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Acute inflammatory response in Nile tilapia fed probiotic *Lactobacillus plantarum* in the diet

Geovana Dotta[1], José Luiz Pedreira Mouriño[1,2], Adolfo Jatobá[1,2], Ricardo Ernesto Burgos Morán[3], Celso Pilati[4] and Maurício Laterça Martins[1*]

*[1]Laboratório AQUOS-Sanidade de Organismos Aquáticos, Departamento de Aquicultura, Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Rod. Admar Gozaga, 1346, 88040-900, Florianópolis, Santa Catarina, Brazil. [2]Laboratório de Camarões Marinhos, Departamento de Aquicultura, Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil. [3]Universidad Politécnica de Catalunya, Campus del Baix Llobregat, Castelldefels, Catalunya, Spain. [4]Laboratório de Patologia Animal, Departamento de Medicina Veterinária, Cento de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Lages, Santa Catarina, Brazil. *Author for correspondence. Email: mlaterca@cca.ufsc.br*

ABSTRACT. The present study evaluated the acute inflammatory response induced by carrageenin (500 μg) injected in the swim bladder of Nile tilapia, after fed or not probiotic supplemented diet. Fifty four fish were distributed in six treatments and three replicates: Group A: Fish fed unsupplemented diet: 0.5 mL saline-injected fish; fish injected with 500 μg carrageenin diluted in 0.5 mL saline; Non-injected. Group B: Fish fed probiotic supplemented diet: saline-injected fish; carrageenin-injected fish; Non-injected. Fifteen days after feeding the fish were injected with carrageenin or saline. After six hours, inflammatory exudate was collected, as well as the blood for hematocrit, red blood cell (RBC) and white blood cell (WBC) counts, differential count of leucocytes and phagocytic activity in the blood. Supplementation with probiotic did not influence the RBC, hematocrit and the numbers of lymphocytes and basophils in the blood. The number of neutrophils was significantly higher in supplemented fish injected with carrageenin. Glucose concentration in supplemented and non-injected fish was higher than that observed in the saline injected ones. Probiotic potentialized the migration of cells to the inflammatory focus in the animals injected with the carrageenin irritant. In fish injected with saline and carrageenin occurred the greatest phagocytic activity in the blood in relation to those treatments.

Keywords: tilapia, probiotic, *Lactobacillus plantarum,* inflammation, haematology, phagocytosis.

RESUMO. Resposta inflamatória aguda em tilápia do Nilo alimentada com probiótico, *Lactobacillus plantarum* na dieta. Este trabalho avaliou a resposta inflamatória aguda induzida por injeção de carragenina (500 μg) na bexiga natatória de tilápia do Nilo suplementada ou não com probiótico na ração. Cinquenta e quatro animais foram distribuídos em seis tratamentos com três repetições: Grupo A: peixes alimentados com ração não-suplementada: Controle (injeção de 0,5 mL de solução salina estéril); Carragenina (injeção de 500 μg de carragenina); Não-injetada. Grupo B: peixes alimentados com ração suplementada com probiótico: Controle; Carragenina; Não-injetada. Após 15 dias de alimentação foi injetado carragenina ou salina. Após 6h, realizou-se a coleta de exsudato e sangue para determinação do hematócrito, contagens totais de eritrócitos, leucócitos, contagem diferencial de leucócitos e atividade fagocitária no sangue. A suplementação com probiótico na ração não influenciou o número total de eritrócitos, o hematócrito e os números de linfócitos e basófilos no sangue dos animais. O número de neutrófilos foi maior nos peixes suplementados com probiótico e injetados com carragenina. A glicose nos peixes suplementados com probiótico não-injetados foi maior do que nos injetados com salina. A suplementação com probiótico potencializou a migração de células para o foco inflamatório nos injetados com o flogógeno carragenina. Em peixes injetados com salina e carragenina, ocorreu maior atividade fagocitária no sangue em relação aos demais tratamentos.

Palavras-chave: tilápia, probiótico, *Lactobacillus plantarum*, inflamação, hematologia, fagocitose.

