Acta Scientiarum

http://www.periodicos.uem.br/ojs/
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v42i1.52473

Probiotic supplementation causes hematological changes and improves non-specific immunity in *Brycon amazonicus*

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ABSTRACT. A commercial probiotic containing *Bacillus subtilis* (109 CFU g^{-1}) was evaluated in caged matrinxã, *Brycon amazonicus*, by measuring hematological parameters and macrophage activity after 42 and 84 days after feeding. The product was added to commercial feed using 2% soybean oil as a protectant. A randomized three-treatment experiment was performed using four replicates per treatment. The groups included: (a) control without probiotic, (b) 5 g kg^{-1} probiotic, and (c) 10 g kg^{-1} probiotic. For hematological analysis, eight fish per treatment were used to determine total cell count (RBC); thrombocytes, differential, and total leukocyte count (TLC); hematocrit (Htc); hemoglobin tax; mean corpuscular volume (MCV); and mean corpuscular hemoglobin concentration (MCHC). Furthermore, plasma cortisol and glucose levels were measured in blood samples. Macrophage phagocytic activity was evaluated by injecting *Saccharomyces cerevisiae* (11,000 cells in a 3 mL volume) into the coelomic cavity incubating for 8 hours. Addition of probiotics to the diet of caged matrinxã altered the Htc, RBC, MCV, MCHC, TLC, lymphocyte, and eosinophil values. We observed increased cortisol and glucose levels and phagocytic activity, but no increase in the phagocytic index. We thus conclude that supplementing caged *Brycon amazonicus* with probiotics improves their non-specific immunity and alters blood profiles.

Keywords: nutrition; *Bacillus*; hematology; cortisol; glucose; fish defense.

Received on February 27, 2020.
Accepted on May 15, 2020.

Introduction

In recent years, research to develop functional feeds and chemical substances that promote feed efficiency and fish growth rate has intensified (Oliveira, Sivieri, Alegro, & Saad, 2002, Dias et al., 2012, Banerjee & Ray, 2017). Among functional foods, probiotics can provide basic nutrition and improve fish health through mechanisms not involved in conventional nutrition (Sanders, 1998; Castro, 2003, Balcázar et al., 2006; Aly, Ahmed, Ghareeb, & Mohamed, 2008, Sutthi, Thaimuangphol, Rodmongkoldee, Leelapatra, & Panase, 2018).

The concept of probiotics has changed over time. Fuller (1989) described probiotics as dietary supplements composed of live microorganisms that benefit host health by achieving a balance in intestinal microorganisms. Probiotics in most products include *Lactobacillus acidophilus*, *Streptococcus faecium*, *Bacillus subtilis*, and in some cases, yeasts (Guzmán, 1992).

A number of studies, mostly involving mammals, have investigated the effect of probiotics on immunity (Coppola & Turnes, 2004). Although their functions and mechanisms are not yet clear (Cross, 2002). Probiotic organisms have been shown to interact with Peyer's patches and intestinal epithelial cells, thereby stimulating IgA production by B lymphocytes and T lymphocyte migration (Perdigón & Ruiz Holgado, 2000). Probiotics have also been shown to favor the non-specific phagocytic activity of alveolar phagocytes, suggesting a systemic effect by secretion of mediators that stimulate the immune system (Cross, 2002).

In aquaculture, tests of activation and improvement of phagocytic migration are being used to verify the immune response of animals during experimental challenge. This methodology described by Silva, Staines, Blazquez, Porto-Neto, & Borges, 2002, Silva, Porto-Neto, Borges, & Jensch-Junior, 2005) verified that the phagocytic index and phagocytic capacity evaluated in Antarctic fish could be used for other aquatic organisms such as snook, *Centropomus parallelus* (Ranzani-Paiva, et al., 2008), the bullfrog *Lithobates catesbeianus* (Dias et al., 2010), and *Brycon amazonicus* (Dias et al., 2011).

Increasing resistance against disease by improving the phagocytic activity of immune cells is of great interest. Increased phagocytic activity against bacterial antigens is induced by the release of dead pathogens or their products and through application of immunostimulators and adjuvants as described by Male Brostoff, Roth, and Roitt (2006). Immunostimulants enhance the enzymatic mobilization of neutrophils (Siwicki, Morand, Klein, Studnicka, & Majewska, 1998), which contain large amounts of peroxidase (Ellis, 1997), a lysosomal enzyme present in phagocytic cells that promotes hydrogen peroxide oxidation during phagocytosis (Oliveira, Moura, Matushima, & Egami, 1997).

Hematology or the study of the blood involves measurement and evaluation of a variety of blood parameters in normal and abnormal conditions (Seriani, Ranzani-Paiva, Souza, & Napoleão, 2011, Silva et al., 2018). Application of hematological analysis in animal research is well accepted and considered a routine procedure in diagnostic methods (Ranzani-Paiva & Souza, 2004), exposure to experimental conditions, biomonitoring, and seasonality among others (Seriani & Ranzani-Paiva, 2012; Seriani, França, Lombardi, Brito, & Ranzani-Paiva, 2015; Corrêa, Abessa, Santos, Silva, & Seriani, 2017). Several authors emphasize that determination of blood and organ abnormalities is a valuable and safe method for evaluating the biological, physiological, and pathological conditions in fish (Ranzani-Paiva & Souza, 2004; Ranzani-Paiva, Felizardo, & Luque, 2005; Seriani et al., 2013; Prado et al., 2015; Seriani et al., 2015; Corrêa et al., 2017; Silva et al., 2018). Thus, knowledge of blood chemistry is fundamental to assess the physiological and nutritional status of fish.

