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Research Article

Effects of dietary mannan oligosaccharide on the growth, survival, intestinal morphometry and nonspecific immune response for Siamese fighting fish (*Betta splendens* Regan, 1910) larvae

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ABSTRACT. A 28-day experiment was carried out to evaluate the effect of dietary prebiotic mannan oligosaccharide (MOS) on the growth, survival, intestinal morphometry and nonspecific immune response of the Siamese fighting fish (*Betta splendens* Regan, 1910) larvae. The MOS supplementation levels evaluated was: 0, 2, 4 and 6 g kg^{-1}. Into 16 plastic aquaria (3 L) 240 larvae (4.21 ± 0.26 mm) were distributed at a density of 5 larvae L^{-1}. All larvae received live food for seven days, followed by a period of eight days of co-feeding (live food + inert diet) and, finally, 13 days of inert diet. At the end of feeding-trial, there were no significant differences between growth, survival, intestinal morphometry and alkaline phosphatase activity of larvae fed the control or MOS supplemented diets. However, the lysozyme activity in larvae fed 2 g MOS kg^{-1} was significantly higher compared to other treatments. For fish farming, the most beneficial effects of prebiotics reside in its ability to enhance the immune system of fish. In this context, this study revealed that, although the MOS supplementation has no effect on growth, survival and intestinal morphometry, 2 g MOS kg^{-1} supplementation for *B. splendens* larvae hint at a possible immunomodulatory effect, as evidenced by the increase in lysozyme activity.

Keywords: *Betta splendens***,** fish nutrition, immunology, lysozyme, mannan oligosaccharides, ornamental fish farming, prebiotic.

Efectos de manan oligosacáridos en la dieta sobre el crecimiento, supervivencia, morfometría intestinal y respuesta inmune inespecífica en larvas de peces luchadores siameses (*Betta splendens* Regan, 1910)

RESUMEN. Se realizó un experimento de 28 días para evaluar el efecto de la suplementación dietética con el prebiótico manano oligosacáridos (MOS) para larvas de peces luchadores siameses (*Betta splendens* Regan, 1910) sobre el crecimiento, la supervivencia, la morfología intestinal y la respuesta inmune inespecífica. Los niveles de suplementación fueron: 0, 2, 4, y 6 g L^{-1}. Se distribuyeron 240 larvas (4,21 ± 0,26 mm) en 16 acuarios de plástico (3 L) a una densidad de 5 larvas L^{-1}. Todas las larvas recibieron alimento vivo durante siete días, seguido de un período de ocho días de co-alimentación (alimento vivo + dieta inerte) y, finalmente, 13 días de dieta inerte. Al final de la prueba de alimentación, no hubo diferencias significativas en el crecimiento, supervivencia, morfología intestinal y actividad de la fosfatasa alcalina entre las larvas alimentadas con la dieta control y las alimentadas con dietas suplementadas con MOS. Sin embargo, la actividad de la lisozima en las larvas alimentadas con 2 g MOS kg^{-1} fue significativamente mayor en comparación con los otros tratamientos. Para el cultivo de peces, los efectos más beneficiosos de los prebióticos residen en su capacidad para mejorar el sistema inmune de los peces. En este contexto, el presente estudio mostró que, a pesar de la administración del prebiótico MOS no tiene ningún efecto sobre el crecimiento, supervivencia y morfología intestinal, el suplemento con 2 g MOS kg^{-1} para larvas de *B. splendens* sugiere un posible efecto inmunomodulador, como se demuestra por el aumento de actividad de la lisozima.

Palabras clave: *Betta splendens*, nutrición de peces, inmunología, lisozima, manan oligosacáridos, cría de peces ornamentales, prebiótico.

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INTRODUCTION

Ornamental fish farming is a growing activity in developing countries and represents a significant source of income for the fish farmers. Siamese fighting fish (*Betta splendens* Regan, 1910) ranks among the most popular and commercialized ornamental fish in the world due to its beauty and variety of colors, hardiness, ease of reproduction and possibility of being created in small aquariums without aeration because of the air breathing attachment system (Faria *et al*., 2006).

Larviculture is one of the most important production stages of the fish production chain. However, it presents many obstacles, such as initial feeding and high mortality rates. Altricial larvae present little yolk reserve and do not have a functional digestive system fully developed at the onset of exogenous feeding, making them dependent on the live food in their initial feeding (Kolkovski, 2001). According to Fosse *et al*. (2013), *B. splendens* larvae have altricial characteristics dependent on live food in the early stages of exogenous feeding.

