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Probiotic feeding improves the immunity of pacus, *Piaractus mesopotamicus*, during *Aeromonas hydrophila* infection

Thaís Heloísa Vaz Farias[a,b], Nycolas Levy-Pereira[a], Lindomar de Oliveira Alves[a], Danielle de Carla Dias[c], Leonardo Tachibana[c], Fabiana Pilarski[a], Marco Antonio de Andrade Belo[b,d,*], Maria José Tavares Ranzani-Paiva[c]

[a] *Aquaculture Center of São Paulo State University (CAUNESP), Rodovia Paulo Donato Castellane, s/n, CEP 14884-900 Jaboticabal, SP, Brazil*
[b] *Department of Preventive Veterinary Medicine, São Paulo State University, Rodovia Paulo Donato Castellane, s/n, CEP 14884-900 Jaboticabal, SP, Brazil*
[c] *Fishery Institute (APTA-SAA), Av. Francisco Matarazzo, 455, CEP 05001-970, São Paulo, SP, Brazil*
[d] *College of Veterinary Medicine, Camilo Castelo Branco University, Av. Hilário da Silva Passos, 950, CEP 13690-000 Descalvado, SP, Brazil*

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ABSTRACT

The utilization of probiotic bacteria have been widely tested and applied in intestinal microflora modulation, through competitive exclusion of the pathogenic bacteria. *Aeromonas hydrophila* is a gram-negative bacterium, responsible for fish outbreaks in farms around the world, and is one of the major loss causes for neotropical fish farmers. 660 pacus (67 ± 7 g) were distributed in 20 tanks ($n = 33$), constituting five groups (four tanks for each treatment): four groups were fed with different levels (2, 4, 8 and 16 g kg^{-1}) of *Bacillus cereus* and *Bacillus subtilis* (1:1, 10^8 CFU g^{-1}), and the fifth group was fed with a control diet (without probiotic). Pacus fed with probiotic showed increment in the ROS production associated to elevated neutrophil and monocyte counts and increased phagocytic activity without affecting the growth parameters. Probiotic fed fish presented higher survival rates, subjected to an i.p. challenge with 10^8 CFU mL^{-1} of *A. hydrophila*. The results demonstrated a dose response effect and the ideal level of the probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}) in *P. mesopotamicus* diets was around 8 g kg^{-1}, as the highest survival rates and immunological responses were found in groups of fish fed with this diet. In addition, too much probiotic should be avoided, as pacus fed diet with 16 g kg^{-1} showed partial suppression of these responses.

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1. Introduction

Aeromonas hydrophila is a gram negative, motile, rod-shaped bacterium, widely known as an opportunist pathogen of farmed fish, with worldwide distribution. In rearing systems, in which occurs stressful conditions, this bacterial agent is responsible for disease outbreaks, as described in east Asia (Xia et al., 2004; Crumlish et al., 2010), Europe (Boran et al.,

* Corresponding author at: Department of Preventive Veterinary Medicine, São Paulo State University, Rodovia Paulo Donato Castellane, s/n, CEP 14884-900 Jaboticabal, SP, Brazil. Tel.: +55 016 99961 7299.
E-mail address: maabelo@hotmail.com (M.A.d.A. Belo).

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2013), middle East (Ahmed and Shoreit, 2001; Sarkar et al., 2012), North America (Pridgeon et al., 2013; Li et al., 2013) and South America (Reque et al., 2010; Silva et al., 2012), being responsible for considerable economic losses in intensive rearing systems.