The genus *Brycon*, a member of the family Characidae, is considered one of the largest genera of neotropical characiforms, accounting for more than 60 nominal species of which about 40 inhabit Central and South America (Howes, et al. 1982). These species have strong teeth, measure up to 50 cm, and have olive-gold coloration with reddish caudal and anal fins (Romagosa, Narahara, Borella, & Verani, 2001). Moreover, they represent a great potential for aquaculture in Brazil, because they are an important food resource for the population, and the quality of their meat also renders them a valuable economic resource. Therefore, our study aimed to evaluate the hematological parameters and the non-specific immune activity of macrophages in matrinxã *Brycon amazonicus* supplemented with probiotics containing *Bacillus subtilis*.

Material and methods

The experiment was conducted in the Southern Region of the Ribeira Valley, in the municipality of Pariquera-Açu in São Paulo State, Brazil. The study included 960 matrinxã fingerlings with an average weight 39.83±8.18 g and mean length 14.60±1.00 cm divided into 12 cages of 2.7 m^3 (1.5 × 1.5 × 1.2 m) installed in three fish ponds, with an area of 600 m^2, an average depth of 1.50 m, and a flow rate of 15 L min^{-1}. The treatments were: T1, Control (32% crude protein ration without probiotic), T2, 5 g of probiotic per kg feed, and T3, 10 g probiotic per kg feed. Blood samples were collected on day 42 and 84 of the experiment.

Eight matrinxãs from each treatment, with an average weight of 262.12±31.95 g and average length of 25.45±0.85 cm were used for hematological analysis. The animals were anesthetized with benzocaine (10 mg L^{-1}). Blood samples were obtained by caudal vein puncture using heparinized syringes. Total red blood cell (RBC) count was determined using a hemocytometer. Hayem solution was used as a diluent and differential white blood cell count was determined. The total leukocyte and thrombocyte numbers were determined as described by Ishikawa, et al. (2008). After sampling, blood smears were prepared on glass slides and colored with May-Grunwald-Giemsa dye. Two thousand cells were analyzed per slide/animal under a compound optical microscope, and leukocytes and thrombocytes were identified and counted. Additional blood smears were used for differential counting of white blood cells neutrophils, monocytes, lymphocytes, basophils, and eosinophils. Hematocrit (Htc) was evaluated by the microhematocrit method. Hemoglobin (Hb) was evaluated by the cyanmethemoglobin method. Absolute RBC indexes including mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated.

Plasma samples were used for analyzing cortisol and glucose levels with a commercially available ELISA and Labtest kit respectively.

Another eight animals from each treatment group were used to assess the macrophage phagocytic activity. The fish were anesthetized with benzocaine. *Saccharomyces cerevisiae* cells were injected into the coelomic cavity at a concentration of 11,000 cells in a volume of 3 mL and incubated for 8 hours. After the incubation period, the animals were euthanized by deepening the anesthetic state. The coelomic cavity was washed with 3 mL of PBS (phosphate buffered saline - pH 7.2) and the fluid was aspirated. The lavage was centrifuged at 1500 rpm

(251.5 × g) for 5 min. and the supernatant was discarded. The sediment was used to determine phagocytic capacity (FC = active macrophages/100 macrophages) and phagocytic index (FI = total yeast cells in macrophages/number of active macrophages) by phase contrast microscopy (400 x), according to the method of Silva et al. 2002 and 2005).

In order to verify the differences between treatments, analysis of variance (ANOVA) followed by Tukey's test (Zar, 1999) was performed. Results were considered significant when $p < 0.05$.

Results

The hematocrit values of both treatment groups showed a significant decrease (p=0.007) when compared with the control group. Animals fed with 5 g kg^{-1} probiotics did not differ from those fed with 10 g kg^{-1} (Table 1). After 84 days (collection 2), there was no statistical difference ($p < 0.05$) between the treatments.

Table 1. Mean values and standard error of the mean (SEM) of the haematologic parameters of matrinxã, *Brycon amazonicus*, treated with probiotic, *Bacillus subtilis*.