Despite its importance in intensive larviculture, the live food use represents a large part of the production costs (Jomori *et al*., 2005). In this context, there is a need to use strategies for reducing or replacing the supply of live food. This reduction can be done abruptly, replacing completely the live food by inert diets, or gradually, starting with a joint feeding period (co-feeding), reducing the live food and gradually increasing the supply of inert diets until it become exclusive. For *B. splendens*, live food can be suppressed after 15 days, with eight days of co-feeding, without affecting the larvae's survival (Fosse *et al*., 2013).

In intensive larviculture, stressful conditions of cultivation may reduce the immune response, facilitate the invasion of opportunistic pathogens and even increase mortality, which cause considerable economic losses and damage the sustainable development (Gómez *et al*., 2007). The antibiotics were used on a large scale as strategies of prevention and treatment of diseases in fish farming. However, the use of antibiotics can result in the development of resistant bacteria, presence of antibiotic residues in meat and destruction of the bacterial population in the cultivated aquatic environment (Marques *et al*., 2005).

Several alternative strategies to antibiotics use have been proposed. One of them and that has generated great interest on the part of researchers is the introduction of prebiotics in fish diets. Prebiotics are non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the intestinal tract, and thus improves host health (Gibson & Roberfroid, 1995). One of the most commonly prebiotics used is mannan oligosaccharide (MOS). In aquaculture, this prebiotic has improved growth, food utilization, survival, nonspecific immune response and disease resistance (Burr *et al*., 2008; Liu *et al*., 2013; Sado *et al*., 2014; Torrecillas *et al*., 2014; Azevedo *et al*., 2015, 2016a). However, few studies have evaluated the MOS supplementation to fish during the larviculture especially for ornamental species.

This study was performed to investigate the effects of a dietary MOS on the growth, survival, intestinal morphometry and nonspecific immune response for *B. splendens* larvae.

MATERIALS AND METHODS

Experimental design and prebiotic

The experiment was conducted in a completely randomized design with four treatments and four replicates, for 28 days. The treatments consisted of different levels of MOS supplementation in inert diets, namely: 0, 2, 4 and 6 g kg^{-1}. As prebiotic, a commercial MOS (ActiveMOS®) derived from the cell wall of yeast *Saccharomyces cerevisiae* was evaluated.

Live food and inert diets

As live food, newly hatched *Artemia franciscana* nauplii were used. The experimental inert diet was prepared from an extruded commercial diet indicated for the initial phases of omnivores and carnivores fish (Table. 1).

The commercial extruded diet was crushed and sieved to present particle size less than 500 µm. For the MOS incorporation, it was diluted in water (10:1 v w^{-1}) and sprayed on the inert diets. After homogenization, the inert diets were oven-dried at 50°C for 24 h, identified and stored in plastic containers kept refrigerated until the beginning of the experiment.

Fish culture and feeding trial

The *B. splendens* larvae were obtained from natural spawning of three pairs. Three days after hatching, be-

Table 1. Commercial extruded diet proximate composition. [a]Value analyzed according to AOAC (2005). [b]Value analyzed by calorimetric bomb (1341 Parr Instrument Company, IL, USA).

Variable	Proximate composition
Dry matter (g kg^{-1})[a]	866.10
Crude protein (g kg^{-1})[a]	421.50
Ether extract (g kg^{-1})[a]	45.85
Crude energy (kcal kg^{-1})[b]	3913.00
Mineral matter (g kg^{-1})[a]	130.30

fore distribution in the experimental units, the larvae were homogenized and a group of 30 larvae were measured (Western® PRO digital caliper) and weighed (Analytical balance AUX 220 Shimadzu, Kyoto, Japan), obtaining initial values of 4.21 ± 0.26 mm and 0.37 ± 0.02 mg respectively. After the initial biometric measurements, two hundred and forty larvae were individually counted and distributed into 16 plastic aquaria with useful volume of 3 L, in a stocking density of 5 larvae L^{-1} of water.

Treatments were investigated under static aerated water conditions. The experimental units were siphoned daily at 14:00 h, renewing two-thirds of the total volume of water. To keep the water's temperature in the proper values heaters were used and the photoperiod was kept in 12 h. Water temperature, dissolved oxygen (AT-170, Alfakit Ltda, Florianópolis, SC, Brazil), pH (mPA 210P, MS TECNOPON, Piracicaba, SP, Brazil) and ammonia (HI83203, Hanna Instruments Inc., Rhode Island, USA) maintained at, respectively, 26.90 ± 1.33ºC, 4.89 ± 0.09 mg L^{-1}; 6.89 ± 0.24, and 0.02 ± 0.01 mg L^{-1}.