Introduction

The research on the action of probiotic bacteria in aquaculture is due to continued search for alternative strategies to prevent diseases (GOMEZ-GILL et al., 2000). Schrezenmeir and De Vrese (2001) proposed the use of probiotic term to designate products that contain viable micro-organisms in adequate amount responsible for

altering the mucosal microbiota by colonizing of an organ of the host with positive effects on their health.

Probiotics especially from lactic-acid bacteria have been used in diet for fish against infections (VERSCHUERE et al., 2000). Among the micro-organisms used as probiotic are *Bifidobacterium* and *Lactobacillus* (COLLINS et al., 1998; SANDERS; KLAENHAMMER, 2001; VIEIRA et al., 2008). *Lactobacillus* is considered the most safety organism by Collins et al. (1998) and Lee et al. (1999). The effects of lactic-acid bacteria against furunculosis caused by *Aeromonas salmonicida* were related by Gildberg and Mikkelsen (1998) in Atlantic cod *Gadus morhua* and by Nikoskelainen et al. (2001) in rainbow trout *Oncorhynchus mykiss* infected by vibriosis.

One of the most important primary defense of fish against pathogens is the inflammatory process. According to Martins et al. (2001, 2008), the inflammatory response in cultivated fish, the knowledge of hematological characteristics and phagocytic activity under stressfull stimuli of infections play an important role for diagnosis of fish health. Inflammatory response in Brazilian freshwater fish was evaluated after carrageenin injection in the swim bladder of Nile tilapia (*Oreochromis niloticus*) (MATUSHIMA; MARIANO, 1996) and in pacu (*Piaractus mesopotamicus*) (MARTINS et al., 2000, 2001). On this view, Bozzo et al. (2007) studied the cellular component of the inflammatory response induced by injection of thioglycolate, *Escherichia coli* lipopolyssacharide and heat-inactivated *Aeromonas hydrophila* in the swim bladder of pacu. Other studies tested colloidal carbon in *Tautogolabrus adspersus* (MACKMULL; MICHELS, 1932) lipopolyssacharide (LPS) of *Escherichia coli*, complete Freund adjuvant (CFA), turpentine and carrageenin (WHITE et al., 1981; JENKINS; KLESIUS, 1998), *Edwardsiella ictaluri* (HÉRBERT et al., 2000) and peptidoglican (KONO; SAKAI, 2001).

In the studies of Flores-Quintana and Moraes (2001), the acute inflammatory process is composed by metabolic and cellular changes such as an increase in the vascular permeability, white blood cell count and mainly neutrophils and their precursors. Not only the inflammatory exudate analysis (MARTINS et al., 2001), but also the phagocytic activity (Cai et al., 2004) and the glass cover slip implant in subcutaneous tissue (PETRIC et al., 2003) are techniques to verify the magnification of an inflammatory process when fish are exposed to several conditions. Closely to an inflammatory response analysis, is the knowledge of hematological variables that can reveal the fish health and cell behavior in the blood (BLAXHALL, 1972; REHULKA, 2002; MARTINS et al., 2004; 2008; GHIRALDELLI et al., 2006).

The evaluation of inflammatory response in fish associated to probiotic supplementation provides assistance to the development of culture methods actually considered as handling practices ecologically correct by improving the disease resistance (GATESOUPE, 1999).

In order to determine the effects of the addition of probiotic bacteria on the inflammatory and hematological response in Nile tilapia, fish were fed diets containing *Lactobacillus plantarum* isolated from tilapia after carrageenin or saline injection in the swim bladder.

Material and methods

Experimental conditions

Fifty four Nile tilapia with 184.94 ± 27.09 g mean weight and 21.47 ± 1.26 cm total length from Fundação 25 de Julho (Joinville/Santa Catarina State) were distributed in 18 aquaria of 100 L with constant aeration, biological filter and acclimated for 7 days before experiment. Six treatments with 3 replicates were as follows: non-injected and probiotic supplemented fish (NI); non-injected and unsupplemented fish (NI); supplemented fish injected with 500 μL de sterile saline solution 0.65% (IS); unsupplemented fish injected with saline (IS); supplemented fish injected with 500 μg carrageenin (Marine Colloids) diluted in 500 μL saline (IC); unsupplemented fish injected with 500 μg carrageenin (Marine Colloids).