Also known as hemorrhagic septicemia, the motile *A. hydrophila* infection is characterized by ulcerative lesions on the skin surface, local hemorrhages in gills and opercula, exophthalmia, liquid accumulation in the coelomic cavity, anemia and liver and kidney lesions (Crumlish et al., 2010; Silva et al., 2012). Aeromoniosis is one of the main diseases in farmed pacu (*Piaractus mesopotamicus*), a neotropical freshwater species of great importance in Brazilian finfish aquaculture, and this teleost fish has demonstrated to be a good bioindicator of water quality and has been used as model for ecotoxicity studies during the process of chemical registrations in Brazil (Castro et al., 2014a). Among the chemicals available for bacterial disease treatment, oxytetracycline and florfenicol are most frequently used in aquaculture (Rigos and Troisi, 2005), and, in some countries, they are the only drugs allowed for fish treatment. The lack of control in antibiotic use, especially for prevention, can trigger undesirable effects in fish, terrestrial animals and environment, which could threaten human health (Cabello, 2006). The use of immunostimulants, as dietary supplements, can improve the innate defense of fish which provide resistance to pathogens, such as vitamins (Belo et al., 2012, 2014), minerals (Castro et al., 2014b, 2014c), essential fatty acids (Sakabe et al., 2013) and yeast *Saccharomyces cerevisiae* (Reque et al., 2010; Castro et al., 2014b). In this context, many studies regarding probiotic-use in farmed animals have reported improvements not only on the productivity indexes in fish, but also in promoting the welfare and maintenance of host health, especially through gut microbiota modulation, inhibition of pathogen colonization in the intestinal epithelium and stimulation of the innate immune system (Yanbo and Zirong, 2006; Zhou et al., 2009; Ai et al., 2011), when used at the appropriate levels.

In aquaculture, the inclusion of exogenous *Bacillus* in fish diets have shown a positive overall effect, improving the productive parameters and immune responses and increasing the resistance to pathogens (Raida et al., 2003; Salinas et al., 2005; Merrifield et al., 2010; Nayak, 2010; Sun et al., 2010; He et al., 2011). In this context, the present study aimed to evaluate the effect of diets containing 2, 4, 8 and 16 g kg^{-1} of probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}) on productivity performance, innate immune parameters and resistance to experimental infection by *A. hydrophila* in pacus, *P. mesopotamicus*.

2. Materials and methods

2.1. Fish and feeding

P. mesopotamicus juveniles, from a commercial fish farm (Águas Claras, Mococa-SP), were maintained for 15 days in 500 L plastic tanks and fed with basal diet (commercial diet) in order to allow them to acclimate to experimental conditions. 660 fish (67 ± 7 g) were distributed in 20 tanks ($n = 33$; four tanks for each treatment) with continuous water flow (~16 L min^{-1}) and artificial aeration. No differences were observed among the water quality parameters throughout the trial period (temperature: 26.6 °C, pH: 7.9 ± 0.09, dissolved oxygen: 6.7 ± 0.59 mg L^{-1} and ammonia: 0.12 ± 0.11 mg L^{-1}).

Five experimental diets were prepared: one without probiotic (control diet) and four with different levels (2, 4, 8 and 16 g kg^{-1}) of the commercial probiotic PAS-TR™ (*Bacillus cereus* and *Bacillus subtilis* in a 1:1 proportion, lyophilized at a concentration of 10^8 CFU g^{-1}). Count of colony forming units of *Bacillus cereus* and *Bacillus subtilis* incorporated in *Piaractus mesopotamicus* diets was performed before the start of the experiment and the reisolation results showed 1.6×10^1, 1×10^5, 7×10^5, 4.2×10^6 and 1.2×10^7 CFU g^{-1} for diets supplemented with 0 (control), 2, 4, 8 and 16 g kg^{-1} of the probiotic, respectively. Every week during the feeding period, the probiotic was incorporated in the pellets via soybean oil (20 g kg^{-1} of diet), mixed manually and the diets were stored at 4 °C (refrigerated). The control diet was made with the same commercial feed (32% crude protein, 7% crude fiber and 6.5% fat, Pirá 32® – Guabi Company), also mixed with soybean oil, but without probiotic. The fish were fed a quantity equal to 3% of their body mass, twice a day, at 9:00 and 17:00, for 60 days.

The protocol for the animal experiment was approved by Ethics Committee on the Use of Animals of São Paulo State University under the protocol n°22.518/10.

2.2. Blood samples and NBT assay

During the feeding period (at days 7, 15, 30 and 60), ten fish per treatment were anesthetized in a benzocaine solution (100 mg L^{-1}) for blood collection from the caudal vein, afterwards the fish were euthanized by deepening anesthesia. White blood cell (WBC) counts were made using a hemocytometer with Natt and Herrick solution. Blood smears for differential leukocyte counts were stained with a combination of May-Grünwald Giemsa and Wright's Method (Belo et al., 2013). For nitro blue tetrazolium (NBT) assay, 0.5 mL of blood was transferred to 2 mL plastic tubes containing 15 μL of heparin (5.000 IU mL^{-1}). The respiratory burst of leukocytes was measured according to Biller-Takahashi et al. (2013). Briefly, 100 μL of heparinized blood was mixed with 100 μL of an NBT-buffered solution (Sigma, St. Louis, MO, USA). The solution was homogenized and incubated in a dark room for 30 min at 25 °C. After the incubation, 50 μL of the solution was added in 5 mL tubes containing 1 mL *n*,*n*-dimethyl-formamide, (DMF, Sigma, St. Louis, MO, USA) and centrifuged at 3000 g for 5 min. The optical density of the supernatant was measured using a spectrophotometer with a wave length of 540 nm.