Fish mortality was recorded before each feeding and during the cleaning of the experimental units. Feeding protocol was adapted from Fosse *et al*. (2013). All larvae received live food for seven days, followed by a period of eight days of co-feeding (live food + inert diet) and, finally, 13 days of inert diet, resulting in 21 days of MOS supplementation.

It was adopted a food frequency both for live food as inert diet, four times a day at 8:00, 11:00, 13:00 and 17:00 h. The amount of live food provided was of 75 nauplii larva day^{-1} during the first seven days. From the eighth day 300 nauplii larva day^{-1} were offered. During the co-feeding period, primarily, inert diet was provided and, later, after two minutes, the live food. The amount of live food has been reduced gradually over the last three days of co-feeding to 75%, 50% and 25% of the total. The larvae were hand-fed with inert diets to apparent satiation.

Sampling

At the end of the experiment, larvae were subjected to fasting for 12 h. Twelve larvae per treatment were euthanized, and from this total, six larvae were fixed in 10% neutral-buffered formalin for 48 h and then transferred into 70º GL ethanol until histological examination and six larvae were frozen and held at -20°C for alkaline phosphatase (AKP) and lysozyme (LYZ) activities.

Growth performance and survival

To evaluate the growth performance and survival, larvae were analyzed as final length (FL) and final weight (FW). From final biometric data the specific growth rate (SGR) [(ln final length - In initial length)/trial period x 100] and survival rate (SUR) [(dead individuals/initial number of individuals) x 100] were calculated.

Intestinal morphometry

Fish larvae were embedded in paraffin, sliced transversely into 5 µm sections and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (E200, Nikon, Tokyo, Japan) equipped with a camera for image capture. Intestinal villus height (VH) and width (VW) were measured with an analysis software package Infinity Analyze® according Azevedo *et al*. (2016b).

AKP and LYZ activities in larvae homogenates

To analyze the AKP and LYZ activities, the larvae are pooled, macerated and homogenized using phosphate-buffered saline (0.5 N and pH 6.2), centrifuged at 12000 *g* for 7 min and the supernatants were used. AKP activity was obtained by Bowers and McComb method modified, using laboratorial kit (Labtest Diagnostica, Minas Gerais, and Brazil) and reading in microplate reader Multiskan GO®. LYZ activity was determined by turbidimetric assay based on lysis of Gram-positive *Micrococcus lysodeikticus* bacteria according to Ellis (1990). For this analysis, 25 µL of supernatant were added to 250 µL of solution (0.2 mg mL^{-1} in Na_2HPO_4 0.05 M, pH 6.2) of freeze-dried *M. lysodeikticus* (Sigma, St. Louis, MO, USA) in a 96 microplate wells. The reduction of optical density in 450 µm was evaluated between 0.5 and 10.0 min at 26°C in microplate reader Multiskan GO®. An activity unit of lysozyme (U mL^{-1}) was defined as the amount of enzyme that caused a 0.001 decrease in optical density per minute.

Statistical analysis

Date on growth, survival, intestinal morphometry, AKP and LYZ activities are presented as means ± SD. The SAS® software version 9.0 was utilized to conduct one-way ANOVA. When there were significantly differences, the Tukey test was applied. The data expressed as percentage were transformed using the formula y = arcsine $\sqrt{x}$ for later evaluation. The *P*-value was fixed at 0.05.

RESULTS

At the end of feeding-trial, there were no significant differences between growth parameters and survival rate of larvae fed the control or MOS supplemented diets ($P > 0.05$) (Table. 2).

Table 2. Growth and survival of Siamese fighting fish (*Betta splendens* Regan, 1910) larvae fed different levels of MOS supplementation. FW: final weight, FL final length, SGR: specific growth rate, SUR: survival rate.

Variable	MOS (g kg^{-1})				*P*-value
	0	2	4	6	
FW (mg)	35.97 ± 1.84	35.25 ± 0.79	36.98 ± 1.27	36.96 ± 1.23	0.3035
FL (mm)	11.99 ± 0.61	11.75 ± 0.26	12.33 ± 0.42	12.32 ± 0.41	0.3035
SGR (% day^{-1})	4.99 ± 0.24	4.89 ± 0.11	5.12 ± 0.17	5.12 ± 0.16	0.2992
SUR (%)	80.00 ± 10.00	80.00 ± 8.16	75.00 ± 12.91	92.50 ± 9.57	0.1792

No significant difference was observed in VH (P = 0.0987), VW (P = 0.3321) (Fig. 1), and AKP activity (P = 0.6461) (Fig. 2) of control group and MOS supplemented fed larvae.

