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Probiotic supplementation in diet and vaccination of hybrid surubim (*Pseudoplatystoma reticulatum*♀ x *P. corruscans*♂)

Suplementação de probiótico na dieta e vacinação de surubim híbrido (*Pseudoplatystoma reticulatum*♀ x *P. corruscans*♂)

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ABSTRACT

*A supplementary diet with the probiotic bacteria **Weissella cibaria** on the efficacy of surubim hybrid immunization against a specific hemorrhagic septicemia caused by **Aeromonas hydrophila** was evaluated on the following treatments: fish fed a supplemented probiotic diet, vaccinated fish and vaccinated fish fed a supplemented probiotic diet, and untreated fish (control). Fish from the probiotic treatments were fed a diet containing **W. cibaria** for 41 days. On the 15th day of the experiment, fish from vaccine treatments were intraperitoneally vaccinated, with posterior oral booster for four days. One week after the oral booster, three fish from each experimental unit were sampled. The probiotic supplementation increased the number of thrombocytes and lysozyme concentration compared with surubim that did not receive **W. cibaria** in the diet. On the other hand, the vaccination increased agglutination titer, lysozyme concentration, and antimicrobial activity compared with surubim that were not vaccinated. However, there was no interaction between diet with probiotics and vaccination in the surubim hybrid in the analysed parameters.*

Key words: *fish, vaccine, lactic acid bacteria, **Aeromonas hydrophila**.*

RESUMO

*A dieta suplementada com a bactéria probiótica **Weissella cibaria** sobre a eficácia da imunização de surubim híbrido contra a septicemia hemorrágica específica, causada por **Aeromonas hydrophila**, foi avaliada nos seguintes tratamentos: peixes alimentados com a dieta suplementada com probiótico, peixes vacinados, peixe vacinadas que receberam um probiótico na dieta e peixes não tratados (controle). Peixes dos tratamentos probióticos foram alimentados com uma dieta contendo **W. cibaria** por 41 dias. No 15º dia do experimento, os peixes dos tratamentos de vacinas foram vacinados intraperitonealmente, com posterior reforço oral por quatro dias. Uma semana após o reforço oral, três peixes de cada unidade experimental foram amostrados. A suplementação de probiótico aumentou o número de trombócitos e concentração lisozima em comparação com surubins que não receberam **W. cibaria** na dieta. Por outro lado, a vacinação influenciou no aumento do título de aglutinação, a concentração de lisozima e a atividade antimicrobiana, em comparação com os surubins que não foram vacinados. No entanto, não houve interação entre a dieta com probióticos e vacinação no híbrido surubim nos parâmetros analisados.*

Palavras-chave: *peixes, vacina, bactérias ácido-láticas, **Aeromonas hydrophila**.*

INTRODUCTION

In surubins (***Pseudoplatystoma corruscans*** Spix and Agassiz 1829 and ***P. reticulatum*** Eigenmann and Eigenmann 1889) culture farms outbreaks of bacterial diseases are frequent during the winter because of the temperature change during the day. The clinical signs of septicemia caused by ***Aeromonas hydrophila*** were described previously by CAMPOS (2004) and further, strains of ***A. hydrophila*** were isolated and characterized by SILVA et al. (2012) as the main surubim hybrid pathogen. Aiming bacterial control, many producers use antibiotics in fish farming, however the use of these antibiotics leads to the selection of resistant bacteria (SASAN et al., 2011). To avoid antibiotic using, prophylactic methods such

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as vaccine and probiotics have been developed and have been shown to be promising tools for fighting fish bacterial diseases (MOURIÑO et al., 2012).

Probiotic bacteria are widely used in aquaculture in different species (MOURIÑO et al., 2012). They are well known as promote fish health and enhance survival (JATOBÁ et al., 2011), improve immunological parameters (JATOBÁ et al., 2012) and protect fish against possible pathogenic bacteria infection (BALCÁZAR et al., 2007). Among the different methods of vaccine preparation, the inactivated vaccines by heat or formalin are mostly used (MAGNADOTTIR, 2010). These vaccines can contain only inactivated cells (bacterin) or inactivated extracellular products as well (toxoids) which are secreted by the bacteria (DA COSTA et al., 2008). The intraperitoneal injection is one of vaccine's administration routes that provide the best results, although it is required high labour costs. This method of administration can be improved by using a booster (second vaccine application) to enhance vaccine durability, antibody production, and immune memory cells (CHENG et al., 2010). The oral vaccine is a good way to provide the booster, because it is practical, low cost, and viable in culture farms (SILVA et al., 2009).

