ASSESSMENT OF DIETARY BOVINE LACTOFERRIN IN ENHANCEMENT OF IMMUNE FUNCTION AND DISEASE RESISTANCE IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Abstract

Lctoferrin (LF) is an iron-binding glycoprotein plays an important role in the nonspecific immune system in fish and mammals. Nile tilapia (*Oreochromis niloticus*) was fed bovine LF supplemented diets (0, 200, 400 and 600 mg/kg diet) for 4 weeks. The effect of dietary LF on immune function and resistance to *Aeromonas hydrophilla* challenge was determined. The level of LF in diet had a significant force on survival following *A. hydrophilla* challenge with fish fed the 600 mg/kg LF diet having significantly superior survival than control fish. There was increase in activity of non-specific and specific immune parameters (lymphocytes count, macrophage oxidative burst, serum lysozyme and bactericidal activities against *A. hydrophilla*) with addition of LF to diets. While there was not a corresponding increase in activity of total leukocytic count and neutrophil adhesion test. The increased resistance to *A. hydrophilla* with increasing dietary concentration of LF seemed to correspond with an enhancement of non-specific immune functions. In conclusion, dietary LF supplementation could be enhances the non-specific immunity and diseases resistance in Nile tilapia (*Oreochromis niloticus*).

INTRODUCTION

Immunostimulants are increasingly viewed as an integral part of disease prevention and management in modern aquaculture. A large number of putative immunostimulants have been investigated in fish and include killed pathogens and their products, fungal β-glucans, chitin, unidentified animal extracts, synthetic chemicals and plant molecules and nutrients (Sakai, 1999, Gannam, and Schrock, 2001 and Scott and Secombes 2005). An immunostimulant is defined as a 'chemical or drug, or action that elevates the non-specific defense mechanisms or the specific immune response (Anderson *et al.*, 1992). Recent interest in the application of immunostimulants has been heightened as a result of the increasing economic impact of infectious disease.

Bacterial fish pathogens constitute one of the major limiting factor which impair the fish health and cause great economic losses. *Aeromonas hydrophilla,* the causative agent of motile *Aeromonas* septicemia, has a world wide distribution, infecting fishes, birds and mammals and causing heavy mortalities (Stoskoph, 1993).

This problem is exacerbated by the lack of chemotherapeutic agents that are approved for use in food fish and no efficient vaccine. Moreover, the prophylactic application of immunostimulants prior to situations where there is an increase in disease risk, including transport, handling and a change in environmental conditions (Raa, 1996).

Lactoferrin is a family of an iron-binding glycoprotein that is closely related to the plasma iron-transport protein transferrin and consists of a single chain peptide with a molecular weight of 87kDa, with 2 iron binding sites per molecule (Sakai *et al.,* 1993, Brock, 2002 and Weinberg, 2003). Lactoferrin has a wide range of effects on the immune system, both in vivo and in vitro (Brock, 2002). Bovine lactoferrin administered orally to rainbow trout at a dose of 100mg kg/body weight for 3 days resulted in an increase in phagocytic and chemiluminescent activities of pronephros cells. Moreover, this coincided with enhanced resistance to intraperitoneal challenges of *Vibrio anguillarum* and *Streptococcus* sp. (Sakai *et al.*, 1993). Meanwhile, Lygren *et al.,* (1999) and Welker *et al.*, (2007) conducted a study in which Atlantic salmon and *O. niloticus* were fed on bovine lactoferrin. Lysozyme activity in serum and head kidney, complement-mediated haemolysis and phagocytosis were all unaffected by this treatment.

Thus, immunostimulant use is increasingly seen as an integral part of the multifaceted approach to disease control. The efficacy of the dietary LF on the immune function and disease resistance is not well recognized. Therefore, the goal of this study was to determine the effects of the orally administered LF to *O. niloticus* on the non-specific immune response under experimental infection with *A. hydrophila.*

MATERIALS AND METHODS

Experimental fish

A total number of 284 apparently healthy Nile tilapia (*Oreochromis niloticus*) weighing 100 ± 5gm was obtained from a local commercial fish farm. They were maintained in glass aquaria (100X60X70 cm) filled with dechlorinated tap water which continuously aerated. The fish were supplied with a commercial fish ration without LF, They were acclimatized to the laboratory conditions for 15 days before the start of the experiment. Moreover, a 12 h dark: 12 h light photoperiod was provided. 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 of 25% protein was supplemented with lactoferrin at the concentrations of 0, 200, 400 and 600 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

Two hundred and eighty four *O. niloticus* fish were randomly allotted into four equal groups. Group (Gp. 1) was the control. Groups (2-4) were fed on ration containing lactoferrin at the doses of 200, 400 and 600 mg/kg ration respectively daily for four consecutive weeks. All the fish were fed twice daily at the rate of 2% of their body weight throughout the period of the experiment. At the end of experimental trial blood samples were taken for hematological and immunological assay and challenge infection test was performed. Prior to handling, sampled fish were euthanized in 200 mg/l MS-222.