2.3. Phagocytic parameters

At the end of the feeding period, 6 fish per treatment (captured randomly in 4 tanks) were used to determine phagocytic activity. One milliliter of a solution containing baker's yeast (*Sacharomyces cerevisiae*) at concentration of 8.000 cells mm^{-3} was inoculated in the coelomic cavity of juvenile pacu. After 2 h (time period determined in previous assay), the fish were anesthetized in benzocaine solution (1 g L^{-1}) and euthanized by spinal transection (cervical portion). Through a ventral incision, the inoculation region was washed with 1 mL of saline solution (1%). The resulting solution containing phagocytic cells was aspirated with a Pasteur pipet (3 mL) and centrifuged at 250 g for 5 min in 5 mL plastic tubes. The supernatant was dispensed, and the resting cells (in the bottom of the tube) were placed between a glass slide and coverslip to observe the phagocyte count using a contrast phase microscope (400 X). The Phagocytic Index (PI = number of phagocytized particles/number of phagocytizing leukocytes) and Phagocytic Rate (PR = number of phagocytizing leukocytes/100 leukocytes) were calculated, according to Silva et al. (2005).

2.4. Bacterial challenge

The *Aeromonas hydrophila* strain used in this assay was acquired from the Laboratory of Aquatic Organisms Pathology (LAPOA) strain collection, of the Aquaculture Center of UNESP (CAUNESP, Jaboticabal, SP, Brazil). The strain was isolated from a hemorrhagic septicemia-diagnosed fish into differential medium for the selective isolation of motile aeromonads (R-S agar) according to Shotts and Rimler (1973). Using an LC_{50} assay, the concentration required to kill 50% of fish was 1.0×10^8 CFU mL^{-1}. The bacteria were stored at −70 °C until the day of inoculation. An aliquot of *A. hydrophila* was thawed and inoculated in TSB broth at 25 °C for 48 h. The bacteria were subsequently centrifuged and washed three times in PBS solution, and the concentration was adjusted to 1.0×10^{-8} CFU.

After 60 days of probiotic feeding, 20 fish of each treatment (five per tank) were anesthetized in aqueous solution of benzocaine (1:10,000), and 1 mL of the solution containing the bacteria was inoculated in each fish's coelomic cavity. Mortality and clinical signs were observed every 12 h during the 15 days after the challenge. The diagnosis of *A. hydrophila* infection was made by the isolation of these bacteria in pure culture of the head kidney and by clinical signs observations. Survival rate was calculated in accordance with the following equation:

$$\text{Survival(Sur)} = \left[1 - \frac{\text{number of dead fish}}{20}\right] \times 100$$

2.5. Statistical analysis

The phagocytosis data was analyzed using MIXED procedure (SAS, 2008) and the analysis of variance for comparing the different experimental groups were estimated on the basis of Tukey–Kramer test ($p < 0.05$), and were tested the linear and quadratic effects by orthogonal polynomial contrasts. Survival data was analyzed by LIFETEST procedure (SAS, 2008). For this analysis the data was transformed into LOGIT in COFTYPE option, and the comparison between treatments was performed using the Wilcoxon test and the interaction treatments vs. time was used to HAZARDRATIO option in PHREG procedure. Correlations between respiratory burst activity values and absolute number of neutrophil plus monocyte counts in the blood were made using Spearman Test (GraphPad Prism® software, version 5.0), since the assumption of data normality was previously tested, but both parameters did not pass in the Kolmogorov–Smirnov normality test.

3. Results

3.1. Productivity parameters

After 60 days of the feeding trial, probiotic inclusion in juvenile *P. mesopotamicus* diets had no effect on overall weight gain, daily weight gain, feed conversion ratio, specific growth rate and consumption when compared with the control group. No mortality was observed during the experimental period (before the bacterial challenge) (Table 1).

