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Haematological changes in Nile tilapia experimentally infected with *Enterococcus* sp.

Martins, ML.[a]**, Mouriño, JLP.*[a,b]*, Amaral, GV.*[a]*, Vieira, FN.*[b]*, Dotta, G.*[a]*, Bezerra, AJM.*[a,b]*, Pedrotti, FS.*[a,b]*, Jerônimo, GT.*[a]*, Buglione-Neto, CC.*[b] *and Pereira-Jr., G.*[a]*,*

[a]Laboratório de Pesquisas em Sanidade de Organismos Aquáticos, Dapartamento de Aqüicultura, CCA, Universidade Federal de Santa Catarina – UFSC, Rod. Admar Gonzaga 1346, CEP 88040-900, Florianópolis, SC, Brazil

[b]Laboratório de Camarões Marinhos, Departamento de Aqüicultura, CCA, Universidade Federal de Santa Catarina – UFSC, Beco dos Coroas, Barra da Lagoa, CEP 88062-601, Florianópolis, SC, Brasil

*e-mail: mlaterca@cca.ufsc.br

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Abstract

This study evaluated the haematological changes in Nile tilapia experimentally infected with 1 x 10^3 and 1 x 10^6 colony-forming units (CFU)/mL of *Enterococcus* sp. in the swim bladder. The experiment consisted of four treatments in triplicates: non-injected fish (NI); fish injected with 1 mL of sterile saline solution 0.65% (SAL); fish injected with 1 x 10^3 and 1 x 10^6 CFU/mL of *Enterococcus* diluted in 1 mL sterile saline. Twenty-four hours after injection, the fish were anesthetized and the blood collected. The haematological tests included red blood cell (RBC) and white blood cell (WBC) counts, hematocrit, number of total thrombocytes, and differential counting of WBC. Fish injected with 1 x 10^6 CFU/mL of *Enterococcus* showed a higher number of thrombocytes than the other treatments. White blood cell and lymphocyte numbers increased significantly in fish injected with 1 x 10^6 CFU/mL of *Enterococcus* when compared to non-injected control. There was significant increase in the number of neutrophils in saline injected fish and reduced number of monocytes after injections with 1 x 10^6 CFU/mL of *Enterococcus*. Hematocrit increased in fish injected with 1 x 10^3 and 1 x 10^6 CFU/mL of *Enterococcus*.

Keywords: tilapia, *Enterococcus*, infection, haematology.

Alterações hematológicas em tilápia do Nilo infectada experimentalmente com *Enterococcus* sp.

Resumo

Este estudo avaliou alterações hematológicas em tilápia do Nilo infectada experimentalmente com 1 x 10^3 e 1 x 10^6 unidades formadoras de colônia (CFU)/mL de *Enterococcus* sp. na bexiga natatória. O experimento consistiu de quatro tratamentos com três repetições cada: peixes não injetados (NI); peixes injetados com 1 mL de solução salina a 0,65% esterilizada (SAL); peixes injetados com 1 x 10^3 e 1 x 10^6 CFU/mL de *Enterococcus* diluída em 1 mL de solução salina esterilizada. Vinte e quatro horas após as injeções, os peixes foram anestesiados com benzocaína e o sangue coletado. Os testes hematológicos incluíram as contagens totais de eritrócitos (RBC), de leucócitos (WBC) e de trombócitos, o percentual do hematócrito e a contagem diferencial de leucócitos. Peixes injetados com 1 x 10^6 CFU/mL de *Enterococcus* mostraram maior número de trombócitos no sangue do que os dos outros tratamentos. O número de leucócitos totais e o número de linfócitos foram significativamente mais altos após injeção de 1 x 10^6 CFU/mL de *Enterococcus*, quando comparados ao controle não injetado. Houve aumento no número de neutrófilos nos injetados com salina e redução no número de monócitos nos injetados com 1 x 10^6 CFU/mL de *Enterococcus*. O hematócrito aumentou nos animais injetados com 1 x 10^3 e 1 x 10^6 CFU/mL de *Enterococcus*.

Palavras-chave: tilápia, *Enterococcus*, infecção, hematologia.

1. Introduction

Bacterial diseases are among the most important causes of economic losses in cultured tilapia. *Aeromonas* spp., *Pseudomonas fluorescens*, *Vibrio anguillarum*, *Flavobacterium columnare*, *Edwardsiella tarda*, *Streptococcus* spp. and *Enterococcus* sp. are commonly found in the facilities (Plumb, 1997). They are often sub-clinical and without apparent signs. Under predisposing factors such as poor water quality, high ammonia as a result of high stocking density and feeding, ectoparasites, inadequate handling and stressful condi-

tions, the microorganism found a portal of entry into the fish host (Moraes and Martins, 2004).