1st Collection			
Variables	Control group	Probiotic group (5 g Kg^{-1})	Probiotic group (10 g Kg^{-1})
RBC	335.06±31.14^{b}	313.81±30.32^{bc}	379.56±29.85^{a}
Hematocrit	42.50±1.56^{a}	36.75±4.30^{b}	39.68±3.45^{b}
Haemoglobin	9.07±0.52	8.85±0.76	8.82±0.74
MCV	127.78±12.25^{ab}	117.46±12.00^{b}	102.90±15.82^{c}
MCHC	21.37±1.36^{c}	24.25±2.39^{b}	22.83±1.21^{bc}
Leukocyte	30943.65±10273.27^{a}	18688.26±6798.01^{b}	18425.55±4060.10^{b}
Lymphocytes	23736.90±11478.06^{a}	13206.92±4993.80^{b}	14709.70±2604.72^{b}
Neutrophils	2408.30±2119.17	3020.28±3748.92	2604.19±1271.42
Monocytes	1810.50±943.39^{a}	1328.26±676.16^{ab}	975.35±618.18^{b}
Eosinophils	97.98±148.43	142.21±176.41	135.75±175.79
Basophils	696.64±1677.73	206.11±310.59	0.00±0.00
Thrombocytes	18781.10±7104.82	21894.11±7923.75	23484.88±5082.83
2nd Collection			
Variables	Control group	Probiotic group (5 g Kg^{-1})	Probiotic group (10 g Kg^{-1})
RBC	263.00±12.21	280.00±38.00	268.62±37.65
Hematocrit	43.56±1.78	44.50±1.65	42.75±1.03
Haemoglobin	9.66±0.20	9.90±0.39	9.34±1.00
MCV	166.11±13.01	161.53±23.17	162.14±25.06
MCHC	22.21±1.01	22.28±1.24	21.84±1.99
Leukocyte	30446.89±12904.67	35295.21±7181.15	29427.80±8602.17
Lymphocytes	22218.67±12298.27	26095.88±4408.32	19557.13±5155.12
Neutrophils	3459.79±1842.01	5691.47±2959.17	5770.04±3783.60
Monocytes	2283.92±1819.26 ^{a}	2415.88±906.70 ^{a}	1216.61±406.20 ^{ab}
Eosinophils	794.94±476.14	2014.67±1591.70	2884.01±2127.05
Basophils	5.57±10.33	0.00±0.00	0.00±0.00
Thrombocytes	28869.50±10342.51	30858.42±9681.52	33985.56±14354.37

Different letters indicate significant difference ($p < 0.05$); RBC: Red blood cell; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration.

Concerning the RBCs, the RBC counts were increased in fish that received 10 g of probiotic kg^{-1} feed, when sampled at 42 days ($p = 0.001$). At the second collection (84 days), the RBC values were decreased in all treatments compared to the collection 1. RBC counts did not differ between treatments on day 84. At the second collection, the RBC counts decreased and Ht increased possibly due to an increase in the size of the RBCs as inferred by the high MCVF value at day 84. The MCV values increased as the probiotic concentration in diet was increased. At the first collection, the control group showed MCHC values smaller than those of fish fed with 5 g kg^{-1} of probiotics, but did not differ from the probiotic treatment with 10 g kg^{-1}. In the second collection, there were no significant differences ($p < 0.05$) between the treatment groups. In this study, statistically significant differences in MCV and MCHC were noted only at the 1st collection (Table 1).

The absolute values of TLC are presented in Table 1. At the 1st collection (42 days), TLC values are greater in animals fed with probiotics compared to those in the controls (p=0.004). At the second collection (84 days) the TLC value of leukocytes was still significantly different ($p = 0.45$).

The lymphocyte count in probiotic-fed fish was decreased compared to the control group at the first collection (p = 0.02). At the second collection, there was no significant difference between the groups (p = 0.29). The values reported in our experiment are higher than the 5680 Lf. mm^{-3} of blood reported by Dias, Affonso, Oliveira, Marcon, and Egami (2008) for this species.

The cortisol values are depicted in Figure 1. Although the values do not demonstrate a significant difference between treatments at the first collection (p=0.16), the second collection indicates that fish fed with a probiotic-supplemented diet showed increased cortisol levels with the 10 g treatment dosage. At the second collection, decreased cortisol values were found in animals that received a diet with 5 g of probiotics, but those in animals fed with 10 g remained elevated when comparing the first and second collections. The glucose levels are shown in Figure 2. Lower values were observed in animals from the control group when compared with other treatments, at both collections. In the first collection, higher glucose levels were found in fish receiving 5 g of probiotic (p=0.002), whereas at the second collection, higher values were observed in fish receiving the 10 g probiotic dosage (p=0.007). Our glucose values corroborate those reported by Carneiro and Urbinati (2002) and Hoshiba, Gonçalves, and Urbinati (2009) ranging from 40 to 120 mg dL^{-1} and 80 to 115 mg dL^{-1}, respectively.

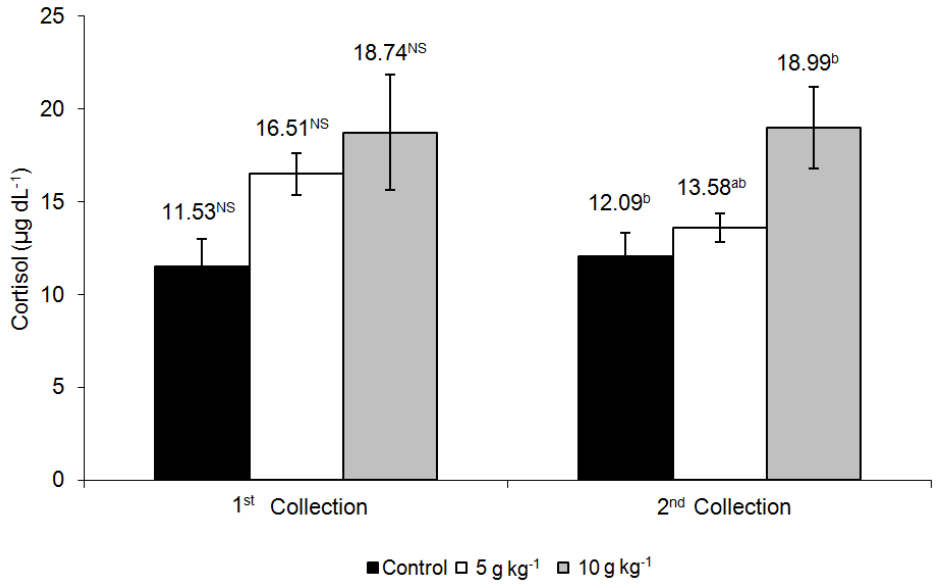

Figure 1. Plasma cortisol levels in matrinxã *Brycon amazonicus* fed different doses of probiotic (different letters indicate statistically significant differences).

