table 1. Hematological parameters of silver catfish fed a diet containing β-glucan. Blood samples were collected prior to (day 0) or after feeding with β-glucan (day 28). The data represent the mean ± SEM (n=7). Significant differences within the same treatment group are indicated by asterisk ($p<0.05$)

Parameter	Treatment					
	Control		β-glucan 0.01%		β-glucan 0.1%	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Hematocrit (%)	40.0 ± 2.0	37.0 ± 2.0	40.0 ± 1.0	49.0 ± 0.9	40.0 ± 1.0	41.0 ± 0.9
Hemoglobin (g/l)	11.0 ± 0.7	10.0 ± 0.5	10.0 ± 0.4	10.0 ± 0.53	11.0 ± 0.4	11.0 ± 0.3
Erythrocyte (106/µl)	1.6 ± 0.08	1.1 ± 0.06*	1.4 ± 0.07	1.0 ± 0.05*	1.6 ± 0.05	1.2 ± 0.05*
Leucocytes (µl)	265.3 ± 19.0	191.6 ± 18.1	311.0 ± 23.0	167.0 ± 10.6	270.0 ± 26.0	234.0 ± 21.0
Neutrophils (µl)	10.924 ± 2.9	6.800 ± 1.4	10.367 ± 2.1	7.293 ± 1.3	9.169 ± 2.5	8.943 ± 2.7
Monocytes (µl)	2.270 ± 870	696 ± 232*	1.885 ± 190	420 ± 140*	4.100 ± 1.9	1.321 ± 385
Lymphocytes (µl)	106.235 ± 14.0	94.175 ± 14.2	104.613 ± 22.0	75.350 ± 7.2	144.507 ± 22.0	113.742 ± 13.85
Thrombocytes (µl)	148.500 ± 16.000	93.140 ± 8.470	203.779 ± 22.166	82.770 ± 5.543*	116.945 ± 11.358	113.815 ± 9.831

studies indicated that β-glucan triggered proinflammatory cytokines in exposed macrophage meliorating phagocytic activity, respiratory burst, serum lysozyme, myeloperoxidase, serum bactericidal activity and complement natural hemolytic activity (Hawlisch & Köhl 2006).

In our work, although serum levels of bacterial agglutinins and myeloperoxidase were not affected by adding β-glucan on the diet, the natural complement hemolytic activity was significantly improved in fish fed β-glucan. The complement cascade in teleost fish is one of the major natural defense mechanism toward parasites, fungi, virus and bacteria (Magnadóttir 2006, Alvarez-Pellitero 2008). The activation of complement, either the classical or alternative pathway, leads to the production of several soluble compo-

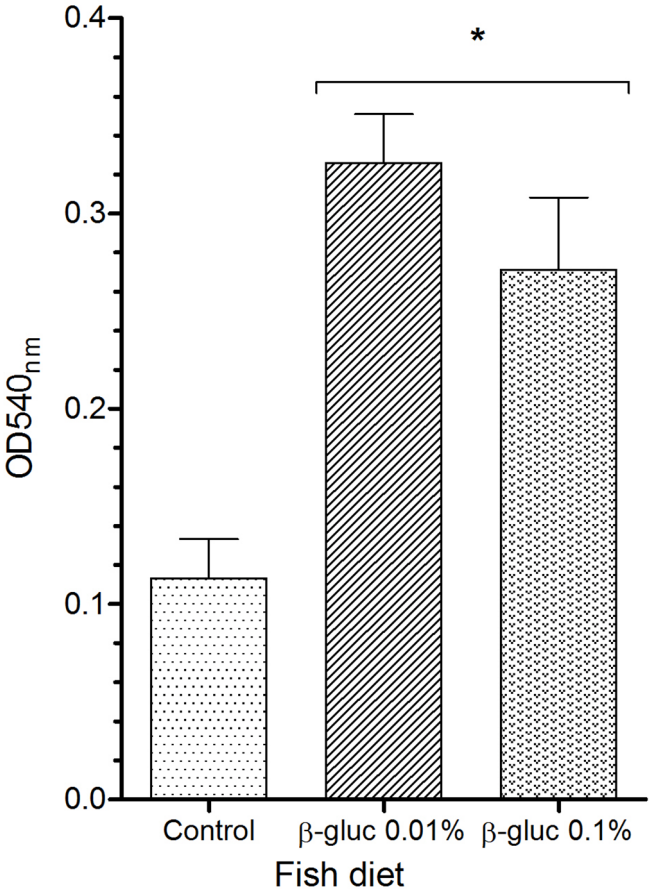

Fig.1. Increased natural complement hemolytic activity in silver catfish fed a diet containing β-glucan. The results are expressed as the mean ± SEM (n=30). Significant differences from the control group (p<0.05) are indicated by asterisk.