Evaluating the influence of probiotic feeding on the intraperitoneal vaccination effectiveness in fish is important in order to assess possible interactions between vaccine and probiotics in improving fish health. Thus, the present study aimed to evaluate dietary supplementation with the probiotic ***W. cibaria*** on the efficacy of the hybrid surubim immunization against hemorrhagic septicemia caused by ***A. hydrophila*** based on immunological and hematological parameters.

MATERIAL AND METHODS

A total of 192 healthy hybrid surubim (***P. corruscans*** x ***P. reticulatum***) were obtained from the Piraí farm located in Mato Grosso do Sul state, Brazil. The pathogenic hemolytic strain ***A. hydrophila*** (CPQBA 228-08 DRM) was isolated during mortality in Mato Grosso do Sul state by SILVA et al (2012) and the probiotic strain ***W. cibaria*** (CPQBA 001-10 DRM 02) was isolated from healthy and asymptomatic hybrid sorubins (MOURIÑO et al., 2012).

For vaccine preparation, the ***A. hydrophila*** strain was grown in the same condictions and following the same methodology used by SILVA et al. (2012). The vaccination was by intraperitoneal rote (i.p.) injection utilizing an insulin syringe (1mL) with a needle (13x0.45mm Injex®).

For oral booster, the same vaccine formulation and concentration ($2x10^8$ CFU mL^{-1}) was used as described above. Thus, 100mL of vaccine was sprayed using a sterile plastic spray bottle for each kilogram of commercial diet or the diet containing probiotic following methodology previously described by (PEREIRA et al., 2015).

The probiotic bacteria ***W. cibaria*** was grown in vials tubes containing MRS broth (Difco®), and incubated at 35°C for 48h. After that, 100mL of the probiotic at concentration of $1x10^8$ CFU mL^{-1} was sprayed (using a sterile plastic spray bottle) on each kilogram of the commercial diet. The diet was dried at 30°C for 12h. This process was repeated every 15 days to achieve a high probiotic concentration in the diet.

To ascertain the ***W. cibaria*** concentration in the diet, 1g of the diet with probiotic was macerated in a sterile mortar with 1mL of saline sterile solution (NaCl 0.65%) and then serially diluted nine times in test tubes at 1:10 factor. The dilutions 10^{-5} to 10^{-9} were plated in petri plates containing MRS agar. The plates were incubated at 35°C for 48h. This process was repeated every time the diet with probiotic was prepared. The probiotic concentration in diet was 5.53×10^6 CFU g^{-1}.

Fish with an initial weight of 44.35±3.07g (mean ± standard deviation), were distributed in 24 tanks with 100L capacity (8 fish per tank) and fed commercial diet (Supra Aqualine) four times a day, approximately 3% of fish biomass. The water quality parameters were monitored daily. When necessary, the water was exchanged from 20% to 60% of the system. The tanks were maintained in a closed-water recirculating system with mechanical and biological filters under constant heat at 28°C and UV sterilization and photoperiod was 24h of darkness according (PIAIA et al., 1999).

The treatments were as follows: (1) fish fed a supplemented probiotic diet; (2) vaccinated fish; (3) vaccinated fish fed a supplemented probiotic diet; and (4) untreated fish (control). There were six repeatitions per each treatment. After the acclimation period, the fish from the groups 1 and 3 received the commercial diet with the probiotic. The others two groups received the same commercial feed without probiotic. The feed was maintained for 41 days, sufficient time for probiotic ***W. cibaria*** colonization (MOURIÑO et al., 2012). After 15 days from the beginning of the experiment, the fish from groups 2 and 3 were vaccinated against ***A. hydrophila*** with 0.01mL of vaccine per gram of fish. Fifteen days after the first vaccination, the oral booster began and lasted four consecutive days. The diet containing

vaccine was also provided four times a day (3% of the biomass). The vaccination protocol and sampling was carried out according PEREIRA et al. (2015).