Fish were fed twice a day, and after 15 days injected with carrageenin or saline in the swim bladder for evaluation 6 hours after injection. During this period the water quality was maintained in pH 7.40 ± 2.50, dissolved oxygen 5.09 ± 3.04 mg L^{-1} and temperature 21.70 ± 2.80ºC.

Preparing the probiotic supplemented diet

The diet was aspersed with *Lactobacillus platarum* from the MRS culture medium at a concentration of 1 x 10^9 colony forming units (CFU) mL^{-1}, in the proportion of 100 mL kg^{-1} dry ration containing 32% crude protein. The mixture was incubated during 24h at 35ºC, and the bags opened for drying for 24h at 35ºC. Unsupplemented diet was submitted to the same procedure except for the MRS aspersion. The isolation of lactic-acid bacteria and the viability of its use followed the procedures of Vieira et al. (2008) and Jatobá et al. (2008).

Injection and collection of exudate cells

After feeding period the animals were anesthetized by immersion in benzocaine (50 mg L^{-1}) (protocol approved by Ethic Commitee no. 23080.009404/2006-15/CEUA/UFSC) for injection of 500 μg carrageenin diluted in 500 μL sterile saline solution 0.65% or only saline according to Martins et al. (2009). After injection, the fish were maintained in the aquaria for 6h until the sacrifice. The interior of the swim bladder was washed with 0.5 mL complete phosphate buffered saline (PBS) containing 0.01 mL EDTA 5%, and cell suspensions were centrifuged at 150 G gravity for 10 min. before staining. With the aid of a Pasteur pipette, the content was collected into centrifuge tubes maintained in ice, diluted to 1:4 and the total number of leucocytes (number μL^{-1}) counted in Neubauer chamber. Afterwards, the cells were centrifuged at 150 G for 10 minutes, and the supernatant discarded. A drop of serum obtained from the same fish species was added to the precipitate and smears of the exudate cells made for the differential count of macrophages, lymphocytes, granulocytes and thrombocytes. After drying, the smears were fixed with methyl alcohol (three minutes) and stained with Giemsa according to the method proposed by Martins et al. (2001).

Hematological analysis

Six hours after injection the animals were anesthetized and the blood was withdrawn from the caudal vessel into a syringe containing a drop of 10% EDTA solution. The blood was utilized to measure hematocrit (GOLDENFARB et al., 1971); total count of red blood cells (RBC) in a haemocytometer; the total count of white blood cells (WBC), and total number of thrombocytes by indirect method (ISHIKAWA et al., 2008). 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.

Phagocytic activity

Leukocyte phagocytic function followed the method of Cai et al. (2004) slightly modified as follows: after blood collection, 0.5 mL of blood was dropped into centrifuge tubes, added 0.25 mL of 1 x 10^6 *Enterococcus* suspension and shaked. The tubes were kept at 28ºC in a water bath for 30 min., and shaken every 10 min. After this time, in order to centrifuge as recommended by Cai et al. (2004), the blood smears were done in duplicates just after incubation, dried, fixed in methylic alcohol and stained by Giemsa/May-Grunwald (ROSENFELD, 1947). The number of leukocytes that engulfed bacteria was counted in percentage in relation to total leukocyte number in the smear from the phagocytosis assay.

Statistical analysis

The animals were randomly distributed among six treatments (supplemented and saline injected fish, un-supplemented and saline injected, supplemented and carrageenin injected, un-supplemented and carrageenin injected, supplemented non-injected, un-supplemented non-injected) with 3 replicates (3 fish per aquarium forming an experimental unit). The averages were compared by the Tukey test, at 5% probability and the percentage from the differential count of the blood and exudate cells were transformed in arc sin ($\sqrt{P+0.5}$).

Results

Probiotic supplementation did not influence ($p > 0.05$) on the RBC, hematocrit percentage and the numbers of lymphocytes and basophils in the blood (Tabela 1). But an inflammatory reaction in the swim bladder was noted after carrageenin injection (Figura 1). On the other hand, the addition of probiotic provoked decreased ($p < 0.05$) WBC count in the blood of non-injected (NI) and in saline injected fish (IS). Moreover, fish fed probiotic and saline injected (IS) showed lower number ($p < 0.05$) of circulating leukocytes than the other treatments.

