Common snook fed in alternate and continuous regimens with diet supplemented with *Bacillus subtilis* probiotic

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Abstract – The objective of this work was to evaluate the addition of *Bacillus subtilis* probiotic to the feed of common snook (*Centropomus undecimalis*) fingerlings, in alternate and continuous regimens. Six hundred and sixty fish, with average length of 5.90±0.88 cm and weight of 1.92±0.28 g, were stocked in 12 cages of 1.0 m^3, with 55 fish each. The experimental design was completely randomized, with three treatments and four replicates. The treatments consisted of diet with the addition of probiotic, provided in alternate regimen for 7 days and in continuous regimen; besides a control without probiotic in the feed. Zootechnical performance, body composition, immune response, and blood parameters were evaluated. No significant differences were observed in zootechnical performance indexes and in body composition of fish treated with probiotic, when compared to the control. Fish from the alternate regimen showed an increment in respiratory burst and a lower total erythrocyte count than fish from the continuous regimen and the control. Fish from the continuous regimen did not differ from those of the control. The addition of *Bacillus subtilis* does not increase growth rates of common snook fingerlings; however, it has an immunostimulant action when supplied in alternate regimen.

Index terms: *Bacillus subtilis*, *Centropomus undecimalis*, hematology, immunostimulant, marine aquaculture, respiratory burst.

Robalo-flecha alimentado em regimes alternado e contínuo com dieta suplementada com o probiótico *Bacillus subtilis*

Resumo – O objetivo deste trabalho foi avaliar a adição de *Bacillus subtilis*, como probiótico, na ração de juvenis de robalo-flecha (*Centropomus undecimalis*), em regimes de alimentação alternado e contínuo. Foram estocados 660 peixes com comprimento médio de 5,90±0,88 cm e peso médio de 1,92±0,28 g, em 12 tanques-rede de 1,0 m^3, com 55 peixes cada um. Utilizou-se o delineamento experimental inteiramente casualizado, com três tratamentos e quatro repetições. Os tratamentos consistiram de ração adicionada de probiótico, fornecida em regime alternado de 7 dias e em regime contínuo; além de controle sem probiótico na ração. Avaliaram-se: desempenho zootécnico, composição corporal, resposta imune e parâmetros hematológicos. Não houve diferença significativa nos índices zootécnicos e na composição corporal dos peixes tratados com probiótico, em comparação ao controle. Os peixes do regime alternado apresentaram incremento na explosão respiratória e menor contagem total de eritrócitos do que os do regime contínuo e do controle. Os peixes do regime contínuo não diferiram dos do controle. A adição de *Bacillus subtilis* não promove o crescimento de juvenis de rebalo-peva; no entanto, tem ação imunoestimulante quando fornecido em regime alternado.

Termos para indexação: *Bacillus subtilis*, *Centropomus undecimalis*, hematologia, imunoestimulante, piscicultura marinha, explosão respiratória.

Introduction

Probiotics may be defined as live microbial preparations that promote enhancements in the health and well-being of the hosts (Gatesoupe, 1999; Verschuere et al., 2000; Schrezenmeir & Vrese, 2001). The use of probiotics has been reported in aquaculture (Balcàzar et al., 2007; Wang, 2007; Aly et al., 2008; Dias et al., 2012) and in marine fish farming (Carnevali et al., 2006; Son et al., 2009; Barbosa et al., 2011), showing their efficacy as growth promoters, immune stimulants, and bioremediators in water quality. According to Jatobá et al. (2008), probiotics can be a viable alternative to decrease the chemicals used in aquaculture.

Bacillus subtilis has been tested as a probiotic in fish culture. In in vitro studies, Aly et al. (2008)

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demonstrated that it inhibits the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*; stimulates the immunity of Nile tilapia (*Oreochromis niloticus*), when added to the feed; and is effective in promoting the growth of treated animals, in comparison to the controls. Dias et al. (2012) observed an increase in the reproductive ability and in the phagocytic activity of matrinxã (*Brycon amazonicus*), when *B. subtilis* was added to the diet at a concentration of 10^{10} colony-forming units (CFU) per kg.

When analyzing microbial balance in common snook (*Centropomus undecimalis*) larvae, Kennedy et al. (1998) found that the exclusion of certain vibrio populations resulted in better survival rates, besides increasing the immunological capacity of the animals treated with a strain of *B. subtilis* isolated from the fish itself, when compared with untreated fish (control).

Appropriate parameters for probiotic regimens, including routes of administration, posology, and period of treatment must to be evaluated, in order to determine the ideal strategies for particular species and farming conditions (Nayak, 2010). According to Merrifield et al. (2010), there are only two viable ways of administration: via water or food. In addition, these authors suggest three possible probiotic regimens: short-term, only when needed; alternating short periods with and without the probiotic; and continuous probiotic exposure.