LYZ activity in larvae fed 2 g MOS kg^{-1} was significantly (P = 0.0001) higher compared to other treatments (Fig. 3).

DISCUSSION

The results of this study showed that MOS supplementation did not change the growth and survival of *B. splendens* larvae. Similarly, MOS supplementation did not improve the growth and survival in larvae of cobia *Rachycentron canadum* (Salze *et al*., 2008), white sea bream *Diplodus sargus* (Dimitroglou *et al*., 2010), Nile tilapia *Oreochromis niloticus* (Schwarz *et al*., 2011) and *Trichogaster leeri* (Azevedo *et al*., 2016b). However, in contrast to these results, there was growth improvement of turbot *Psetta maxima* larvae fed diets supplemented with oligofructose (Mahious *et al*., 2006) and in the survival of common carp *Cyprinus carpio* larvae fed diets supplemented with short-chain fructooligosaccharides (Hoseinifar *et al*., 2015). The disparities in the results could be explained due to the different products evaluated, methods, time and level of supplementation, cultivation conditions and differences in fish's morphology and intestinal microbiota.

In larviculture of ornamental fish, one of the most important factors is the survival at the end of this phase. Considering that the experimental conditions were appropriate (nutrition, density and management) for the larvae survival, the benefits of MOS supplementation may not have been evidenced on this variable. Future experiments with major periods and stressful situations are necessary to evaluate the effect of MOS supplementation on this parameter.

The structural knowledge of intestinal mucosa can provide important information for studies on fish nutrition. A greater value at the VH suggests a better integrity of the intestinal mucosa, allowing its best development and, consequently, greater efficiency in the absorption process. In this study, there was no influence of MOS supplementation on the intestinal morphometry. A similar result was obtained with juve-

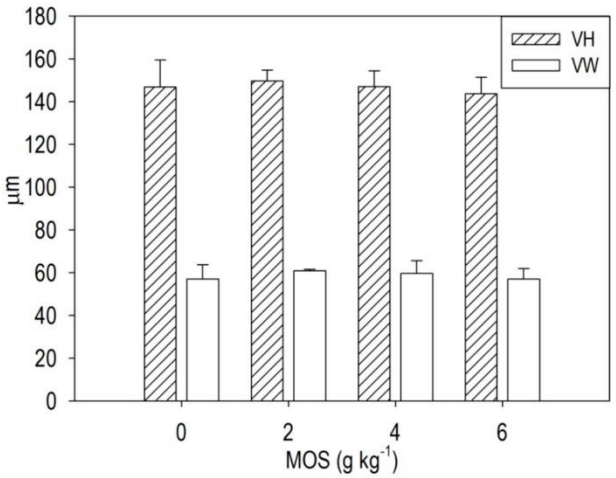

Figure 1. Villus height (VH) and villus width (VW) of Siamese fighting fish (*Betta splendens* Regan, 1910) larvae fed different levels of mannan oligosaccharide (MOS).

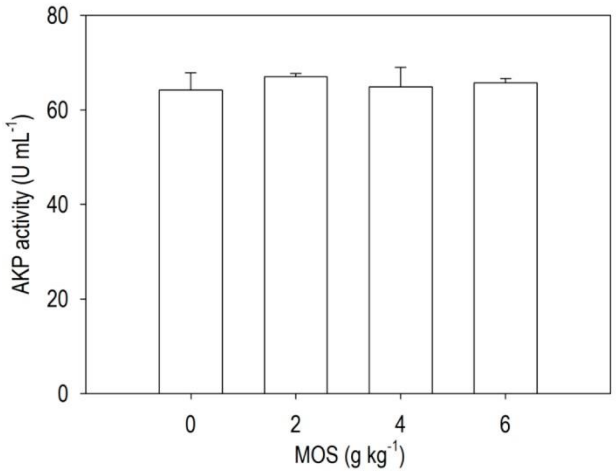

Figure 2. Alkaline phosphatase (AKP) activity of Siamese fighting fish (*Betta splendens* Regan, 1910) larvae fed different levels of mannan oligosaccharide (MOS).