A week after the end of the oral booster, three fish from each experimental unit were sampled. They were anaesthetized with benzocaine (0.1g L^{-1}), and the blood was collected by puncture of the caudal vessel in 3mL syringes (21G) containing 10% anticoagulant (EDTA), according to the ethic committee of Universidade Federal de Santa Catarina. A small amount of blood was used for the haematological analysis, and the other part was separated for phagocytosis analysis. A pool of blood was made from the remainder of the blood from each experimental unit to obtain the plasma by centrifugation.

The collected blood was used to make the blood smears stained with Giemsa/ May Grunwald (ROSENFELD, 1947) for differential leukocyte counts, as well as to obtain the total leukocytes and thrombocytes number. An aliquot was used to determine the haematocrit (GOLDENFARB et al., 1971) and the remainder stored in glass vials on ice to quantify the total number of erythrocytes (RBC) by Neubauer chamber. The total numbers of thrombocytes and leukocytes (WBC), as well as differential leukocytes was obtained from blood smears by the indirect method (ISHIKAWA et al., 2008).

The vials tubes containing the blood pool (from three fish) from each experimental unit were centrifuged at 1400g for 10 min to obtain blood plasma and were stored at -20°C. The protein in the blood plasma was measured using the Total Protein kit according to the manufacturer's instructions (Lab Test®, Lagoa Santa, MG, Brazil). The total immunoglobulin concentration was measured after infection (AMAR et al., 2000).

The lysozyme activity of the blood plasma was determined using the methodology adapted by SANKARAN AND GURNANI (1972), The initial and final absorbance of the samples were measured in a microplate reader (Expert Plus Asys®) at 492nm and the rate of reduction in absorbance of the samples was converted to lysozyme concentration (µg mL^{-1}).

The title serum agglutination as well as the blood serum antimicrobial activity were prepared according methodology described by SILVA et al. (2009). To perform the agglutination activity the bacteria ***Aeromonas hydrophila*** (CPQBA 228 08) was used. The antimicrobial activity was performed using the same strain of ***A. hydrophila*** as (Gram negative) mentioned before, further the strain of ***Enterococcus durans*** (Gram positive ATCC 19492).

To determine the percentage of leukocyte phagocytosis, 0.5mL of blood and 0.25mL of a suspension of $1x10^6$CFU mL^{-1} formalin-inactivated ***A. hydrophila*** were added in centrifuge tubes, which were kept at 28°C for 30 min and homogenized every 10 min. After hatching, the blood was seeded in blood smears, in duplicate, and the slides were stained with Giemsa/ May Grunwald (ROSENFELD, 1947). The number of phagocytic leukocytes was counted by percentage to the total number of leukocytes in the smear analysis (MARTINS et al., 2004).

Data were submitted for normality and the Bartlett test was performed to check the homogeneity of variance. The agglutination results were converted to log2. Data that did not have homogeneous variances were transformed into log10 (x+1) and subsequently subjected to a 2x2 factorial analysis of the variance supplemented by the Tukey test for mean separation. All analyses were subjected to a significance level of 5%.

RESULTS AND DISCUSSION

The water quality parameters including, dissolved oxygen (8.06±0.56mg L^{-1}), total ammonia (1.5±0.5mg L^{-1}), nitrite (0.67±0.65mg L^{-1}), nitrate (1.96±1.44mg L^{-1}), pH (7.83±0.39) and temperature (27.5±2.46°C) were within the fish culture limit (BOYD, 1990). Fish mortality was verified throughout the trial period. The survival at the final of the experiment was: 95.84% fish fed a supplemented probiotic diet; 97.92% vaccinated fish; and 100% vaccinated fish fed a supplemented probiotic diet; and 93.75 untreated fish (control).

There were no interaction between the factors vaccination and probiotic feeding on the haematological parameters. The lymphocyte number in blood from the two vaccinated groups was lower when compared to unvaccinated fish (Table 1). Comparing with other studies, intraperitoneal vaccination in Nile tilapia (***O. niloticus***) led to an increase in lymphocyte number, after 7 and 21 days post-vaccination, and also after 21 days when the fish were orally vaccinated (SILVA et al., 2009). In this case, the decrease of lymphocytes could be due to their migration to the tissues as a response to the vaccination.