3.2. NBT assay and leukocyte counts

Positive correlation ($p = 0.0002$) was observed between respiratory burst activity values and absolute number of neutrophil plus monocyte counts in the 168 blood samples (Table 2), demonstrating the increase of ROS production associated to high number of these leucocytes. Fish fed diet without probiotic (0 g kg^{-1}) or supplemented with 16 g kg^{-1} did not show statistical significance for this correlation (Table 2), while pacu fed with 2 and 8 g kg^{-1} of probiotic showed 70.08 and 31.15% of positive correlation between both parameters. Although fish fed 4 g kg^{-1} has positive correlation of 25.77%, these findings were not statistically significant (Table 2).

Table 1
Production parameters of *Piaractus mesopotamicus* after 60 days on diets containing different levels of probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}).

Productivity parameters[a]	Probiotic (g kg^{-1})					SE[b]	CV[b]	p[b]
	0	2	4	8	16			
W_i (g)	64.81	64.01	72.48	66.51	68.16	3.97	11.83	0.5942
W_f (g)	140.10	136.10	131.09	134.15	133.06	6.30	9.35	0.8772
WG (g)	75.28	72.06	58.61	67.64	64.90	4.59	13.58	0.1494
DWG (g day^{-1})	1.25	1.19	0.98	1.13	1.07	0.07	13.57	0.1494
DC (g day^{-1})	2.50	2.52	2.57	2.38	2.57	0.05	3.95	0.1222
FCR	2.00	2.09	2.78	2.13	2.40	0.21	18.23	0.1021
SGR (g day^{-1})	1.28	1.25	0.97	1.17	1.12	0.07	12.44	0.0591

[a] W_i is the weight (initial); W_f is the weight (final); WG is the weight gain (WG = $W_f - W_i$); DWG is the daily weight gain (DWG = WG/t; where t = time in days); DC is the daily consumption; FCR is the feed conversion ratio (FCR = feed intake/weight gain); SGR is the specific growth rate {SGR = [(ln W_f − ln W_i)/t] *100, where t = time in days, ln = natural logarithm}.
[b] SE is the standard error; CV is the coefficient of variation; p is the probability of significance.

Table 2
Correlation analysis between respiratory burst activity values and absolute number of neutrophil plus monocyte counts in the blood of pacus fed diets with different levels of probiotic.

Correlated parameters[a]	Experimental sampling[b]	Correlation analysis	
		ρ[c]	Prob > $\lvert\rho\rvert$[c]
Leukocytes × Burst	All animals	0.2848	0.0002
	All treated animals	0.3434	<0.0001
	0 g kg^{-1}	0.01905	0.9135
	2 g kg^{-1}	0.7008	<0.0001
	4 g kg^{-1}	0.2571	0.0771
	8 g kg^{-1}	0.3115	0.0474
	16 g kg^{-1}	−0.01009	0.9570

[a] Leukocytes = absolute number of monocyte plus neutrophil counts in the blood; Respiratory burst activity (NBT assays).
[b] Correlation between all animals (n = 168); only animals fed with probiotics (n = 133); pacus fed 0 g kg^{-1} (n = 35); 2 g kg^{-1} (n = 32); 4 g kg^{-1} (n = 29); 8 g kg^{-1} (n = 41); 16 g kg^{-1} (n = 31).
[c] ρ is the coefficient of Spearman correlation; Prob. > $\lvert\rho\rvert$ is the significance probability of ρ value.

3.3. Phagocytic activity

Phagocytic index increase was observed in treated fish (Fig. 1), where the highest value was expressed by the fish that were fed with 8 g kg^{-1}. A dose–response effect was observed in pacus fed up to 8 g kg^{-1} of probiotic (Fig. 1). Fish fed diet without probiotic (0 g kg^{-1}) presented significant (p = 0.0036) decrease in the phagocytic index only when compared to pacu fed with 8 g kg^{-1} (Fig. 1). Increase in the phagocytic rate (Fig. 2) was observed in fish fed with 4, 8 or 16 g kg^{-1} of the probiotic, although with no statistical differences in relation to the control group. The 2 g kg^{-1} group presented statistically lower values when compared to other treated fish.