There are several studies on fish bacteria identification, experimental infection or disease resistance (Azad et al., 2001; Al-Harbi and Uddin, 2004; Cai et al., 2004) but little relates the haematological parameters to bacterial experimental infection. The haematological parameters are an important tool of diagnosis that reveals the state of health of fish (Blaxhall, 1972; Rehulka, 2002; Martins et al., 2004a). For example, decreased red blood cells and hematocrit were found in coho salmon (*Oncorhynchus kisutch*) infected with *V. anguillarum* (Harbell et al., 1979); in Asian cichlid fish (*Etroplus suratensis*) with epizootic ulcerative syndrome (Pathiratne and Rajapakshe, 1998); in rainbow trout (*Oncorhynchus mykiss*) with ulcerous dermatitis (Rehulka, 1998); in rainbow trout experimentally infected with *Aeromonas sobria* and *A. caviae* (Rehulka, 2002); in carp (*Cyprinus carpio*) experimentally infected with *A. hydrophila* (Harikrishnan et al., 2003) and in Nile tilapia experimentally infected with *Streptococcus iniae* (Chen et al., 2004). On the other hand, an increase in the white blood cells and glucose was observed by Haney et al. (1992) in chum salmon (*Oncorhynchus keta*) with erythrocytic necrosis virus.

The anaerobic Gram-positive bacteria *Enterococcus* sp. can be isolated from the water, plants and from the excretion of animals and humans as a commensal microorganism. It is responsible for considerable economic losses in cultured yellowtail (*Seriola quinqueradiata*) (Kusuda and Salati, 1993), turbot (*Scophthalmus maximus*) (Toranzo et al., 1995) and tilapia (Plumb, 1999). Its presence is common in integrated fish culture (Petersen and Dalsgaard, 2003). Clinical signs and pathological manifestations are similar to streptococcosis and consist of exophthalmia, muscular haemorrhages, acute branchitis, suppurative inflammation in the eyes and necrosis of the spleen and kidney (Plumb, 1999).

This study evaluated the haematological changes in Nile tilapia (*Oreochromis niloticus* L.) experimentally infected with two concentrations of *Enterococcus* sp. in the swim bladder originally isolated from an outbreak of Nile tilapia.

2. Material and Methods

Sixty fish with 269.0 ± 63.6 g weight and 23.4 ± 2.0 cm length were distributed in 12 aerated aquaria of 150 L capacity acclimated for 10 days before assay and fed with commercial diet. During this period, the water temperature was maintained at 22.0 ± 0.2 °C, pH 7.0 ± 0.2 and ammonia 0.5 ± 0.1 $mg.L^{-1}$.

The bacterium was originally isolated from the Nile tilapia raised in cages in the São Francisco River, AL. From the liver, heart and gills of diseased tilapia the isolation of bacterial colonies by using the streaked plate method was prepared in Streptococcus KF agar (Difco) and incubated at 28 °C for 24 hours. The colonies were confirmed as *Enterococcus* sp. according to the API 20E Kit (Biomeriux) method. To prepare the inoculum the bacteria was diluted in tubes containing infusion of heart and brain (BHI) (Difco, Detroit, MI) to reach the concentration of $1x10^6$ CFU/mL estimated by serial-dilution method (1:10) according to Madigan et al. (2000).

The experiment consisted of four treatments in triplicates: non-injected fish; fish injected with 1 mL of sterile saline solution 0.65% in the swim bladder according to the Matushima and Mariano (1996) and Martins et al. (2004b) method; fish injected with 1×10^3 CFU/mL of *Enterococcus* and fish injected with 1×10^6 CFU/mL of *Enterococcus* diluted in 1 mL saline solution in the swim bladder.

Twenty-four hours after injection, the fish were anesthetized with benzocaine solution (50 $mg.L^{-1}$) and the blood was withdrawn from the caudal vessel into a syringe containing a drop of 10% EDTA solution (Ethic Committee n° 23080.007045/2006-61/UFSC). The blood was utilized to measure hematocrit (Goldenfarb et al., 1971); glucose (Accu-Chek Advantage 2 Roche) according to Azevedo et al. (2006); total count of red blood cells (RBC) with haemocytometer, total count of white blood cells (WBC), and total number of thrombocytes by indirect method (Martins et al., 2004a). For differential counting of leucocytes, the smears were stained by Giemsa/May-Grunwald (Rosenfeld, 1947) in which a hundred cells were counted for the establishment of each cell contents.