The supplemented probiotic diet increased thrombocyte number in the circulating blood of the hybrid surubim (Table 1). This result is in agreement with those obtained by JATOBÁ et al. (2011) where an increase in thrombocyte number in ***O. niloticus*** fed with lactic acid bacteria ***L. plantarum*** was reported. In vaccinated fish, it is expected that the white blood cells counts would be altered. However, in this case, the supplementation of probiotic could not improve the cells count. Future works should be performed to evaluate the haematological parameters, in the

Table 1 - Hematological parameters (mean and standard error) of hybrid surubim (***Pseudoplatystoma reticulatum***♀, Spix and Agassiz x ***P. corruscans***♂, Eigenmann and Eigenmann) fed diet containing probiotic (Probiotic); vaccinated against ***A. hydrophila*** and fed diet containing probiotic (Probiotic + vaccine) and vaccinated fish (Vaccine) against ***A. hydrophila***; and untreated fish (Control), after 41 days of treatment.

Treatments	RBC ($x10^6$ mL^{-1})	Thrombocytes ($x10^3$ mL^{-1})	WBC ($x10^3$ mL^{-1})	Lynphocytes ($x10^3$ mL^{-1})	Monocytes ($x10^3$ mL^{-1})
Control	1.64 ± 0.07	36.9 ±1.21[a]	79.5 ±5.95	63.9 ±5.23[b]	1.91± 0.03
Probiotic	1.85 ± 0.02	44.1 ±4.68[b]	83.0 ±8.08	81.8 ±5.08[b]	1.32 ± 0.01
Vaccine	1.66 ± 0.03	23.8 ±4.31[a]	83.4 ±4.50	60.1 ±5.33[a]	1.30 ± 0.02
Probiotic + vaccine	1.67 ± 0.06	46.4 ±5.04[b]	86.4 ±4.75	57.3 ±8.14[a]	1.52 ± 0.03
P of probiotic factor	0.068446	0.001678	0.595889	0.229583	0.550917
P of vaccine fator	0.170268	0.203834	0.547952	0.030445	0.525978
P of interaction	0.077059	0.074783	0.972808	0.103493	0.213258
	Eosinophils ($x10^3$ mL^{-1})	Basophils ($x10^3$ mL^{-1})	Neutrophils ($x10^3$ mL^{-1})	LG - PAS ($x10^3$ mL^{-1})	Hematocrit (%)
Control	0.67 ± 0.23	21.8 ± 0.35	3.02 ± 0.81	0.00 ± 0.00	24.9 ± 1.40
Probiotic	0.70 ± 0.29	15.7 ± 0.30	3.07 ± 0.55	0.00 ± 0.00	26.0 ± 0.80
Vaccine	0.29 ± 0.13	22.9 ± 0.50	2.81 ± 0.61	0.07 ± 0.07	23.3 ± 0.57
Probiotic + vaccine	0.41 ± 0.10	23.9 ± 0.57	3.77 ± 0.70	0.00 ± 0.00	24.3 ± 0.82
P of probiotic factor	0.729999	0.570656	0.468069	0.329257	0.281385
P of vaccine fator	0.124594	0.310845	0.719312	0.329257	0.101812
P of interaction	0.824305	0.437895	0.515520	0.329257	0.914649

[*]Different letters indicate significant difference by Tukey test (P<0.05).

same conditions, however after a bacterial challenge. It is important to notice that the probiotic and the vaccine had influence in different cells groups. As well as the haematological parameters, the use of probiotic and vaccine did not presented interaction in the fish immunology in the present work, however we can see some differences when analysed them separately. Fish fed the supplemented probiotic diet and vaccinated fish had a higher plasma lysozyme concentration (Table 2), corroborating the study of MERRIFIELD et al. (2010) in ***O. mykiss***. The present study shows that both, supplemented diet and vaccinated fish had a higher plasmatic lysozyme than untreated fish. It was expected that vaccinated fish would present an increase in the concentration of total immunoglobulins in plasma, because these defence proteins are activated after immunization, as observed by MIKKELSEN et al. (2011) in ***Gadus morhua***, L. vaccinated against ***Vibrio anguillarum***. However, the increase in immunoglobulins observed in those studies was after infection, thereby enhancing the immune response and consequently the concentration of total and specific immunoglobulins.