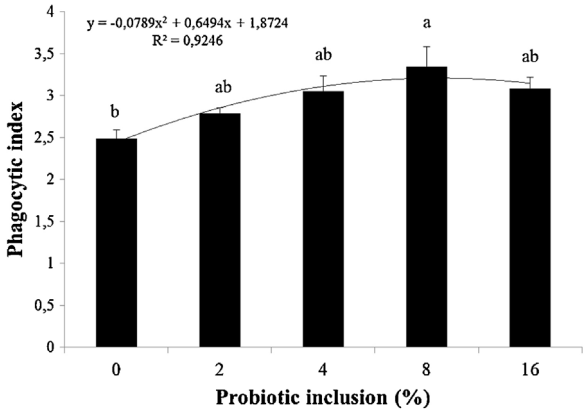

Fig. 1. Means values (n = 6, ±SE) for phagocytic index of *Piaractus mesopotamicus* fed with different levels (0, 2, 4, 8 and 16 g kg^{-1}) of probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}) for 60 days (p = 0.0036, quadratic effect by orthogonal polynomial contrasts).

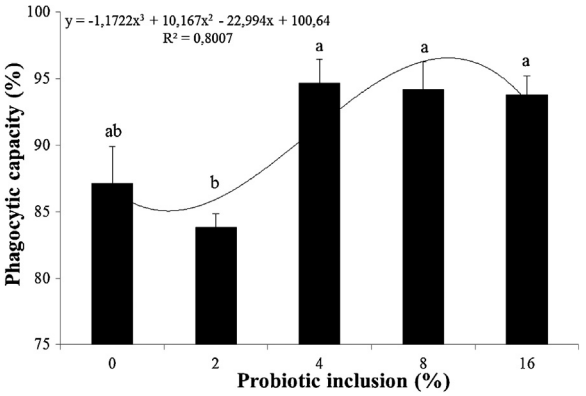

Fig. 2. Means values ($n = 6$, ±SE) for phagocytic capacity of *Piaractus mesopotamicus* fed with different levels (0, 2, 4, 8 and 16 g kg^{-1}) of probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}) for 60 days ($p = 0.0008$, quadratic effect by orthogonal polynomial contrasts).

3.4. Bacterial challenge

All treated groups on diets containing the probiotic showed higher survival rates compared to the control group when challenged with *A. hydrophila* (Fig. 3). Clinical signs such as hemorrhagic petechiae on skin and fins, ascites, and isolation from the rest of the group started to appear 24 h post-challenge, and the first death occurred after 48 h post-challenge. After the sixth day, stabilization of the disease was observed, with no additional mortality. The experimental infection by *A. hydrophila* was confirmed through reisolation of the bacteria from the head kidney of the dying fish.

4. Discussion

There was no influence of probiotic inclusion on pacu productivity parameters after 60 days of feeding. However, some studies have shown positive effects of the supplementation of fish feeds with *Bacillus* sp. (Aly et al., 2008; Son et al., 2009; Dias et al., 2012). This may be due to an improvement in the digestive activity by vitamin synthesis and bacterial enzyme liberation, promoting better absorption/utilization of nutrients (Gatesoupe, 1999). Experimental conditions, mainly dissolved oxygen amount, temperature, pH and environmental microflora, seem to be the main reasons for the discrepancies in the literature regarding probiotic inclusion in fish feeds, influencing the establishment, proliferation and function of probiotic bacteria in the host intestinal tract (Das et al., 2008; Mehrim, 2009). In addition, the results of Apun-Molina et al. (2009) and Ridha and Azad (2012) indicate that, in order to promote fish growth, there is a necessity of a period longer than 60 days of probiotic feeding.