Table 2. Weight gain in silver catfish fed with a diet containing β-glucan. All fish where weighted and measured prior to and after the end of the experiment (42 days) to evaluate weight gain (%) and specific growth rate (SGR). Values represent the mean ± S.E.M (n=50)

Parameter	Experimental group		
	Control	β-glucan 0.01%	β-glucan 0.1%
Initial weight (g)	20.3±0.3	20.2±0.4	20.3±0.3
Final weight (g)	67±1.8	66±2	72±1.6
Weight gain (%)	230±8.8	227±10	252±7.9
SGR (%/day-1)	1.2±0.03	1.2±0.03	1.3±0.02

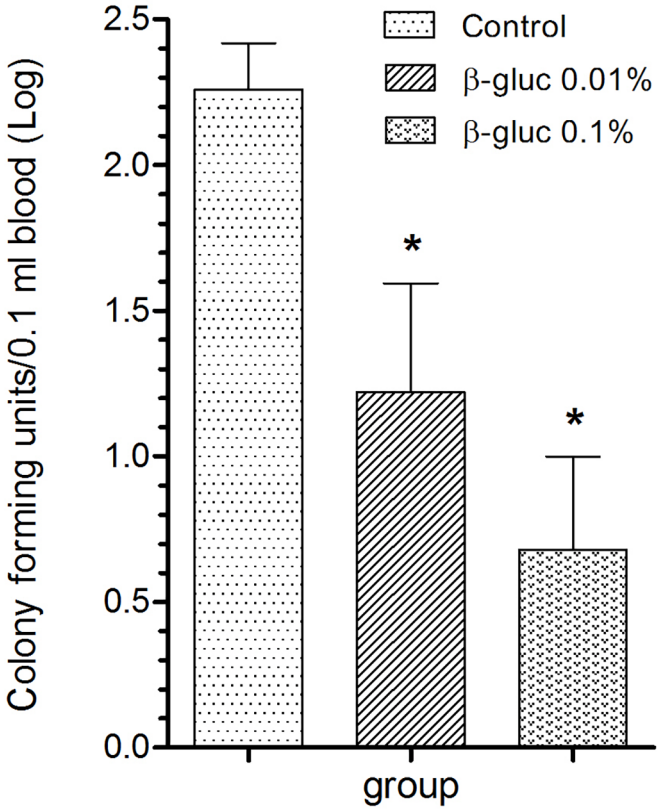

Fig.2. Number of colony forming units (CFU) in the blood of silver catfish challenged with *Aeromonas hydrophila* ($2x10^8$ CFU/fish). Blood samples were collected aseptically 24h after challenging and cultured in BHI plates (0.1ml blood/plate). Data are represented as the mean ± SEM (n=10) of the natural logarithm of the number of colonies observed in each plate. Significant differences from the control group (p<0.05) are indicated by asterisk.

nents involved in chemiotaxis, opsonization, phagocytosis and pathogen destruction (Magnadóttir et al. 2005). Inclusion of β-glucan into the diet of carps (*Labeo rohita* and *Cyprinus carpio*) also improved complement activity (Misra et al. 2006) and heightened expression of complement genes in several tissues (Pionnier et al. 2013). Furthermore, the activity of the alternative complement cascade might also be improved by adding *Saccharomyces cerevisiae* to the diet of *Epinephelus coioides* (Chiu et al. 2010). These indicate that complement *per se* might be capable of controlling initial infection by specific pathogens.

In contrast, the production of anti-BSA antibodies in fish fed β-glucan and vaccinated with BSA+montanide was similar to fish from the control groups (data not shown). Indeed, feeding β-glucan to fish hardly improves the production of total or specific immunoglobulins, as observed in Nile tilapia, *Oreochromis niloticus* (Whittington et al. 2005), common carp (Selvaraj et al. 2006), rainbow trout (Skov et al. 2012, Ghaedi et al. 2015) and gilthead sea bream, *Sparus aurata* (Guzmán-Villanueva et al. 2014).