The snook (*Centropomus* spp.) is the most valued fish in the market of the state of São Paulo, Brazil (Companhia de Entrepostos e Armazéns Gerais do Estado de São Paulo, 2013); however, the depletion of its natural stocks has been reported in the estuary of Cananéia-Iguape, by Mendonça & Katsuragawa (2001). Currently, in Southeast Brazil, there is a tendency to preserve the fish in their natural environment, with capture restrictions and protected seasons, and to develop new technologies that support their rearing in captivity.

The objective of this work was to evaluate the addition of *Bacillus subtilis* probiotic to the feed of common snook fingerlings, in alternate and continuous regimens.

Materials and Methods

The experiment was performed in the estuary of Cananéia-Iguape, in the municipality of Ilha Comprida, in the state of São Paulo, Brazil (24°53'20"S, 47°48'01"W). The region encompasses a large area of preserved mangroves and is influenced by marine and continental waters, mainly from the Ribeira de Iguape River, as well as other watercourses. Because it is an estuary region, there is great variation in water salinity, turbidity, and in tidal speed.

The trial was conducted in a completely randomized design, with three treatments and four replicates. Twelve cages with 1.3-m^3volume each, measuring 1.0x1.0x1.3 m, with 5.0x5.0-mm mesh were used. The cages were installed in three floating structures measuring 2.7x3.0 m, with four cages each. The cages were placed in a sheltered bay to avoid interferences in the experiment. The experimental period was of 191 days, from July to December 2012.

The common snook fingerlings were obtained from the Laboratory of Marine Acquaculture (Lapmar) of Universidade Federal de Santa Catarina, located in the municipality of Florianópolis, in the state of Santa Catarina, Brazil. Fish were transported in polyethylene bags filled with properly-oxygenated water. A total of 660 fish were acclimated to the experimental site for 45 days before trial conduction. Fifty-five fish with average length of 5.90±0.88 cm and average weight of 1.92±0.28 g were stocked in each cage and fed twice per day, until apparent satiety.

Diets were prepared with commercial feed (powder), previously moisturized with water in 2:1 feed/water ratio. Feed nutritional composition, in g kg^{-1}, was: 120 humidity (max.); 450 total protein (min.); 85 total fat (min.); 45 total fiber (max.); 140 minerals (max.); 15 calcium (min.); 38 calcium (max.); and 10 phosphorus (min.). This mixture was then pelleted and dried up to 12% humidity; pulverized into 1.0, 2.0, or 4.0-mm particles (feed pellet size was increased according to fish growth, during the experiment); and, finally, sprayed with 2% soybean oil. The prepared feed was used as a control diet. The probiotic diet was prepared similarly to the diet described above, but was sprayed with soybean oil containing lyophilized *B. subtilis* (C-3102) at a concentration of $5.0x10^9$ CFU per kg of feed. These diets were prepared once a month and kept under refrigeration at 3–7ºC, as recommended by Aly et al. (2008).

The evaluated treatments were: control diet without probiotics, provided continuosly; probiotic diet, provided alternately with the control diet during

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7 days, in alternate regimen (T1); and probiotic diet exclusively, provided during the entire 191 days of the experiment, in continuous regimen (T2).

The alternate regimen adopted in T1 was determined based on data obtained in a previous study performed under laboratory conditions with fat snook (*Centropomus parallelus*) fingerlings. During the continuous probiotic diet, the phagocytic activity in fish increased during the initial 7 days but decreased afterwards. Therefore, the weekly alternation of diets was adopted due to this higher phagocytic activity in *C. parallelus* fingerlings. The phagocytic activity was determined according to Silva et al. (2002, 2005).

After being prepared and stored for 31 days, 100-g samples of the feed were sent to the Laboratory of Uniquímica-Ltda., in the municipality of São Paulo, in the state of São Paulo, Brazil, to check *B. subtilis* content. Samples were homogenized with sterile saline solution (1:1 ratio) and placed in a 65ºC water bath for 35 min. After a cool-down period, the samples were serially diluted four times (500 µL in 100 mL), inoculated in typical soy agar (TSA) medium, and incubated at 37ºC for 24 hours. Morphological checking of *B. subtilis* colonies was performed, and the positively identified ones were counted. Microbiological analyses showed *B. subtilis* probiotic concentration of 5×10^9 CFU kg^{-1} in the feed, indicating bacterial viability for conservation at 3–7°C; the presence of *B. subtilis* was not observed in the control feed, as expected.