The results were submitted to the variance analysis (ANOVA) and F test ($P < 0.05$) and the means to the Tukey test ($P < 0.05$).

3. Results

During the assay no mortality was observed after experimental infection. Glucose and the red blood cell counts were not significantly different among the treatments (Table 1). Fish injected with 1×10^6 CFU/mL of *Enterococcus* showed significantly higher ($P < 0.05$) number of thrombocytes than the other treatments. White blood cells significantly increased in fish injected with 1×10^6 CFU/mL of *Enterococcus* when compared to non-injected control. Although the hematocrit percentage decreased in fish non-injected and injected with saline, in fish injected with 1×10^3 and 1×10^6 CFU/mL of *Enterococcus* increased significantly ($P < 0.05$) as shown in Table 1.

Differential leucocyte counts were characterized by predominance of lymphocytes (Table 2). Three types of leucocytes, namely lymphocytes, neutrophils and monocytes were identified in the circulating blood of Nile tilapia. The number of lymphocytes in fish injected with 1×10^6 CFU/mL of *Enterococcus* was significantly ($P < 0.05$) higher than that of the other treatments. There was a significant increase in the number of neutrophils in saline injected fish. On the other hand, a significantly reduced number ($P < 0.05$) of mono-

Table 1. Haematological parameters in Nile tilapia non-injected (NI), injected with saline, with 1 x 10^3 and 1 x 10^6 CFU/mL of *Enterococcus* in the swim bladder. Total counts of red blood cells (RBC), white blood cells (WBC) and thrombocytes (TCT), and hematocrit (HTC). Letters in the column indicate significant difference among treatments ($P < 0.05$)

Treatments	Glucose ($mg.dL^{-1}$)	RBC ($x10^6.\mu L^{-1}$)	TCT ($x10^3.\mu L^{-1}$)	WBC ($x10^3.\mu L^{-1}$)	HTC (%)
NI	80.5 ±33.6[a]	0.74 ± 0.5[a]	7.8 ± 2.5[a]	15.0 ± 7.8[b]	15.5 ± 3.6[a]
Saline	83.5 ± 37.9[a]	0.83 ± 0.7[a]	8.8 ± 4.8[a]	20.8 ± 10.7[ab]	13.3 ± 5.1[a]
1 x 10^3	86.0 ± 55.6[a]	0.86 ± 0.7[a]	10.2 ± 4.9[a]	19.0 ± 8.4[ab]	20.9 ± 8.2[b]
1 x 10^6	100 ± 69.1[a]	1.0 ± 0.5[a]	11.3 ± 5.4[b]	27.3 ± 14.8[a]	21.7 ± 7.2[b]

Table 2. Differential counting of leucocytes in the blood of Nile tilapia non-injected (NI), injected with saline, with 1 x 10^3 and 1 x 10^6 CFU/mL of *Enterococcus* in the swim bladder. Letters in the column indicate significant difference among treatments ($P < 0.05$)

Treatments	Lymphocytes ($x10^3.\mu L^{-1}$)	Neutrophils ($x10^3.\mu L^{-1}$)	Monocytes ($x10^3.\mu L^{-1}$)
NI	26.5 ± 9.7[a]	6.5 ± 4.6[a]	14.3 ± 7.7[ab]
Saline	28.1 ± 12.5[a]	14.7 ± 9.1[b]	12.5 ± 4.0[ab]
1 x 10^3	34.0 ± 13.7[a]	5.6 ± 5.2[a]	17.3 ± 10.6[a]
1 x 10^6	48.1 ± 17.1[b]	2.9 ± 2.3[a]	9.9 ± 3.7[b]

cytes was found after injection with 1 x 10^6 CFU/mL of *Enterococcus*.

4. Discussion

Tilapia is one of the most cultivated freshwater fish worldwide. This study was designed to determine if injection with bacteria isolated from diseased tilapia is responsible for haematological changes. The variation degree on the haematological response is an important tool to fish health diagnosis and may vary according to stressor stimulus, treatment, parasitic or infectious diseases (Silveira-Coffigny et al., 2004; Chen et al., 2004; Martins et al., 2004a; Rehulka, 2002).

In this study, the values of hematocrit, RBC and thrombocytes were lower than those related in tilapia by Hrubec et al. (2000), Tavares-Dias et al. (2000), Barros et al. (2002) and Ghiraldelli et al. (2006) in normal conditions. This assay showed that the total number of thrombocytes, lymphocytes and hematocrit were affected by the bacterial injection. The aim of this study was not to determine mortality rate, but to verify the effects of experimentally injected bacteria on blood parameters.