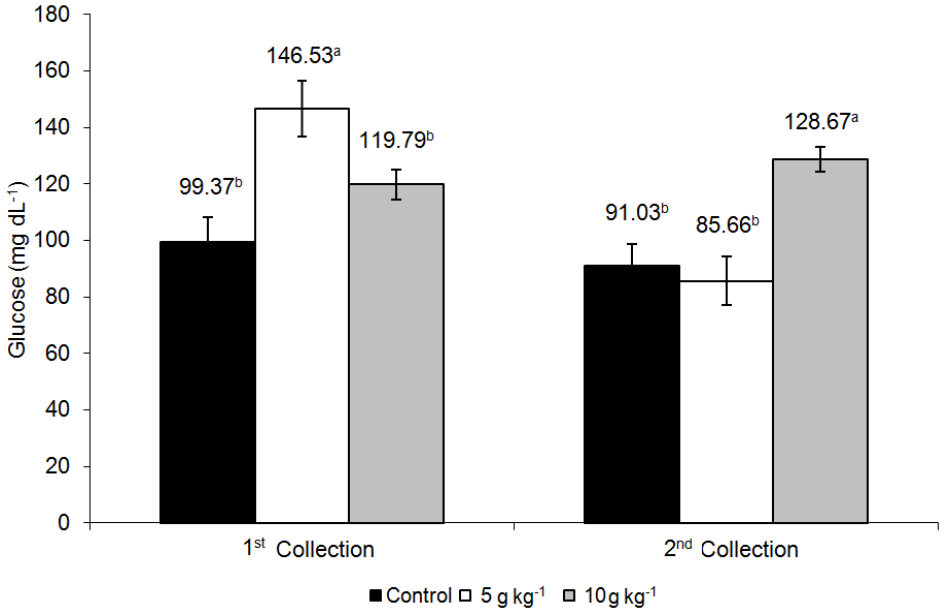

Figure 2. Plasma glucose levels in matrinxã *Brycon amazonicus* fed with different doses of probiotics (Equal letters indicate no significant difference).

Hyperglycemia is reported under stress, and in this case it occurs because of the energy mobilization induced by catecholamines and cortisol (Mommsen, Vijayan, & Moon, 1999). Increase in plasma glucose at the initial exposure times (between 6 and 24 hours) shows a tendency toward mobilization of energetic reserves due to the effect of catecholamines on the liver and skeletal muscle, causing glycogen breakdown to provide energy (Mazeaud & Mazeaud, 1981; Bonga, 1997).

The phagocytic index and macrophage activity of matrinxã are shown in Figure 3 and 4. The values indicating phagocytic capacity showed significant differences (p=0.0001) in animals that received a diet supplemented with probiotics. Evaluation of the phagocytic index showed no difference (p=0.07) between the treatment values or between the collection 1 (42 days) and 2 (84 days).

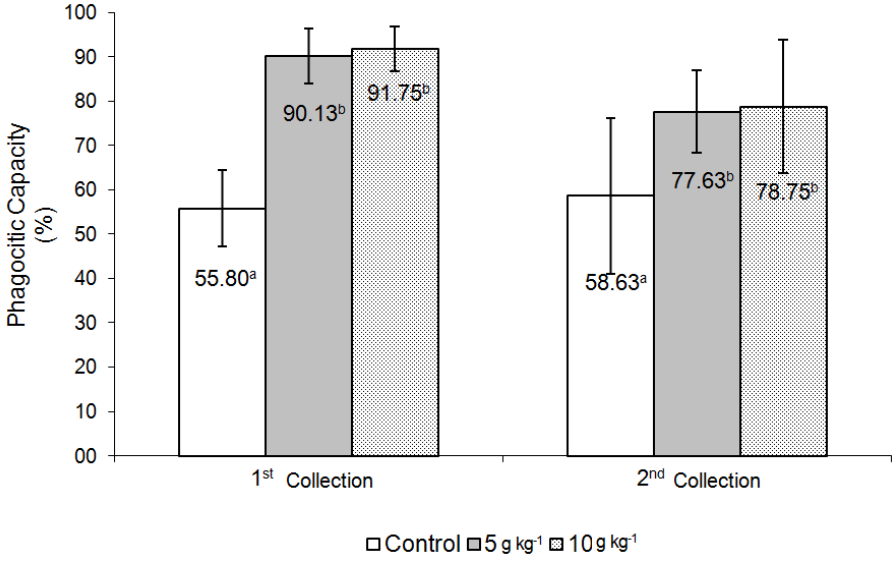

Figure 3. The phagocytic index in matrinxã *Brycon amazonicus*. The phagocytic index showed no difference (p = 0.07) between the treatment values or between the 1st (42 days) and 2nd collection (84 days). NS: no significant (p > 0.05).

