EFFECTS OF DIETARY BOVINE LACTOFERRIN ON NON-SPECIFIC IMMUNE RESPONSE OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Abstract

Lactoferrin (LF) is a multifunctional iron-binding protein mainly present in the milk and other exocrine secretions of mammals. It belongs to the iron transporter family and plays an important role in the nonspecific immune response, antimicrobial and antioxidant actions. This study was carried out on 125 *Oreochromis niloticus* (divided into five equal groups) to evaluate the protective effects of the LF in the ration of the Nile-tilapia exposed to crowding stress (gps. 2-5) and given LF at the concentrations of 0, 100, 200 or 400 mg/kg ration, respectively. After 1, 2 and 4 weeks of administration, the total leukocytes count, respiratory burst index and lymphocyte transformation index were measured as a monitor for the cellular immune response. Moreover, the serum lysozyme as an indicator of humoral immune responses.

The results showed that, the LF feeding at 200 mg/kg diet for 2 weeks enhanced the cellular innate immune response. The total leukocytic count and serum lysozyme were significantly increased with 400 mg/kg ration for 4 weeks. The crowding stress produced a significant increase in the plasma cortisol and glucose. On the other hand, neither the plasma glucose nor the cortisol concentrations were affected in the dietary LF groups.

It is therefore concluded that, dietary LF supplementation could be considered as immunostimulant for overcrowded farmed Nile tilapia (*Oreochromis niloticus*).

INTRODUCTION

The intensive aquaculture systems expose the fish to repeated stressors, which have deleterious effects on the production and immune status. Mass mortalities have become a serious problem due to the intensification of the aquaculture allover the world (Stoskoph, 1993). Lactoferrin (LF) is 80 kDa iron-binding glycoprotein. It is expressed in various secretions and tissues of mammals (Weinberg, 2003). LF is secreted from activated polymorphonuclear leukocytes during inflammation (Larkins, 2005). LF appears to play several biological roles including antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and anticancer effects, besides immunomodulatory activities (Nemet and Simonovits, 1985, Sakai *et al.,* 1993, Hagiwara *et al.,* 1997, Wang *et al.,* 2000 and Weinberg, 2003).

However, the results of LF administration have varied depending on the species of fish and test conditions (Esteban *et al.,* 2005). LF administration enhanced the tolerance against high stocking density stress in rainbow trout (Kakuta, 1997). Juvenile ayu fed on dietary LF had a higher tolerance to stress when exposed to low dissolved oxygen, formalin and copper sulfate solutions (Kakuta *et al.,* 1998). The resistance of Asian catfish (*Clarias batrachus*) to *Aeromonas hydrophila* and activity of several non-specific immune parameters were increased in fish fed 100 mg LF/kg feed, but the specific immunity was not affected (Kumari *et al.,* 2003). Esteban *et al.,* (2005) found enhanced innate immune cellular activity, mainly respiratory burst and natural cytotoxic activity in gilthead sea bream (*Sparus auratus* L.) feed human LF 0, 50, 100 or 200 mg/ kg diet for 2 weeks.

On the other hand, the dietary administration of LF to the Atlantic salmon failed to decrease the mortality rate when challenged with *Aeromonas salmonicida* or Infectious salmon anemia virus (Lygren *et al.,* 1999). Welker *et al.,* (2007) did not find any increase in the activity of the non-specific or specific immune parameters (plasma lysozyme and spontaneous haemolytic complement activities or agglutination antibody titer against *Staphylococcus iniae*) of *Oreochromis niloticus* with the addition of bovine

LF to the basal diet at 0, 200, 400, 800, or 1600 mg/kg diet taken twice daily for 8 weeks.

The feasibility of studying the disease and infection is facilitated by various stressors, handling, intensive culture and overcrowding biomass (Davis *et al.,* 2002, Wedemeyer, 1996 and Welker *et al.,* 2007) respectively. It is, therefore, important to enhance the tolerance against stress for cultured fish.

Oreochromis niloticus is among the most important cultured species in Egypt and allover the world. The use of immunostimulants has been suggested to be an effective mean to control fish mortality in increased biomass in aquaculture. The efficacy of the dietary LF on the immune function and disease resistance is not well established (Welker *et al.,* 2007). Therefore, the objective of this study was to determine the effects of the orally administered LF to *O. niloticus* on the non-specific immune response under the over crowding stress.