Probiotics feeding increased reactive oxygen species (ROS) production associated to elevated neutrophil and monocyte counts. However, fish fed with 16 g kg^{-1} of probiotics showed no significant correlation between ROS production and leukocyte counts. Respiratory burst is the rapid release of ROS and plays an important role in the immune system (Castro et al., 2014b). It is a crucial reaction which occurs in phagocytes to degrade internalized particles and bacteria (John et al., 2002) and according to Gimbo et al. (2015), the nitroblue tetrazolium (NBT) assay is indicative of oxidative radical production from neutrophils and monocytes for use in defense against pathogens. Some studies have shown ROS production increases in fish

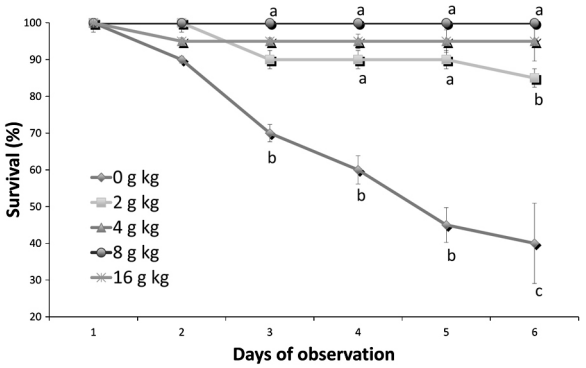

Fig. 3. Means values ($n = 20$, ±SE) of the survival percentual of *Piaractus mesopotamicus* challenged with *Aeromonas hydrophila* strain after 60 days on diets containing different levels (0, 2, 4, 8 and 16 g kg^{-1}) of probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}). Values followed by the same letter do not differ by the Tukey–Kramer test ($p > 0.05$), and lowercase letters compare the different treatments within each experimental day.

on dieting different levels of the probiotic *Bacillus*, which was also affected by the time of administration (Panigrahi et al., 2004, 2005; Newaj-Fyzul et al., 2007; Giri et al., 2012).

The baker's yeast (*S. cerevisiae*) inoculum stimulated an inflammatory response in the coelomic cavity of pacus. Granulocytes as neutrophils and macrophages derived from monocytes are phagocytes involved in the internalization and destruction of pathogens and particles (such as yeasts) in the site of inflammation (Claudiano et al., 2013; Belo et al., 2014). The inclusion of *Bacillus cereus* and *Bacillus subtilis* (1:1, 10^8 CFU g^{-1}) in *P. mesopotamicus* diets increased the phagocytic index, only the 8 g kg^{-1} treated fish showed a statistically higher value when compared to the control group, but the regression analysis showed a dose–response effect between probiotic level of inclusion and number of phagocytized particles (yeasts). The inclusion of PAS-TR™ in *P. mesopotamicus* diets increased the phagocytic capacity (with the exception of the 2 g kg^{-1} group), although with no statistical difference compared to the control group. Some studies also showed improvements in the phagocytic activity of other fish species such as *Oreochromis niloticus* (Aly et al., 2008), *Labeo rohita* (Kumar et al., 2008), *Solea senegalenses* (Diaz-Rosales et al., 2009), *Brycon amazonicus* (Dias et al., 2012) on diets containing the probiotic *Bacillus*.

Improvements on immune status and higher resistance to *A. hydrophila* in *P. mesopotamicus* fed with different inclusion levels of *B. cereus* and *B. subtilis* in diets were observed. The manipulation of the intestinal microbiota by the inclusion of beneficial bacteria in aquatic organism diets have been noted as a viable alternative to antibiotic use as a prophylactic to disease outbreaks. Studies with the probiotic bacteria of group *Bacillus* have shown a positive overall effect, efficiently increasing host resistance to infectious agents through modulation of the immune system (Kumar et al., 2008), as observed in this study.

The probiotic inclusion in fish feed seems to be very effective in increasing the resistance of juvenile *P. mesopotamicus* to experimental infection with *A. hydrophila*. Several authors have seen a positive overall effect following the administration of probiotics as enhancers of fish resistance to pathogenic bacteria (Brunt and Austin, 2005; Vendrell et al., 2008; Sharifuzzaman and Austin, 2009; Gildberg et al., 1995; Vijayabaskar and Somasundaram, 2008). We hypothesize that the enhancement in fish defenses against this pathogenic bacterium comes from the systemic immunostimulation caused by the interaction between the probiotic *Bacillus* and the host's intestinal innate immune system.

5. Conclusion

The results demonstrated a dose response effect and the ideal level of the probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}) in *P. mesopotamicus* diets was around 8 g kg^{-1}, as the highest survival rates and immunological responses were found in groups of fish fed with this diet. In addition, too much probiotic should be avoided, as pacus fed diet with 16 g kg^{-1} showed partial suppression of these responses.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical approval

The experiment design was approved by Ethics Committee on the Use of Animals of São Paulo State University under the protocol n°22.518/10.

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