The values of total length, total weight, and survival rate, S = (final fish count × 100)/initial fish count, were measured during the initial, intermediate (87 days), and final (191 days) biometries. The values of apparent feed conversion, AFC = feed consumption/weight gain, were obtained from feeding data, average weight gain, WG = final weight - initial weight, and survival (S) from each of the replicates. Fulton’s condition factor (K) (Le Cren, 1951) and specific growth rate, SGR = ((ln final weight - ln initial weight)/time) × 100, were also calculated.

After 191 days, two fish from each replicate were euthanized via deep sedation in 100 mg L^{-1} benzocaine solution (Coyle et al., 2004), followed by medullar sectioning. The fish were then tagged, frozen, and sent to the Food Technology Institute, located in the state of São Paulo, Brazil, for analysis of body composition. Samples from each treatment were ground and homogenized for determination of: humidity at 105ºC until weight was stable, ether extract (Soxhlet), total protein content (Kjeldahl N × 6.25), and ash at 500ºC, according to Williams (1984).

At the end of the experimental period, another five fish from each treatment were euthanized with benzocaine solution (Coyle et al., 2004), and blood was collected from the caudal vein, with heparinized syringes and needles. The blood was immediately analyzed for: red blood cell count (RBC), performed with a Neubauer chamber; hematocrit (Ht), using the hematocrit method; and hemoglobin (Hb), via the cyanmethemoglobin method. A blood smear was prepared and stained by the May-Grünwald-Giemsa method, according to Rosenfeld (1947), to perform the counts of differential and total leukocytes and of platelets, as in Hrubec & Smith (2000).

To determine respiratory burst, after blood collection, cephalic kidneys were extracted, macerated with the plunger of a syringe against a 50-µm mesh, and diluted in RPMI-1640 culture medium, with 20% bovine serum, 0.5% glutamine, and antibiotics. The cellular suspension obtained was transferred to 10-mL Falcon tubes, with 50 µL heparin, and the cell concentration was adjusted to 10^7 phagocytes per µL of culture medium, using a Neubauer chamber. This medium was incubated for 2 hours in 96-well plates (400 µL each), sampled in duplicate. After the incubation period, the supernatant was discarded, each sample received 100 µL RPMI medium, and the supernatant was discarded again. Then, each well received 100 µL nitroblue tetrazolium (NBT), phorbol myristate acetate (PMA), and RPMI medium; subsequently, the plate was incubated for another hour to perform phagocytosis of NBT. The supernatant was once more discarded, and the wells were rinsed twice with 100 µL phosphate-buffered saline (PBS). Then, 100 µL methanol (70%) were added to each well for macrophage lysis and consequent release of the formazan granules. To solubilize the precipitate, 120 µL KOH 2 mol L^{-1} and 140 µL dimethyl sulfoxide (DMSO) were added to each well. The plate was then placed in a spectrophotometer, and the absorbance (A) at 630 nm was measured.

Data were subjected to analysis of variance (Anova), and means were compared by the Tukey test, at 5% probability, using the SAS software (Cary, NC, USA). All data are presented as averages±standard deviation.

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Results and Discussion

Feed supplemented with *B. subtilis* probiotic bacteria did not significantly alter the zootechnical growth index in treated and untreated common snook (Table 1), possibly due to the fact that the used bacilli strain does not stimulate the digestion and absorption of nutrients in this fish species. Barbosa et al. (2011) also did not observe significant differences in the zootechnical index values after feeding fat snook with diet supplemented with *Lactobacillus plantarum* isolate of the Nile tilapia digestive tract.

Kennedy et al. (1998) reported a higher survival rate for common snook larvae in water containing *B. subtilis* at a concentration of 10^9 CFU mL^{-1}. Moreover, Carnevali et al. (2006) found that European sea bass (*Dicentrarchus labrax*) presented an average increase of 81% in weight after 59 days of treatment with *Lactobacillus delbrueckii* probiotic. These authors supplemented the feed with probiotic bacteria directly isolated from the digestive tract of European sea bass. The adopted procedures suggest that probiotic strains isolated from the same species could improve the zootechnical index in saltwater fish, since the bacteria were acclimated to the digestive tract of the host and could perform their functions as a probiotic more efficiently.

No significant differences were observed in the corporal index among groups (Table 2), although the fish from the alternate regimen have apparently shown lower accumulation of muscle and body fat. Barbosa et al. (2011) also did not find any improvement in the body composition of fat snook fed with L. *plantarum*.

Table 1. Growth parameters of common snook (*Centropomus undecimalis*) fed diets with and without (control) *Bacillus subtilis* probiotic, in alternate and continuous regimen, during 191 days[(1)].