The effect of β-glucan on blood cells is controversial. We found that monocytes and thrombocytes were reduced by the end of the feeding trial in the control group and in the group fed with 0.01% β-glucan. High variation found within

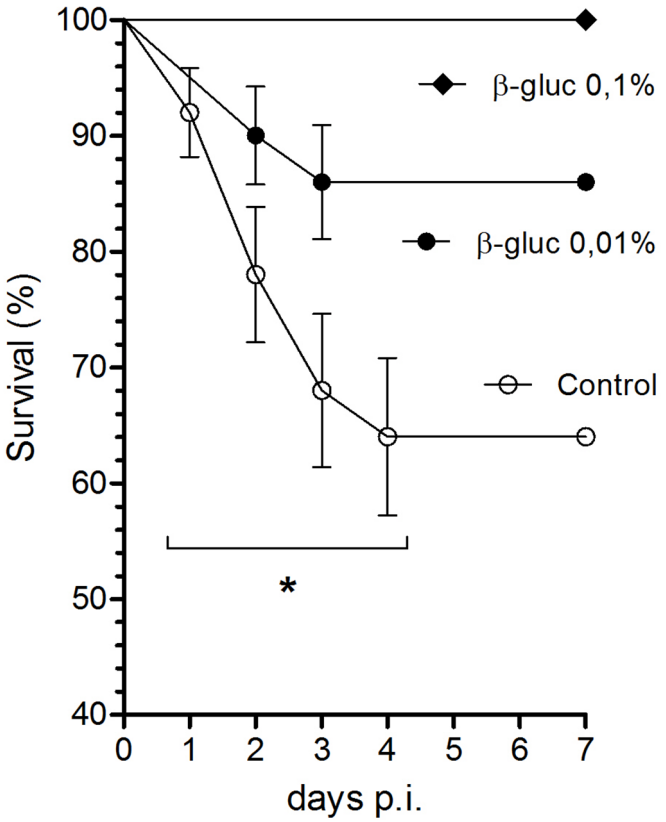

Fig.3. Effect of β-glucan on survival rate of silver catfish challenged with an intraperitoneal injection of *Aeromonas hydrophila* ($2x10^8$ CFU/fish). The number of death fish in each group was annotated daily up to the seventh day. All groups consisted of 40 fish and the data is expressed as daily survival rate ± SEM ($p<0.05$) of replicate tanks. Significant differences within groups are indicated by asterisk.

fish from the same group difficults a better analysis. Nonetheless, adaptation of fish to the tank environment might contribute to alter blood cells and even decrease the circulation of immune cells due to cortisol release (Gabriel et al. 2011). In addition, at least one study indicated that Indian carps (*Labeo rohita*) had reduced total leukocytes counts after a two week feeding trial with β-glucan (500mg/Kg of feed)(Misra et al. 2006). In fish fed with 0.1% β-glucan, the number of monocytes and thrombocytes were not altered at the end of the experiment and it would be tempting to attribute this effect to β-glucan. However, we are aware that a larger number of fish should be used to assume that β-glucan would prevent the stress effect on blood cells.

Nonetheless, the beneficial effect of adding β-glucan to silver catfish diet has been unequivocally demonstrated by challenging fish with an intraperitoneal injection of *A. hydrophila*. Fish fed with β-glucan (0.01% and 0.1%) had significantly less bacteria in blood at 24 h p.i. and a significantly higher survival rate. In fact, silver catfish fed 0.1% β-glucan had a survival rate of 100%. The beneficial effect on resistance to challenge with specific pathogen has been reported in several fish species (Misra et al. 2006, Welker et al. 2007, Garcia & Villarroel 2009, Pionnier et al. 2013). β-glucan is thought to stimulate the complement cascade, phagocytosis, serum lysozyme and bactericidal activity. Combined, these mechanisms suffice the control of inoculated pathogens and prevent a widespread dissemination that could cause organs failure. In our work, indeed, fish fed β-glucan had a significantly lower bacteremia that corresponded to lower clinical signs, lesions and mortality.

Several studies indicated that β-glucan improved fish weight gain (Whittington et al. 2005, Welker et al. 2007, Garcia & Villarroel 2009, Chiu et al. 2010, Guzmán-Villanueva et al. 2013, Ghaedi et al. 2015) . However, in our study, the addition of β-glucan to the diet had no effect on SGR. Differences in the feeding regimen and fish species should account for this observation. In at least one study, β-glucan improved the secretion of digestive enzymes and this could be related to improved growth (Guzman-Villanueva et al. 2013).

In summary, although we could not demonstrate a beneficial effect on blood cells and some innate immune parameters, the addition of β-glucan to the diet improved natural complement hemolytic activity, reduced bacteremia levels and, most importantly, increased fish resistance to challenge with *A. hydrophila*. Taken together, we would strongly recommend the use of β-glucan on silver catfish diet aiming to improve overall health.

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Conflict of interest.- The authors have no competing interests.

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