Figure 1. Inflammatory response in the swim bladder of Nile tilapia fed probiotic supplemented diet and injected with 500 μg carrageenin six hours after injection.

Glucose levels in probiotic supplemented fish and non-injected (NI) were significantly higher ($p < 0.05$) than that observed in saline injected (IS) (Table 1). The differential counting of leukocytes in carrageenin injected fish (IC) either supplemented or not with probiotic showed an increase ($p < 0.05$) in the monocyte number when compared to saline injected ones. Interestingly, the number of neutrophils was significantly higher in probiotic supplemented and carrageenin injected fish (IC).

Regarding the inflammatory response, the probiotic supplementation caused an increase ($p < 0.05$) in WBC and thrombocytes count after carrageenin injection (IC), as well as in macrophage and lymphocyte number on the inflammatory exudate in relation to unsupplemented fish (IC). It was also observed a significant increase in WBC count in supplemented fish both saline and carrageenin injected. Table 2 shows lower WBC number in the exudate of carrageenin (IC) injected fish than saline injected (IS). The number of macrophages on the inflammatory exudate of unsupplemented fish (IS) was lower ($p < 0.05$) than that related to the other treatments (Table 2).

Probiotic supplementation in the diet in both saline and carrageenin injected fish increased ($p < 0.05$) the phagocytic activity as shown in Table 2.

Discussion

Hematological parameters are widely used as an important tool for fish health diagnosis under stressful conditions normally found in intensive culture. Moreover, the analysis of inflammatory exudates may help the comprehension of the inflammatory process in fish.

In contrary to an increase in the number of WBC observed by Selvaraj et al. (2005) in carp (*Cyprinus carpio*) intraperitoneally injected with beta-glican of *Saccharomyces cerevisae* the present results did not show difference among saline and carrageenin injected fish. It must be emphasized that differently from that related by Selvaraj et al. (2005), unsupplemented fish injected with both saline and carrageenin did not show significant difference in WBC and monocytes in relation to non-injected fish. Similarly to that reported by Selvaraj et al. (2005), it can be thought that the increased number of monocytes in carrageenin-injected tilapia was due to the fact that these cells are directly involved on phagocytosis of foreign particles or organisms.

Table 1. Hematological characteristics of Nile tilapia supplemented or not with probiotic in the diet, non-injected (NI) and six hours after injection with saline (IS) or carrageenin (IC) in the swim bladder. Different letters indicate significant difference among the treatments ($p < 0.05$). RBC: red blood cell, WBC: white blood cell.

Treatments	RBC (x 1000 μL^{-1})	Hematocrit (%)	WBC (x 1000 μL^{-1})	Thrombocytes (x 1000 μL^{-1})	Glucose (mg dL^{-1})	Monocytes (n° μL^{-1})	Lymphocytes (n° μL^{-1})	Neutrophils (n° μL^{-1})	Basophils (n° μL^{-1})
Supplemented diet (NI)	1198.89±70.74	30.36±5.83	54.52±2.23 b	15.4±1.02 b	150.33±77.8 a	19.57±2.03 bc	17.65±3.68	16.99±2.36 b	0.47±0.62
Unsupplemented diet (NI)	1455.56±44.39	26.53±2.46	67.65±4.14 a	26.82±0.59 a	96.33±19.53 b	27.02±3.34 a	15.67±3.37	23.63±2.34 b	0.61±0.26
Supplemented diet (IS)	1220±104.77	27.33±1.63	37.86±6.35 bc	15.23±0.51 b	48.78±4.11 c	11.29±3.22 c	13.88±3.4	12.14±3.85 b	0.15±0.16
Unsupplemented diet (IS)	1497.78±471.1	27.56±0.25	47.01±19.54 b	23.17±12.2 b	109.89±7.75 b	14.09±3.49 c	15.2±8.87	17.71±7.2 b	0±0
Supplemented diet (IC)	1328.89±166.21	28.28±0.49	59.6±17.75 b	18.44±5.02 b	111.78±6.99 b	18.23±4.79 bc	14.4±5.72	24.41±8.11 a	0.88±1.15
Unsupplemented diet (IC)	1213.33±113.72	28.31±1.42	53.52±2.09 b	16.01±3.83 b	124.89±6.05 b	19.8±1.95 b	9.54±1.6	22.46±1.99 b	0.15±0.26