Parameter	Control	Alternate	Continuous
Total length (cm)	9.7±0.9	9.4±0.9	10.1±0.7
Total weight (g)	7.9±1.1	6.5±1.4	8.7±1.5
Weight gain (g)	6.0±1.1	4.6±1.4	6.8±1.5
Apparent feed conversion	15.4±3.8	19.6±5.26	12.6±1.77
Specific growth rate (%)	0.73±0.7	0.63±0.1	0.78±0.1
Fulton factor[(2)]	0.87±0.1	0.78±0.1	0.83±0.1
Survival (%)	68.6±11.3	71.36±12.8	70.90±18.8

[(1)]No significant differences were observed among treatments by analysis of variance, at 5% of probability. [(2)]Fulton factor (Le Cren, 1951).

The values of respiratory burst, expressed as optical density (630 nm), were 0.470, 0.997, and 0.417, respectively, for control, alternate regimen, and continuous regimen, showing a higher oxygen consumption in the alternate regimen during the phagocytosis process. The alternate probiotic regimen improved the immune system of the fish, consequently yielding a higher protection against natural pathogens (Bricknell & Dalmo, 2005). Geng et al. (2011) also reported an increase in the respiratory burst in cobia (*Rachycentron canadum*) that daily received a diet supplemented with a mix of chitosan and *B. subtilis.* However, Díaz-Rosales et al. (2006) and Cerezuela et al. (2012) did not observe significant differences in the respiratory burst of sea bream (*Sparus aurata*) fed with probiotics.

The continuous feeding of probiotics did not increase the immune system activity of common snook, since the values of respiratory burst in fish from this group were similar to those of the control. This data is in agreement with those of Bricknell & Dalmo (2005), who observed that continuous exposure to elevated concentrations or continued exposure to probiotics may fail in stimulating the fish's immune system due to the resistance induced by the host. Merrifield et al. (2010) reported that the continuous use of immunostimulant substances could decrease the activity of the immune system and, in some cases, even trigger immunosuppression.

Among the analyzed blood parameters, treatments did not alter the total amount of hemoglobin or leukocyte and thrombocyte counts (Table 3). Barbosa et al. (2011) found higher rates of leukocytes, thrombocytes, and lymphocytes in fish treated with probiotics, when compared to the control, contrary to

Table 2. Whole body chemical composition of common snook (*Centropomus undecimalis*) fed diets with and without (control) *Bacillus subtilis* probiotic, in alternate and continuous regimen, during 191 days[(1)].

Treatment	Moisture	Proteins	Ash	Ether extract
	(%)			
Control	75.24	18.48	4.48	1.07
Alternate	75.46	17.43	5.41	0.73
Continuous	76.15	18.14	4.72	0.92

[(1)]No significant differences were observed among treatments by analysis of variance, at 5% of probability. n = 8.

Table 3. Hematological parameters of common snook (*Centropomus undecimalis*) fed diets with and without (control) *Bacillus subtilis* probiotic, in alternate and continuous regimen, during 191 days[(1)].

Parameters	Control	Alternate	Continuous
Red blood cells (10^6 µL^{-1})	4.19±0.33a	3.5±0.52b	4.76±0.6a
Hemoglobin (g dL^{-1})	5.99±0.99	5.18±0.33	5.16±0.48
Leukocytes (10^3 µL^{-1})	24.96±27.1	24.07±23.63	23.13±7.37
Lymphocytes (10^3 µL^{-1})	6.57±7.62	9.31±14.50	9.74±2.89
Neutrophils (10^3 µL^{-1})	12.49±1.23	9.33±7.73	10.63±7.11
Monocytes (10^3 µL^{-1})	5.89±7.97	5.23±6.77	2.66±1.48
Thrombocytes (10^3 µL^{-1})	41.99±24.1	32.28±12.04	1.37±18.07

[(1)]Means followed by equal letters do not differ significantly by the Tukey test, at 5% probability. n = 5.

the data obtained in the present study. However, fish from the alternate regimen presented a significantly lower amount of erythrocytes (RBC) when compared to those of the control and the continuous regimen. Contrasting with these results, animals exposed to probiotics generally present higher RBC and leucocyte counts, due to the immunological capacity of probiotics (Talpur et al., 2014). In the present study, the resistance induced by the continuous probiotic supply may not have interfered with the production of RBC in fish from the continuous regime, as opposed to the observed in the fish from the alternate regime, which presented anemia.

Conclusions

1. Adding *Bacillus subtilis* to the feed of common snook (*Centropomus undecimalis*) fingerlings stimulates the immune system in the alternate regimen.
2. The addition of *B. subtilis* does not improve the growth rate of the fish, independently of feed regimen.

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