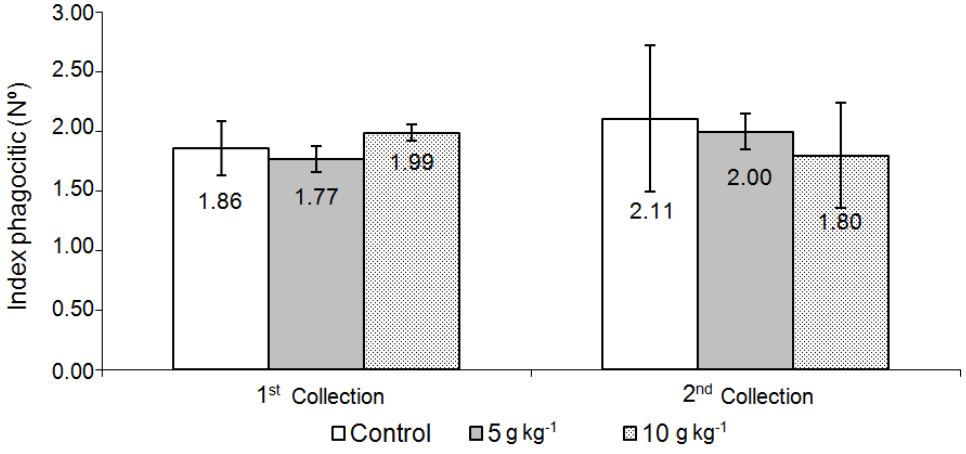

Figure 4. The macrophage activity in matrinxã *Brycon amazonicus*. The capacity showed significant differences (p = 0.0001) in animals that received a diet supplemented with probiotics for 42 (1st) and 84 days (2nd). NS: no significant (p > 0.05).

Discussion

We observed that *B. amazonicus* supplemented with probiotics, showed alterations in their hematological and immunological parameters, characterized as improved non-specific immunity possibly due to nutritional competition, production of antibacterial substances, or as immunostimulants.

Irianto and Austin (2002) and Kumar, Mukherjee, Ranjan, and Nayak (2008) reported that probiotic-supplemented *Oncorhynchus mykiss* and *Labeo rohita* showed altered immunological and hematological parameters due to interaction with monocytes, neutrophils, and NK cells to enhance their innate immune responses in addition to enhancing the number of erythrocytes, granulocytes, and lymphocytes in others fish.

Eosinophils showed significant differences only at the second collection when larger numbers of cells were found in all treatments. An increase in the number of eosinophils is usually related to allergic processes, but occurs frequently in fish. Recent studies on mammals showed that eosinophils are pleiotropic multifunctional leukocytes involved in the initiation and propagation of diverse inflammatory responses, as well as modulators of innate and adaptive immunity (Rothenberg & Hogan, 2006). In mammals, eosinophils constitute a distinct lineage of granulocytes that provide innate immunity (Hogan et al, 2008). The neutrophil, basophil, and monocyte counts showed no significant differences between treatments.

According Ranzani-Paiva and Souza (2004), the body begins to release immature cells with less hemoglobin. These immature cells result in increased MCV and decreased MCHC. These results suggest that at the first collection fish in the control group. It is possible that these results could be associated with improved transport of oxygen and the nutrients involved in immunological process.

Allen (1994) suggested the possibility that MCV is reduced and that MCHC increases without altering the number of erythrocytes and hemoglobin, indicating that the same amount of hemoglobin occupies less volume in the cell. Reduction of hematocrit may also occur due to an ionic balance impairment that triggers increased water retention thus causing hemodilution. Notably, the responses generally vary according to the species under study (Seriani & Ranzani-Paiva, 2012)

The increase in the cortisol and glucose levels in the blood are indicators commonly used to express stress conditions (Bonga, 1997, Wells & Pankhurst, 1999). Overall, this study shows that fish with fed the highest concentration of probiotics showed a faster response to the stressor stimulus.

These results are not necessarily associated with stress. According to Railo, Nikinmaa, and Soivio (1985) and Tetens and Christensen (1987), release of catecholamines directly results in elevated MCV by increasing membrane permeability of erythrocytes and the affinity of hemoglobin with oxygen. Therefore, elevation of MCV is considered as cell hydration, because adrenaline, one of the catecholamines produced during stress, causes an increase in cell size due to retention of sodium and chloride in the intracellular environment, thus increasing their internal concentration and consequently the erythrocyte volume. Nevertheless, it is important to mention that the presence of immature erythrocytes in a stress situation can be high, which culminates in altered MCV, because at this stage of the cell, size is larger but contains less hemoglobin.

The results obtained in the phagocytic capacity test indicate that probiotics influence macrophage activity by increasing migration to local injury and increasing their capacity to phagocytose yeast. Probiotics benefit the production of farmed animals, optimizing the phagocytic response and affording a higher level of immunity against infections. According to Coppola and Turnes (2004), probiotics have an immunomodulatory effect beyond their activity as growth promoters and mucosal microbiota regulators, although their action is poorly understood.

In a study conducted with the bullfrog (*Lithobates catesbeianus*) during fattening, Dias et al. (2010) observed a positive influence on the phagocytic ability of animals supplemented with probiotics. However, there were no significant differences in the phagocytic index values in the present work. Franca et al. (2008) working with the same frog species and the same probiotic bacteria in other growth phases, observed an increase in the phagocytic capacity of macrophages, with a significant difference between animals fed with different treatments compared to the control group.

Probiotics promote the nonspecific phagocytic activity of alveolar macrophages, suggesting a systemic action through secretion of mediators that stimulate the immune system, even though the mechanisms by which probiotics stimulate immunity have not been clarified (Cross, 2002). According to Coppola and Turnes (2004), *Bacillus* sp. can stimulate the immune response and can be used as immunomodulators. The ability of probiotic bacteria to modulate immunity and increase the microbial balance of enteric commensal microorganisms offers a biologically effective alternative to improve health without resorting to the use of allopathic drugs (Kailasapathy & Chin, 2000). According to Gatesoupe (1999) and Chang and Liu (2002), probiotics in aquaculture should be used as antagonists of pathogens that colonize the intestine and increase the host resistance against the pathogen, thus demonstrating that probiotics are more effective at preventing disease than in treatment.