MATERIAL AND METHODS

Experimental fish

A total number of 125 apparently healthy Nile tilapia (*Oreochromis niloticus*) weighing 100 gm was obtained from a local commercial fish farm. They were maintained in glass aquaria filled with dechlorinated tap water which continuously aerated. The fish were supplied with a commercial fish ration without LF. Moreover, a 12 h dark: 12 h light photoperiod was provided. They were acclimatized to the laboratory conditions for 15 days before the start of the experiment. The water temperature was kept at 24±2 °C throughout the experiment. About half of the water was changed daily in all the experimental aquaria. The fecal matters were siphoned out once daily. The biomass of the fish in each aquarium was measured at the beginning of the experiment and after each sampling to adjust the daily ration.

Rations

A standard commercial ration was supplemented with lactoferrin at the concentrations of 0, 100, 200 and 400 mg/kg ration. The dietary ingredients were thoroughly mixed in a mixer and extruded through a 2.5-mm diameter in a meat grinder. The pellets were air-dried at room temperature (28°C), broken into small pieces, sieved to obtain appropriate size, and stored at −5 °C until used.

Experimental design

A total number of 125 *O. niloticus* was randomly allotted into five equal groups. Group (Gp. 1) was the control. Groups (2-5) were subjected to stress of overcrowdiness by reducing the volume of the water to the half. Gps. (1 & 2) were fed on the control ration (0 lactoferrin). Gps (3-5) were fed on ration containing lactoferrin at the doses of 100, 200 and 400 mg/kg ration respectively. All the fish were fed twice daily at the rate of 2% of their body weight throughout the period of the experiment. Prior to handling, sampled fish were euthanized in 200 mg/l MS-222.

Hematological and immunological studies

Five fish were randomly sampled from each group at the end of 1st, 2nd and 4th week of the experiment. Blood samples were collected by heart puncture in air-dried, heparinized sterile test tubes (500 U sodium heparinate /ml) to study the non-specific defense mechanism, total and differential leukocytic count, neutrophil adhesion test and lymphocyte transformation test. The remaining whole blood samples were centrifuged at 3000 rpm for 5 minutes and plasma was stored at -80 °C to be used for plasma lysozyme assay. The peritoneal macrophages were isolated to assay the macrophage oxidative burst. The total and differential leukocytic counts were performed in duplicate for each sample according to Stoskoph, (1993). The following haematological and immulological techniques have been carried out:

a- Neutrophils glass- adherence: using nitroblue tetrazolium (NBT) assay was determined according to Anderson *et al.,* (1992). Briefly, within 15 minutes after blood samples were collected, one drop of blood using heparinized capillary

hematocrit tubes was placed onto a 22-mm square coverslip. The coverslips were placed individually in Petri-dishes humid chambers and incubated for 30 minutes at room temperature (25°C) to allow the neutrophils to stick to the glass. After incubation, the coverslips were gently washed with phosphate buffer solution (PBS) at of pH 7.4 and the cells were transferred upside down to a microscope slide containing a 50 µl drop of 0.2% filtrated NBT solution (Fluka Buchs, Co. Switzerland). After other 30 minutes of incubation, the positive, dark-blue stained cells were counted under the microscope. Two coverslips were examined for each fish. Three random fields were counted for each slide. The six fields were averaged. The mean and standard error of the fish lots were calculated.

b- Lymphocytes transformation index: was determined according to Barta (1984). Briefly, equal whole blood Rosewell Park Memorial Institute (RPMI) mixture for lymphocytes was isolated on cell separating medium Hiostopaque (Sigma). The RPMI (Sigma) was mixed with 40% bovine fetal serum. The harvested cells were washed 3 time with balanced Hank's salt solution (Sigma) without calcium and magnesium but with Hepes. The absence of calcium prevents the clotting of the lymphocytes which were isolated from heparinized blood. The viable lymphocytes concentration was standardized at $2X10^6$ /ml. Flat-bottom microtiter plates were set up with 200µ/l (100µ/l RPMI serum mixture and 100 µ/l lymphocyte cell suspension). Five µ/l of mitogen Phytohemagglutinin (PHA) 1mg/ml (in sterile PBS) was added to each well. Non stimulated cultures were prepared in the same manner except without addition of mitogen. All assays were performed in triplicate for each sample. The microtiter plates were incubated at 37°C in a humid atmosphere containing 5% CO2 for 48 hours. Each culture medium was transferred in a micro-tube and centrifuged at 400g for 10 minutes. The supernatant was collected and glucose concentration was determined with semi-automatic spectrophotometer (BM-Germany 5010) using a standard (100 mg/dl) glucose solution (Werner *et al.,* 1970). The blast transformation index (TI) was calculated as follows: TI %=[(MG–SG)/MG] X100, where MG=glucose

concentration in the non stimulated culture medium and SG=glucose concentration in the sample after incubation (Khokhlova *et al.,* 2004).