Table 2. Mean values and standard deviation of white blood cells (WBC) and thrombocytes counts and differential count of leukocytes on the inflammatory exudate of Nile tilapia supplemented or not with probiotic in the diet non-injected (NI) and six hours after injection with saline (IS) or carrageenin (IC) in the swim bladder. Different letters indicate significant difference among the treatments ($p < 0.05$).

Treatments	WBC (n° μL^{-1})	Thrombocytes (n° μL^{-1})	Macrophages (n° μL^{-1})	Lymphocytes (n° μL^{-1})	Granulocytes (n° μL^{-1})	Phagocytosis (%)
Supplemented diet (IS)	94111,1±31157,9 a	5609,7±3994,1 a	41695,7±11195,8 a	17038,8±8610,3 a	29766,9±15201	34±3,84 a
Unsupplemented diet (IS)	35555,6±8441,5 c	1918,6±740,5 b	12202,8±3703,9 c	7395,3±2049,1 b	13269±3408,7	21±1,9 b
Supplemented diet (IC)	60888,9±21677,8 b	4972,5±2726,7 b	25636,6±7743,3 b	9039,3±4413,2 b	21240,4±6962,6	34±4,67 a
Unsupplemented diet (IC)	50555,6±17752,4 bc	2629,5±498,4 b	25170,6±7762,5 b	7454,2±1639 b	16071,1±8514,7	18±1 c
Supplemented diet (NI)						17±0,69 c
Unsupplemented diet (NI)						21±1,2 bc

The lack in leukocyte response in supplemented saline/carrageenin-injected tilapia is in agreement with the findings of Selvaraj et al. (2005) in carp orally fed *S. cerevisae*. As above supported, the feasible way of probiotic supplementation would be via oral.

Enhanced WBC number in indian carp (*Labeo rohita*) supplemented with *Bacillus subtilis* and vitamin C (NAYAK et al., 2007) and in tilapia supplemented with *L. plantarum* (JATOBÁ et al., 2008) differed from the present result, where WBC decreased in carrageenin injected fish when compared to non-injected. Nevertheless, the decreased WBC number in supplemented fish injected with both saline and carrageenin might be explained by the fact that the probiotic supplementation must have favored the migration of cells to the injected place. Based on this fact, it can be inferred that the reduction in monocytes number in saline and carrageenin injected fish were due to cell migration for phagocytosis.

Kumar et al. (2008) studying indian carp supplemented with *B. subtilis* observed increased number of granulocytes and monocytes but a decrease in lymphocyte number. This assay is in agreement with the results of Kumar et al. (2008) on the neutrophill number in supplemented and carrageenin injected fish. Contrarily to that observed by Kumar et al. (2008) and Jatobá et al. (2008), in this work circulating lymphocytes were not affected by probiotic supplementation. By supporting the above statements, it can be concluded that probiotic supplementation has enhanced the fish immune system.

This study showed no influence of the probiotic supplementation on RBC and hematocrit as related by Jatobá et al. (2008). However, experimental conditions, fish species and their genetic varieties could influence on the hematological response.