In conclusion we observed that (a) the supplementation with probiotics for 42 days altered the blood parameters of matrinxã, especially in the Htc, RBC, MCV, MCHC, and the absolute number of leukocytes, lymphocytes, and eosinophils; (b) *B. subtilis* as probiotic in diet at 5 g kg^{-1} dry ration and did improve non-specific immunity in matrinxãs; and finally, (c) alterations in cortisol could represent acute stress and increase glucose as energy recruitment for phagocytosis. In addition, alteration in glucose and cortisol levels could be due to sensitivity to management, caused by high-density designs in cages and the stress of acclimatization, but without influencing hematological parameters. Finally, we believe that probiotics with

B. subtilis could be used in aquaculture as immunostimulators and recommend further experiments to clearly establish the role of *B. subtilis* in the immune system and to replace antibiotics with probiotics for ecological considerations as well sustainability.

Acknowledgements

This study was supported financially by the São Paulo Research Foundation (*Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP*), Process No. 2008/05823-7. We would like to thank for coworkers of Polo Regional Vale do Ribeira/Pariquera-Açu in São Paulo State, Brazil, by help in fish collection and handling. Finally, we would like to thank EDITAGE for the manuscript review.

References

Allen, P. (1994). Changes in the haematological profile of the cichlid *Oreochromis aureus* (Steindachner) during acute inorganic mercury intoxication. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *108*(1), 117-121. doi: 10.1016/1367-8280(94)90097-3

Aly, S. M., Ahmed, Y. A. G., Ghareeb, A. A. A., & Mohamed, M. F. (2008). Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. *Fish & Shellfish Immunology*, *25*, 128-136. doi: 10.1016/j.fsi.2008.03.013

Balcázar, J. L., Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., & Múzquiz, J. L. (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, *114*, 173-186. doi: 10.1016/j.vetmic.2006.01.009

Banerjee, G., & Ray, A. K. (2017). The advancement of probiotics research and its application in fish farming industries. *Research in Veterinary Science*, *115*, 66-77. doi: 10.1016/j.rvsc.2017.01.016

Bonga, S. E. W. (1997). The stress response in fish. *Physiological Reviews, 77*(3), 591–625. doi: 10.1152/physrev.1997.77.3.591

Carneiro, P. C. F., & Urbinati, E. C. (2002). Transport stress in matrinxã, *Brycon cephalus* (Teleostei: Characidae), at different densities. *Aquaculture International*, *10*, 221-229. doi: 10.1023/A:1022166411089

Castro, J. C. (2003). Uso de aditivos e probióticos em rações animais. In C. M. Ferreira, M. J. T. R. Paiva, P. C. Teixeira, F. M. França & D. C. Dias (Eds.), *I Simpósio brasileiro de ranicultura e II ciclo de palestras sobre ranicultura do Instituto de Pesca* (p. 12-18). São Paulo: Boletim Técnico do Instituto de Pesca.

Chang, C.-I., & Liu, W.-Y. (2002). An evaluation of two probiotic bacterial strains, *Enterococcus faecium* SF68 and *Bacillus toyoi*, for reducing edwardsiellosis in cultured European eel, *Anguilla anguilla* L. *Journal of Fish Diseases*, *25*, 311-315. doi: 10.1046/j.1365-2761.2002.00365.x

Coppola, M. M., & Turnes, C. G. (2004). Probiotics and immune response. *Ciência Rural, 34*(4), 1297-1303. doi: 10.1590/S0103-84782004000400056

Corrêa, S. A. S., Abessa, D. M. S., Santos, L. G., Silva, E. B., & Seriani, R. (2017). Differential blood counting in fish as a non-destructive biomarker of water contamination exposure. *Toxicological & Environmental Chemistry*, *99*(3), 482-491. doi: 10.1080/02772248.2016.1189554

Cross, M. L. (2002). Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunology & Medical Microbiology, 34*(4), 245-253. doi: 10.1111/j.1574-695X.2002.tb00632.x

Dias, D. C., De Stéfani, M. V., Ferreira, C. M., França, F. M., Ranzani-Paiva, M. J. T., & Santos, A. A. (2010). Haematologic and immunologic parameters of bullfrogs, *Lithobates catesbeianus*, fed probiotics. *Aquaculture Research*, *41*(7), 1064-1071. doi: 10.1111/j.1365-2109.2009.02390.x

Dias, M. T., Affonso, E. G., Oliveira, S. R., Marcon, J. L., & Egami, M. I. (2008). Comparative study on hematological parameters of farmed matrinxã, *Brycon amazonicus* Spix and Agassiz, 1829 (Characidae: Bryconinae) with others Bryconinae species. *Acta Amazonica, 38*(4), 799–806. doi: 10.1590/S0044-59672008000400026

Dias, D. C., Furlaneto, F. P. B., Ayroza, L. M. S., Tachibana, L., Romagosa, E., & Ranzani-Paiva, M. J. T. R. (2012). Probiotic in feeding of juvenile matrinxã (*Brycon amazonicus*): economic viability. *Acta Scientiarum. Animal Sciences*, *34*(3), 239-243. doi: 10.4025/actascianimsci.v34i3.13257