c- Plasma lysozyme was determined by the turbidometric assay (Parry *et al.,* 1965). Briefly, the lysozyme substrate was 0.75 mg/ml of gram positive bacterium *Micrococcus lysodeikticus* lyophilized cells (Sigma, St. Louis, MO). The substrate was suspended in 0.1 M sodium phosphate/citric acid buffer, pH 5.8. Plasma or mucus (25 µl) was placed, in triplicate, into a microtiter plate and 175 µl of substrate solution was added to each well at 25 ^{0}C. The reduction in absorbance at 450 nm was read after 0 and 20 minutes using microplate ELISA reader (Bio TEC, ELX800G, USA). The units of lysozyme present in plasma or mucus (µg/ml) were obtained from standard curve made with lyophilized hen-egg-white-lysozyme (Sigma).

Cortisol and Glucose Determination

The cortisol was measured using an ELISA kit manufactured by Alpha Diagnostic International, Inc. (TX, USA). The glucose concentration was determined by kits (Human Co. Germany.) according to the manufacturer's instructions.

Statistical analysis

The data were statistically analyzed by ANOVA test with posthock LSD multiple comparison test using statistical software program State View 4.01 (1993). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Immunostimulants are useful in fish culture because they can improve the immune response and so increase the disease resistance. The use of natural immunostimulants is promising in aquaculture because they are safe for the environment and human health, biocompatible and biodegradable (Ortun *et al.,* 2002). The LF is an iron-binding protein included in several biological functions (Weinberg, 2003).

The result of this work revealed that, the orally administered LF enhanced the non specific cellular immunity and the lymphocyte transformation index at a dose of 400 mg at the end of the 4th week and neutrophils adhesion at a dose 200 and 400 mg at the end of the 2nd & 4th weeks post-administration respectively when compared with the stressed group (Tables, 1-3). Esteban *et al.,* (2005) showed that the LF feeding at a rate of 100 mg/kg diet for 1 week enhanced the cellular immune response, leukocyte peroxidase content, respiratory burst and phagocytic index in the gilthead seabream. Moreover, Sakai *et al.,* (1995) stated that the LF increased the chemiluminescent response and nitroblue tetrazolium reaction of the phagocytic cells in *Oncorhynchus mykiss*. It is worthy to mention that, when the mammalian phagocytes were exposed to a highly purified human LF, they exhibited an increased phagocytic activity and were primed to produce more radical oxygen intermediates (Miyauchi *et al.,* 1998 and Gahr *et al.,* 1991).

The present study, showed a significant increase in the serum lysozyme in fish group received 400mg/ Kg diet at the end of the 4th week post-administration when compared with crowding stress non-fed group (Tables, 1-3). The results were in accordance with Kumari *et al.,* (2003) who reported that the Asian catfish fed 100 mg LF /kg diet for 1 week, showed enhanced serum lysozyme levels. However, no effect of LF administration was found on the humoral non-specific immunity as was described in the Atlantic salmon (Lygren *et al.,* 1999), gilthead sea bream (Esteban *et al.,* 2005) and Nile tilapia (Welker *et al.,* 2007).

Stress-induced increase in cortisol blood level has been reported by Vosyliene (1996) in different species of fishes. In the present study, the LF administration was not associated with any a significant reduction in the measured cortisol and glucose in the Nile tilapia after exposure to the crowding stress (Tables, 1-3). The results were in accordance with Welker *et al.,* (2007), who reported that the LF did not produce any reduction in the elevated blood cortisol and glucose levels in the stressed Juvenile Nile tilapia (*Oreochromis niloticus*) when fed nutritionally complete, practical basal diets supplemented with the bovine lactoferrin at 0, 200, 400, 800, or 1600 mg/kg diet twice daily for 8 weeks. Other species of fish showed an increased resistance to the stress as a response to LF in their diet. The cortisol and glucose concentrations in carp were decreased with increasing the levels of the dietary LF for 14 days (0, 0.01, 0.1, and 1% of diet) after exposure to high rearing density or hypoxia stress (Kakuta *et al.,* 1998).

The results showed leukocytosis, neutrophilia, esinopenia and lymphopenia in all the investigated LF fed fish groups when compared with the non-fed control group (Tables, 1-3). Małgorzata (2005) recorded neutrophilia and lymphopenia in the common carp when subjected for 3 hours to 10, 5, 10 or 20 mg/l of lead, copper, cadmium and zinc, respectively. It is known that cortisol is secreted during the stress reaction. It shortens the life span of lymphocytes and promotes their apoptosis (Wyets *et al.*, 1998 and Verburg *et al.,* 1999). Moreover, it reduces their proliferation, leading to a decrease in both lymphocyte count and activity are often observed due to stress.