Greater migration of leukocytes in carrageenin injected tilapia was in agreement with the inflammatory response found by Martins et al. (2001, 2004, 2006, 2008, 2009) and Bozzo et al. (2007) in tambacu hybrid (*Piaractus mesopotamicus* male x *Colossoma macropomum* female) injected with carrageenin and thioglycolate, in tilapia injected with carrageenin and LPS and in pacu (*P. mesopotamicus*) injected with carrageenin, thioglycolate or inactivated *Aeromonas hydrophila*. Contrarily to that reported by Matushima and Mariano (1996) in tilapia, in the differential count of cells in the inflammatory exudate in this assay, macrophages and granulocytes were the main cells found followed by lymphocytes and thrombocytes. Despite the fact that pacu injected with LPS (FLORES-QUINTANA; MORAES, 2001), similar inflammatory response characterized either by macrophages or lymphocytes were here observed. Increased number of macrophage and thrombocyte in carrageenin injected tilapia was in agreement with Martins et al. (2006, 2008) observations. Similarly to the present result, vitamin C and E supplementation improved the migration of thrombocytes to the inflammatory site (BOZZO et al., 2007). Inflammatory infiltrated cells were also related in vitamin C-supplemented *Cirrhinus mrigala* injected with Freund Complete Adjuvant (FCA) (SOBHANA et al., 2002). On the other hand, chromium supplementation in pacu did not affect the exudate cell component (FLORES-QUINTANA; MORAES, 2001). The present results showed higher number of thrombocytes, macrophages and lymphocytes in the inflammatory exudate that comprove the probiotic action on fish defense system (VERSCHUERE et al., 2000). The evident involvement of thrombocytes as defense cells was also observed in the swim bladder of pacu after different stimuli (BOZZO et al., 2007).

Although it was not related significant difference in migrated granulocytes to the inflammatory site, their number was higher than lymphocytes and thrombocytes. According to Suzuki and Iida (1992) neutrophils migrate more rapidly to an inflammatory site than the macrophages. Greater migration of macrophages followed by granulocytes in this study was in agreement with the observations of Afonso et al. (1998) in peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*).

Matsuyama and Iida (1999) reported the highest migration of neutrophils to the inflammatory swim bladder exudate in tilapia 24 hs after injection with formalin-killed *E. coli*. Contrarily to that observed by Matsuyama and Iida (1999) 6 hs after injection was sufficient to migration of granulocytes to the inflammatory site. As also related by Martins et al. (2004) it was not possible to obtain resident population of leukocytes and thrombocytes in unstimulated or non-injected swim bladder.

Phagocytic activity of circulating blood leukocytes of the present assay varied from that previously found by other authors especially in tilapia (MARTINS et al., 2008). Cai et al. (2004) observed 61 and 39% phagocytosis, respectively in Nile tilapia and blue tilapia (*Oreochromis aureus*). On the other hand, Casas Solis et al. (2007) verified 74.3 to 88.1% phagocytic activity in three species of tilapia and their hybrids. In the present study lower phagocytosis was observed when compared to 56% phagocytosed bacteria from 1×10^6 CFU *Enterococcus* mL^{-1} treatment found by Martins et al. (2008). In

this work, the highest phagocytic activity in probiotic supplemented fish both injected with saline and carrageenin was clearly observed. The difference in phagocytic response depends on the fish species and experimental conditions. In spite of similar water temperature in this assay and of Martins et al. (2008), there was difference in phagocytic activity.

In the studies of Cai et al. (2004) and Casas Solis et al. (2007) fish were maintained at 28ºC. Differently, in this study the water temperature was near to that normally found in Southern Brazil in fish farm. It can be thought that the lower phagocytosis percentage could be related to this fact. This is especially true when high temperature stimulates more efficiently the inflammatory response as reported by Finn and Nielsen (1971). In probiotic supplemented fish injected with saline, a decrease in WBC number was accompanied by an increase in this cell number in the inflammatory exudate. The results suggested the responsivity of tilapia fed *L. plantarum* under this experimental conditions. Moreover, this work was able to comprove the highest migration of leukocytes and thrombocytes to the inflammatory site. In order to verify the effects of circulating hormones on the inflammatory response, further studies must be carried out with this animal model under stressfull condition.

Conclusion

This work has demonstrated that tilapia fed probiotic *L. plantarum* in the diet showed more migration of cells to the inflammatory site, the swim bladder than those not supplemented fish. Besides the reduction in the circulating white blood cell count an increase on these cells on the inflammatory exsudate was found in fish supplemented with probiotic. Probiotic supplementation was also responsible for enhanced phagocytic activity in both saline-injected and carragenin-injected fish.

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