Dias, D. C., Tachibana, L., Seriani, R., Santos, A. A., Ranzani-Paiva, M. J. T., & Romagosa, E. (2011). Macrophagic migration time in matrinxã, *Brycon amazonicus*, through inoculation of yeast, *Saccharomyces cerevisiae*, technique. *Acta Amazonica*, *41*(3), 421-424. doi: 10.1590/S0044-59672011000300013

Ellis, A. E. (1997) The leukocytes of fish: Review. *Journal of Fish Biology*, *11*, 453-491. doi: 10.1111/j.1095-8649.1977.tb04140.x

Franca, F. M., Dias, D. C., Teixeira, P. C., Marcantônio, A. S., De Stéfani, M. V., Antonucci, A., ... Ferreira, C. M. (2008). Efeito do probiótico *Bacillus subtilis* no crescimento, sobrevivência e fisiologia de rãs-touro (*Rana catesbeiana*). *Boletim do Instituto de Pesca*, *34*(3), 403-412.

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology*, *66*(5), 365-378.

Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. *Aquaculture*, *180*(1-2), 147-165. doi: 10.1016/S0044-8486(99)00187-8

Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P., ... Rothenberg, M. E. (2008). Eosinophils: Biological Properties and role in health and disease. *Clinical & Experimental Allergy*, *38*(5), 709–750. doi:10.1111/j.1365–2222.2008.02958.x

Hoshiba, M. A., Gonçalves, F. D., & Urbinati, E. C. (2009). Respostas fisiológicas de estresse no matrinxã (*Brycon amazonicus*) após exercício físico intenso durante a captura. *Acta Amazonica*, *39*(2), 445-451. doi: 10.1590/S0044-59672009000200025

Irianto, A., & Austin, B. (2002). Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, *25*(6), 333-342. doi: 10.1046/j.1365-2761.2002.00375.x

Ishikawa, N. M., Ranzani-Paiva, M. J. T., & Lombardi, J. V. (2008). Metodologia para quantificação de leucócitos totais em peixe, *Oreochromis niloticus*. *Archives of Veterinary Science*, *13*(1), 54-63.

Kailasapathy, K., & Chin, J. (2000). Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunology and Cell Biology*, *78*(1), 80-88. doi: 10.1046/j.1440-1711.2000.00886.x

Kumar, R., Mukherjee, S. C., Ranjan, R., & Nayak, S. K. (2008). Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. *Fish & Shellfish Immunology*, *24*(2), 168-172. doi: 10.1016/j.fsi.2007.10.008

Male, D., Brostoff, J., Roth, D. B., & Roitt, I., 2006. Immunology (7th ed.). London, UK: Elsevier.

Mazeaud, M. M., & Mazeaud, F. (1981). Adrenergic responses to stress in fish. In A. D. Pickering (Ed.), *Stress and fish* (p. 49-75). London, UK: Academic Press.

Mommsen, T. P., Vijayan, M. M., & Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*, *9*(3), 211-268. doi: 10.1023/A:1008924418720

Howes, G. N, Podoll, M. H., Howes, G. J., & Howes, J. (1982). Review of the genus *Brycon* (Teleostei-Characoidei. *Bulletin of the British Museum (Natural History), 43*, 1-47. doi: 10.5962/bhl.part.8299

Oliveira, L. W., Moura, W. L., Matushima, E. R., & Egami, M. I. (1997). Detecção citoquímica da mieloperoxidase em eosinófilos e mononucleares azurófilos do sangue de *Caiman crocodilus yacare* (Daudin, 1802) Reptilia, Crocodilia. In *Anais da 49º Reunião Anual da Sociedade Brasileira para Progresso da Ciência* (p. 878). Belo Horizonte, MG: SBPC.

Oliveira, M. N., Sivieri, K., Alegro, J. H. A., & Saad, S. M. I. (2002). Aspectos tecnológicos de alimentos funcionais contendo probióticos. *Revista Brasileira de Ciências Farmacêuticas*, *38*(1), 1-21. doi: 10.1590/S1516-93322002000100002

Ranzani-Paiva, M. J. T., Felizardo, N. N., & Luque, J. L. (2005). Parasitological and hematological analysis of Nile tilapia *Oreochromis niloticus* Linnaeus, 1757 from Guarapiranga reservoir, São Paulo State, Brazil. *Acta Scientiarum. Biological Sciences*, *27*(3), 231-237. doi: 10.4025/actascibiolsci.v27i3.1334

Ranzani-Paiva, M. J. T., Santos, A. A., Dias, D. C., Seriani, R., & Egami, M. I. (2008). Hematological and phagocytic response of the fat snook, *Centropomus parallelus*, reared in net cages, before and after inoculation with *Sacharomyces ceresivisiae*. *Bioikos*, *22*(1).

Ranzani-Paiva, M. J. T. R., & Souza, A. T. S. (2004). Hematologia de peixes brasileiros.In M. J. T. Ranzani-Paiva, R. M. Takemoto & M. A. P. Lizama (Eds.), *Sanidade de organismos aquáticos* (p. 86-120). São Paulo, SP: Varela.

Perdigón, G., & Ruiz Holgado, A. P. (2000). Mechanisms involved in the immunostimulation by lactic acid bacteria. In R. Fuller & G. Perdigón (Eds.), *Probiotics 3: immunomodulation by the gut microflora and probiotics* (p. 213-233). Dordrecht: Springer.