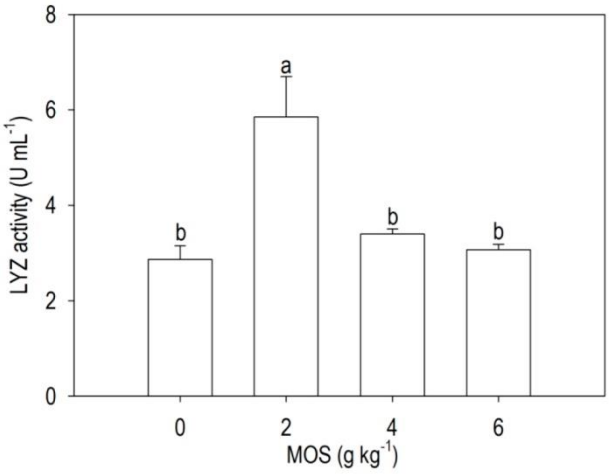

Figure 3. Lysozyme (LYZ) activity of Siamese fighting fish (*Betta splendens* Regan, 1910) larvae fed different levels of mannan oligosaccharide (MOS). Bars assigned with different superscripts are significantly different (P = 0.0001).

niles of European sea bass *Dicentrarchus labrax* (Torrecillas *et al*., 2007) fed MOS. On the other hand, some authors observed positive influence of MOS supplementation on intestinal morphometry in fish larvae (Salze *et al*., 2008; Dimitroglou *et al*., 2010; Schwarz *et al*., 2011; Azevedo *et al*., 2016b).

AKP is an important enzyme that regulates a number of functions in living organisms (Liu *et al*., 2013). In this study, there was no effect of MOS supplementation on the activity of this enzyme. A similar result was obtained for juveniles of red drum *Sciaenops ocellatus* and hybrid striped bass *Morone chrysops* x *M. saxatilis* fed diets MOS supplemented (Anguiano *et al*., 2013). However, MOS supplementation increased the AKP activity in juveniles of gilthead sea bream *Sparus aurata* (Suzer *et al*., 2008) and gibel carp *Carassius auratus gibelio* (Liu *et al*., 2013).

AKP is expressed by mature and active enterocytes (Harpaz & Uni, 1999). Wold *et al*. (2007) reported that the increase of the overall length in the intestinal epithelium of Atlantic cod *Gadus morhua* larvae was positively related to the increase in the AKP activity. Thus, the increase of the AKP activity may indicate increased functionality of enterocytes and, therefore, improve growth. This way, the effect's absence of the MOS supplementation on the AKP activity, together with the intestinal morphometry, can explain partly the absence of effect on the growth of larvae in this study.

One of the most common benefits in the application of immunostimulants for fish is the health promotion through the stimulation of nonspecific immune system and disease resistance (Dagá *et al*., 2013). The results of this study showed that 2 g MOS kg^{-1} supplementation for *B. splendens* larvae increase the LYZ activity. Several authors have reported an increase in the LYZ activity in juvenile fish that received MOS supplemented diets (Staykov *et al*., 2007; Buentello *et al*., 2010; Ye *et al*., 2011; Liu *et al*., 2013). The mechanism of action of MOS as immune modulator probably is based in the activation of pattern recognition receptors and proteins, which trigger the innate immune system in response to a non-self-substance (Torrecillas *et al*., 2014).

LYZ is a lytic enzyme and, therefore, defends the body against pathogenic agents. The increase in LYZ activity suggests elevation of humoral factors that can protect the host against the invasion of pathogens (Harikrishnan *et al*., 2011). LYZ activity in fish can be modulated by various species of lactic acid bacteria (Panigrahi *et al*., 2004; Kim & Austin, 2006; Akrami *et al*., 2013), that, besides the contribution to digestion, can increase mucus production, which is the first line of defense against parasites and bacterial infections through its enzymatic system, where the lysozyme is present (Salinas *et al*., 2008). Once the MOS can be a selective substrate for this group of beneficial bacteria (Gibson & Roberfroid, 1995; Salze *et al*., 2008), the major enzymatic activities, observed in the group supplemented with 2 g MOS kg^{-1}, can be the result of the manipulation in intestinal microbiota.

For fish farming, the most beneficial effects of prebiotics reside in its ability to enhance the immune system of fish (Dimitroglou *et al*., 2011). In this context, this study showed that 2 g MOS kg^{-1} supplementation for *B. splendens* larvae hint at a possible immunomodulatory effect, as evidenced by the increase in LYZ activity. However, additional studies are required to assess the effects of MOS on the immune response of *B. spendens*.

CONCLUSIONS

In conclusion, this study revealed that, although the MOS supplementation has no effect on growth, survival and intestinal morphometry, 2 g MOS kg^{-1} supplementation for *B. splendens* larvae hint at a possible immunomodulatory effect, as evidenced by the increase in LYZ activity.

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