Decreased RBC, hemoglobin and hematocrit in chum salmon infected with *V. anguillarum*, in rainbow trout infected with *Aeromonas/Streptococcus* and in cichlid fish with epizootic ulcerative syndrome were previously reported (Harbell et al., 1979; Barham et al., 1980; Pathiratne and Rajapakshe, 1998). Contrarily to that observed in this study, RBC and glucose did not alter with the injections. It can be suggested that the inoculum neither was sufficient to alter these parameters nor affected the haemopoiesis, as commented by Barham et al. (1980). On the other hand, fish injected with 1 x 10^6 CFU/mL of *Enterococcus* showed increased WBC, hematocrit and total number of thrombocytes. Haney et al. (1992) and Pathiratne and Rajapakshe (1998) have reported increased WBC corroborating our data. Total leucocyte counts suggested severe leucocytosis of 15.0 x 10^3 WBC/µl in control to 27.3 x 10^3 WBC/µl in fish injected with 1 x 10^6 CFU/mL of *Enterococcus*. This fact shows more production of leucocytes in bacterial injected fish enhancing the fish defense mechanisms.

The distribution of different types of leucocytes was also affected. Fish injected with 10^6 CFU/mL of *Enterococcus* were found to have the highest values of lymphocytes in the differential counting besides a decreased number of monocytes. Leucocyte belongs to an important cell involved in the immune response (Ellis et al., 1976). In fact, under severe infection the organism produces more white blood cells. It can be added that lymphocytes have been reported as immunocompetent cells. Contrary to the high number of neutrophils observed in infected fish by Pathiratne and Rajapakshe (1998), in this experiment only the saline solution was responsible for increased number of these cells, possibly due to the stressor effect as also commented by Martins et al. (2002). An interesting result was related to the decreased number of monocytes in 1 x 10^6 *Enterococcus*/mL injected fish. Possibly these cells are being recruited to the lesion site as commented by Matushima and Mariano (1996) and Martins et al. (2006). Contrarily, Rafiq et al. (2001) did not observe any alteration in the differential counts of white blood cells in tilapia challenged with *A. hydrophila*. In carp experimentally infected with *A. hydrophila*, Harikrishnan et al. (2003) have related increased WBC counts, corroborat-

ing our results. According to this author, decreased RBC counts and hematocrit indicate that erythrocytes are being affected or destroyed with the infection. In this study RBC counts did not alter, indicating that the inoculums were not sufficient to cause disease. It must be commented that the injection in the swim bladder could not be as aggressive to the same degree of intraperitoneal injection. Previous study showed the security in the use of the swim bladder as an inflammatory site (Martins et al., 2004a, Bozzo et al., 2007) by dint of the fact that this organ suffers little influence of contamination.

In *O. aureus* infected with *Corynebacterium* sp., Silveira-Coffigny et al. (2004) did not observe alteration in RBC as observed in the present assay. On the other hand, the increased number of lymphocytes was in agreement with these findings. Silveira-Coffigny et al. (2004) comments that the fish injected with *Corynebacterium* sp., a Gram positive bacteria responsible for severe mortality in tilapia aquaculture, only caused sub-clinical effects. The *Enterococcus* strain used in this experiment was isolated from diseased tilapia with the classical clinical signs. According to Petersen and Dalsgaard (2003), *Enterococcus* spp. is a good indicator of antimicrobial resistance. It must be emphasized the importance of this study to evaluate the haematological changes under experimental infection, and the fact that in South Brazil, the use of integrated tilapia culture with pig manure is common (Ghiraldelli et al., 2006). The importance in studying this Enterobacteriaceae was extensively commented by Petersen and Dalsgaard (2003).

Nowadays, thrombocyte has been found to be an important cell involved in fish defenses (Matushima and Mariano, 1996, Martins et al., 2006, Tavares-Dias and Moraes, 2007, Bozzo et al., 2007). Besides the high number of lymphocytes, the injection of *Enterococcus* was found to incite more production of thrombocytes in the circulating blood. In fact, whether thrombocytes are involved in fish cell response, these results could suggest their participation in such event. This is especially true in analyzing the findings with tilapia inflammation (Martins et al., 2004b; Bozzo et al., 2007). In this previous study, thrombocytes were the main cells present in the inflammatory exsudate suggesting their importance in fish cell response.

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