The results of this work suggested that lactoferrin had a direct positive effect on the non specific cellular immunity of Nile-tilapia when mixed with fish ration in overcrowded fish culture facilities.

Table 1. Some Immunological and Hematological Parameters (Mean ± S.E) in Nile tilapia (*Oreochromis niloticus*) treated with Lactoferrin for 1 week.

Fish Groups	Neutrophil Adhesion Cells/HPF	Lymphocyte Transformation Index	Lysozyme µg/ml	Cortisol ng/ml	Glucose mg/dl	TLC $10^3/\mu L$	Neutrophil $10^3/\mu L$	Eosinophil $10^3/\mu L$	Basophil $10^3/\mu L$	Lymphocyte $10^3/\mu L$	Monocyt $10^3/\mu L$
G1	8.42b ±0.64	26.24b ±1.65	9.95 ±0.68	41.58b ±4.2	53.4b ±5.22	33.78b ± 3.46	6.16b ± 0.61	1.22b ± 0.08	0.00	24.82b ± 1.42	1.58 ± 0.16
G2	5.91a ±0.41	15.21a ± 1.34	8.75 ± 0.57	74.51a ±7.45	76.81a ±6.12	51.64a ± 4.14	33.30a ± 4.65	0.57a ± 0.06	0.00	16.12a ± 1.46	1.65 ± 0.18
G3	5.74a ±0.45	14..6 a ± 1.45	8.81 ± 0. 41	71.41a ±6.52	74.24a ±5.14	49.21a ± 3.85	28.81a ± 3.81	0.64a ± 0.07	0.05 ± 0.05	17.84a ± 1.68	1.87 ± 0.26
G4	6.12a ±0.51	15..92 a ± 1.65	9.05 ± 0. 62	78.56a ±8.75	72.29a ±6.10	48.36a ± 3.14	26.78a ± 3.05	0.68a ± 0.05	0.00	19.15a ± 2.48	1.74 ± 0.17
G5	5.68a ±0.42	16.85a ± 1.81	9.12 ± 0. 62	76.42a ±7.35	79.32a ±7.34	46.94a ± 3.76	27.51a ± 3.12	0.66a ± 0.04	0.00	16.95a ± 1.82	1.83 ± 0.19

The same column not followed by the same letter differs significantly ($P<0.05$).

Table 2. Some Immunological and Hematological Parameters (Mean ± S.E) in Nile tilapia (*Oreochromis niloticus*) Treated with Lactoferrin after 2 weeks.

Fish Groups	Neutrophil Adhesion Cells/HPF	Lymphocyte Transformation Index	Lysozyme µg/ml	Cortisol ng/ml	Glucose mg/dl	TLC $10^3/\mu L$	Neutrophil $10^3/\mu L$	Eosinophil $10^3/\mu L$	Basophil $10^3/\mu L$	Lymphocyte $10^3/\mu L$	Monocyt $10^3/\mu L$
G1	8.94b ±0.72	24.14b ±1.52	10.81b ±0.74	45.21b ±4.78	58.1b ±5.10	34.28b ± 3.15	8.91b ± 0.95	1.06b ± 0.09	0.07 ± 0.07	22.5b ± 1.25	1.74 ± 0.15
G2	5.53a ±0.38	15.58a ± 1.41	8.05a ± 0.61	78.31a ±8.35	86.45a ±8.35	49.35a ± 2.47	28.98a ± 3.12	0.54a ± 0.05	0.00	17.95a ± 1.18	1.88 ± 0.12
G3	5.85a ±0.42	15..35 a ± 1.56	8.12a ± 0. 71	73.28a ±7.12	79.12a ±7.15	46.78a ± 3.21	26.41a ± 2.96	0.56a ± 0.04	0.00	18.12a ± 1.29	1.69 ± 0.16
G4	6.21a ±0.51	16.48 a ± 1.52	8.32a ± 0. 52	75.16a ±9.15	75.85a ±6.08	47.82a ± 2.94	26.83a ± 3.45	0.67a ± 0.06	0.00	18.65a ± 1.38	1.67 ± 0.19
G5	6.74c ±0.46	17.25a ± 1.78	8.28a ± 0. 57	77.32a ±8.05	81.41a ±7.92	45.91a ± 3.56	24.45a ± 2.88	0.64a ± 0.05	0.00	19.01a ± 1.15	1.79 ± 0.15

The same column not followed by the same letter differs significantly ($P<0.05$).