The plasma antimicrobial activity of the surubim showed significant difference between treatments against ***A. hydrophila*** (Table 2). According to SILVA et al. (2013) the serum antimicrobial activity of intraperitoneally vaccinated hybrid surubins was higher than that in fish vaccinated by bath or unvaccinated surubim. In fact, these results have shown that vaccination increases the antimicrobial capacity of fish serum, furthermore against the bacterium ***A. hydrophila***, which the vaccine was previously prepared.

Moreover, the agglutination titer was higher in both vaccinated fish groups (Table 2). In the study of SILVA et al. (2013), the serum agglutination titer in vaccinated hybrid surubim by intraperitoneal way, before challenge with ***A. hydrophila***, was also higher than unvaccinated fish. Those results indicate that vaccination induced plasmatic agglutination in fish and enhanced the protection against possible subsequent infections, compared with unvaccinated fish or fish fed a supplemented probiotic diet.

Besides the fact that no interaction was observed in both haematological and immunological parameters, it can be attested that the probiotic and the vaccination helped the fish protection against pathogens however in different parameters. This remark can highlight a positive result, such as the probiotic and the vaccination indeed worked together in different and specific parts of the immune system in the fish, so they complement each other because they increase separate parameters.

Table 2 - Plasmatic immunological parameters: lysozyme, total protein, total immunoglobulin (mean ± standard error), percentage of phagocytic leukocytes, agglutination titer and antimicrobial activity in hybrid surubim (***Pseudoplatystoma reticulatum***♀, Spix and Agassiz x ***P. corruscans***♂, Eigenmann and Eigenmann) fed diet containing probiotic (Probiotic); vaccinated against ***A. hydrophila*** and fed diet containing probiotic (Probiotic + vaccine) and vaccinated fish (Vaccine) against ***A. hydrophila***; and untreated fish (Control), after 41 days of treatment.

Treatments	Lysozyme (μg mL^{-1})	Total protein (mg mL^{-1})	Total imunoglobulin (mg mL^{-1})	Phagocytosis (%)
Control	10.32 ± 0.39[a]	23.64 ± 0.65	0.96 ± 0.39	7.22 ± 0.72
Probiotic	10.71 ± 0.61[b]	24.60 ± 0.59	1.61 ± 0.50	9.75 ± 1.16
Vaccine	10.79 ± 0.47[b]	24.04 ±0.59	1.38 ± 0.34	10.92 ± 1.01
Probiotic + vaccine	12.93 ± 0.26[b]	23.83 ±0.53	1.05 ± 0.30	9.75 ± 1.44
P of probiotic factor	0.018765	0.256662	0.674756	0.558108
P of vaccine factor	0.024562	0.827398	0.853697	0.117067
P of interaction	0.092330	0.655884	0.230215	0.117067
	Agglutination titer	---------- Antimicrobial activity ----------		
	A. hydrophila	***A. hydrophila***	***E. durans***	
Control	7.33 ± 0.21[a]	10.40 ± 0.39[a]	9.80 ± 0.00	
Probiotic	8.17 ± 0.30[a]	10.80 ± 0.19[a]	10.00 ± 0.24	
Vaccine	9.50 ± 0.42[b]	12.20 ± 0.67[b]	10.20 ± 0.58	
Probiotic + vaccine	9.66 ± 0.21[b]	11.60 ± 0.73[b]	9.40 ± 0.37	
P of probiotic factor	0.114263	0.857213	0.425920	
P of vaccine factor	0.000006	0.030488	0.789424	
P of interaction	0.283987	0.374978	0.192460	

*Different letters indicate significant difference by Tukey test (P<0.05).

The ***W. cibaria*** supplementation in diet had no interaction with vaccination in the hybrid surubim against ***A. hydrophila***; however, probiotic and vaccination promoted a response of different immunological and hematological parameters in these fish.

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BIOETHICS AND BIOSSECURITY COMMITTE APPROVAL

This experiment was approved by the Ethics Committee on the use of animals (number 178 / CEUA / PRPE / 2011) at the Universidade Federal de Santa Catarina (UFSC).

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