Prado, L. R., Felix, C., Abessa, D. M., Buruaem, L. M., Abujamara, L. D., Kirschbaum, A. A., & Seriani, R. (2015). Hematological parameters and nuclear abnormalities in peripheral erythrocytes of *Achirus lineatus* (Pleuronectiformes: Achiridae). *Comparative Clinical Pathology*, *24*(1), 169-175. doi: 10.1007/s00580-014-1880-3

Railo, E., Nikinmaa, M., & Soivio, A. (1985). Effects of sampling on blood parameters in the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology*, *26*(6), 725-732. doi: 10.1111/j.1095-8649.1985.tb04312.x

Romagosa, E., Narahara, M. Y., Borella, M. I., & Verani, N. F. (2001). Seleção e caracterização de fêmeas de matrinxã, *Brycon cephalus*, induzidas a reprodução. *Boletim do Instituto de Pesca*, *27*(2), 139-147.

Rothenberg, M. E., & Hogan, S. P. (2006). The eosinophil. *Annual Review Immunology*, *24*, 147-174. doi: 10.1146/annurev.immunol.24.021605.090720

Sanders, M. E. (1998). Overview of functional foods: emphasis on probiotic bacteria. *International Dairy Journal*, *8*(5-6), 341-347. doi: 10.1016/S0958-6946(98)00056-9

Seriani, R., Abessa, D. M. S., Pereira, C. D. S., Kirschbaum, A. A., Abujamara, L. D., Buruaem, L. M., Ranzani-Paiva, M. J. T. R. (2013). Blood parameters of estuarine and marine fish as non-destructive pollution biomarkers. In E. A. Almeida & C. A. O. Ribeiro (Eds.), *Pollution and fish health in tropical ecosystems* (p. 182-205). Boca Raton: CRC Press.

Seriani, R., França, J. G., Lombardi, J. V., Brito, J. M., & Ranzani-Paiva, M. J. T. (2015). Hematological changes and cytogenotoxicity in the tilapia *Oreochromis niloticus* caused by sub-chronic exposures to mercury and selenium. *Fish Physiology and Biochemistry*, *41*(1), 311-322. doi: 10.1007/s10695-014-9984-x

Seriani, R., & Ranzani-Paiva, M. J. T. (2012). Alterações hematológicas em peixes: aspectos fisiopatológicos e aplicações em ecotoxicologia aquática. In A.T. S. Souza, M. A. P Lizama & R. M. Takemoto (Orgs.), *Patologia e sanidade de organismos aquáticos* (p. 221-242). Maringá: Abrapoa.

Seriani, R., Ranzani-Paiva, M. J. T., Souza, Â. T. S., & Napoleão, S. R. (2011). Hematology, micronuclei and nuclear abnormalities in fishes from São Francisco river, Minas Gerais state, Brazil. *Acta Scientiarum. Biological Sciences, 33*(1), 107-112. doi: 10.4025/actascibiolsci.v33i1.7117

Silva, E. B., Corrêa, S. A. S., Abessa, D. M. S., Silva, B. F. X., Rivero, D. H. R. F., & Seriani, R. (2018). Mucociliary transport, differential white blood cells, and cyto-genotoxicity in peripheral erythrocytes in fish from a polluted urban pond. *Environmental Science and Pollution Research*, *25*, 2683-2690. doi: 10.1007/s11356-017-0729-0

Silva, J. R. M. C., Porto-Neto, L. R., Borges, J. C. S., & Jensch-Junior, B. E. (2005). Germicide capacity of macrophages in the Antarctic fish *Notothenia coriiceps* (Richardson, 1844) at 0ºC. *Polar Biology*, *28*(4), 326-328. doi: 10.1007/s00300-004-0704-3

Silva, J. R. M. C., Staines, N. A., Blazquez, F. J. H., Porto-Neto, L. R., & Borges, J. C. S. (2002). Phagocytosis and giant cell formation at 0ºC by macrophage (MØ) of *Notothenia coriiceps*. *Journal of Fish Biology*, *60*(2), 466-478. doi: 10.1111/j.1095-8649.2002.tb00294.x

Siwicki, A. K., Morand, M., Klein, P., Studnicka, M., & Majewska, E. T. (1998). Modulation of nonspecific defence mechanisms and protection against diseases in fish. *Acta Veterinaria Brno, 67*(4), 323–328. doi: 10.2754/avb199867040323

Sutthi, N., Thaimuangphol, W., Rodmongkoldee, M., Leelapatra, W., & Panase, P. (2018). Growth performances, survival rate, and biochemical parameters of Nile tilapia (*Oreochromis niloticus*) reared in water treated with probiotic. *Comparative Clinical Pathology, 27*(3), 597-603.

Tetens, V., & Christensen, N. J. (1987). Beta-adrenergic control of blood oxygen affinity in acutely hypoxia exposed rainbow trout. *Journal of Comparative Physiology B,157*, 667–675. doi: 10.1007/BF00700988

Wells, R. M. G., & Pankhurst, N. W. (1999). Evaluation of simple instruments for the measurement of blood glucose and lactate, and plasma protein a stress indicator in fish. *Journal of the World Aquaculture Society*, *30*(2), 276–284. doi: 10.1111/j.1749-7345.1999.tb00876.x

Zar, J. H. (1999). Biostatistical Analysis(3rd ed.). Upper Saddle River, NJ: Prentice-Hall.