Table 3. Some Immunological and Hematological Parameters (Mean ± S.E) in Nile tilapia (*Oreochromis niloticus*) treated with Lactoferrin for 4 weeks.

Fish Groups	Neutrophil Adhesion Cells/HPF	Lymphocyte Transformation Index	Lysozyme µg/ml	Cortisol ng/ml	Glucose mg/dl	TLC $10^3/\mu L$	Neutrophil $10^3/\mu L$	Eosinophil $10^3/\mu L$	Basophil $10^3/\mu L$	Lymphocyte $10^3/\mu L$	Monocyt $10^3/\mu L$
G1	8.41b ±0.63	23.15b ±1.35	11.05b ±0.81	48.31b ±5.21	56.8b ±5.42	36.56b ± 3.14	8.05b ± 0.71	0.96b ± 0.07	0.00	25.9b ± 1.45	1.65 ± 0.12
G2	5.48a ±0.49	14.45a ± 1.31	7.42a ± 0.53	84.52a ±9.15	81.34a ±8.01	47.25a ± 2.34	26.65a ± 2.32	0.52a ± 0.04	0.00	18.24a ± 1.21	1.84 ± 0.18
G3	6.15ac ±0.37	15..95 a ± 1.76	8.21a ± 0. 56	78.91a ±8.24	78.85a ±7.34	45.34a ± 2.95	23.56a ± 2.81	0.55a ± 0.06	0.00	19.45a ± 1.14	1.78 ± 0.15
G4	6.39ac ±0.54	18..51c ± 1.61	8.18a ± 0. 63	82.35a ±8.95	79.31a ±6.37	46.21a ± 2.73	25.71a ± 2.98	0.61a ± 0.05	0.05 ± 0.05	17.91a ± 1.58	1.91 ± 0.24
G5	6.85c ±0.43	19.02c ± 1.65	8.98c ± 0. 54	79.05a ±8.45	76.29a ±6.12	47.14a ± 2.84	24.68a ± 2.48	0.66a ± 0.07	0.00	20.08a ± 1.65	1.72 ± 0.21

The same column not followed by the same letter differs significantly ($P<0.05$).

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تأثير لاكتوفيرن البقرى علي الاستجابة المناعية الغير متخصصة فى أسماك البلطى النيلي

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يزداد الوعي البيئي يوما بعد يوم بضرورة استخدام المركبات الطبيعية للوقاية والعلاج من الأمراض ولرفع المناعة ضد عوامل الاجهاد. ويعتبر اللاكتوفيرين ذو وظائف كثيرة، فهو عبارة عن بروتين متحد مع الحديد، ويتواجد فى اللبن والأفرازات الأخرى للثدييات. انه ينتمى الى عائلة حاملات الحديد حيث يلعب دورا هاما وحيويا فى رفع المناعة الغير متخصصة، ومضاد للميكروبات ومضاد للأكسدة .

وقد تمت هذه الدراسة لتقييم كفاءة اللاكتوفيرين بعد خلطه بالعلف بتركيزات ٠، ١٠٠، ٢٠٠ أو ٤٠٠ ملجم/كجم علف فى أسماك البلطى النيلي مع تعرضها للأجهاد نتيجة ازدحام الأسماك فى الحوض .

تم الحصول على عدد ٥ أسماك عشوائيا من كل مجموعة عند ١، ٢، ٤أسبوع وتشير النتائج الى ما يلى :

- ان اضافة اللاكتوفيرين الى العليقة عند تركيز ٢٠٠ ملجم/كجم علف لمدة أسبوعين يزيد من الأستجابة المناعية الخلوية، فى حين ان تركيز ٤٠٠ ملجم/كجم يرفع من العدد الكلى لخلايا الدم البيضاء ويزيد من أنزيمات التحلل بصورة معنوية بعد ٤ أسابيع من التغذية.
- يؤدى ازدياد كثافة الأسماك فى الحوض الى زيادة معنوية فى نسبة الكورتيزول والجلوكوز فى امصال الأسماك، على الجانب الآخر لا يوجد اى زيادة فيهما عند وجود اللاكتوفيرين بالتركيزات المختلفة .

ومن هنا يتضح ان اللاكتوفيرين له تأثير جيد فى رفع مقاومة أسماك البلطى النيلي ضد عوامل الأجهاد ومن ثم ضد الأمراض حيث انه يقوى المناعة الخلوية .