Five fish were randomly sampled from each replicate (three replicate per group) at the 2nd and 4th week of 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 and neutrophil adhesion 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 triplicate for each sample according to Stoskoph, (1993). The following haematological and immunological techniques have been carried out:

Immunological studies

a. Bactericidal activity was determined as described by Kampen *et al.,* (2005) with modifications for plasma according to Welker *et al.,* (2007). Twenty microliters of sample plasma or Hank's Balanced Salt Solution for positive controls was added to duplicate wells of a round-bottom 96-well microtiter plate and incubated for 2.5 h with aliquots of a 24 h culture of *A. hydrophila*. To each well, 25 µl of 3-(4, 5 dimethyl thiazolyl-2)- 2, 5-diphenyl tetrazolium bromide (MTT; 2.5 mg/ml) (Sigma) was added and incubated for 10 min to allow the formation of formazan. Plates were again centrifuged at 2000 ×g for 10 min, the supernatant discarded, and the precipitate dissolved in 200 µl of dimethyl sulfoxide (DMSO). The absorbance of the

dissolved formazan was read at 560 nm. Bactericidal activity was calculated as the decrease in the number of viable *A. hydrophila* cells by subtracting the absorbance of samples from that of controls and reported as absorbance units.

b- 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 cover slip. The cover slips 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 cover slips 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 cover slips 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.

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).

d- Macrophage oxidative burst: Peritoneal macrophages were isolated as a method described by Klesius and Sealey (1996) and oxidative burst was assayed according to Rice *et al.,* (1995).

Challenge test

The challenge test was performed at the end of experimental period on 3 replicates where 10 fish from each replicate were randomly collected and inoculated with virulent strain of *Aeromonas hydrophila. A. hydrophila* was previously isolated from naturally infected tilapia and identified according to standard bacteriological tests. Also, polymerase chain reaction test for detection of cytolytic aerolysin and 16S rDNA genes in the isolates was performed as described by Chu and Lu (2005). The

inoculation route was intraperitonealy using 0.5 ml culture suspension of pathogenic *A. hydrophila* containing 108 bacteria/ ml. The challenge trial lasted for 15 days during which the mortality rate was recorded. Dead fish were removed once a day and subjected to bacterial re-isolation.

Statistical analysis

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

Our results revealed that, orally administrated LF has been shown to enhance the non specific cellular immunity, total lymphocytes count, bactericidal activity, serum lysozyme and macrophage oxidative burst were significantly increased at a dose 600mg at 4th week post treatment in compared with the control group. Dietary LF supplementation did not significantly affect neutrophil adhesion cells, the total leukocytes, neutrophils, basophils, monocytes count allover the experiment period between treatment and control group. Absolute esinophils count was significantly increased at a dose of 600 mg at 2nd week only in compared to control group (table 1 & 2). It was observed that the mortalities among the challenged fish are dose related. Mortality rates post challenge infection, were significantly lesser in gp.4 and 3 than in control group (table, 2).

DISCUSSION

Oreochromis niloticus is one of the most important commercial species cultured all over the world. 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). LF has several biological activities including antibacterial, antiprotozoans, antiviral, antineoplastic and immunomodulatory effects (Weinberg, 2003).

Dietary administrated LF was able in enhancing the non specific cellular immunity, absolute lymphocytes count and macrophage oxidative burst at a dose 600 mg for 4th week supplementation. Similarly, rainbow trout fed a commercial diet supplemented with 100 mg/kg diet for 1 week enhanced the cellular immune response, leukocytes peroxidase content, respiratory burst and phagocytic index in gilthead seabream fish (Esteban *et al.,* 2005). Also Sakai *et al.,* (1995) reported increases the chemiluminescent response of phagocytic cells detected in

Oncorhynchus mykiss dietary supplemnted with LF. Kumari and Sahoo (2006) found that LF delivered as feed supplements significantly enhanced most of the non-specific immune parameters (respiratory burst and phagocytic activities) in both healthy and immunosuppressed Asian catfish (*C. batrachus*) compared to their respective controls. Also, Artym and Zimecki (2005) concluded that LF stimulates proliferation and differentiation of T and B cell from their immature precursors in mice. We observed significant increase in bactericidal and lysozyme serum activities with increasing level of the dietary LF. Kumari *et al.,* (2003) reported elevated serum lysozyme levels in Asian catfish fed 100 mg LF /kg diet for 1 week. In contrast to our results, Ward and Conneely (2004) showed that LF is a prominent bactericidal component of the secondary granules of neutrophils. Meanwhile, Esteban *et al.,* (2005) and Welker *et al.,* (2007) recorded no effect of LF administration on bactericidal and lysozyme serum activities in gilthead seabream and *O. niloticus* respectively.

Mortality rates among the challenged *O. niloticus* with *A. hydrophila* were significantly lower in the groups fed the 400 and 600 mg LF diet compared to the control group. Several studies have also observed increased disease resistance in fish fed diets supplemnted with bovine LF. Kumari, *et al.,* (2003) observed increase resistance of Asian catfish (*C. batrachus*) to *A. hydrophila* when fish were fed 100 mg/kg dietary Lf for 1 week. They suggested that the increased resistance in fish administered bovine LF was due to the enhanced function of the non-specific immune system as serum lysozyme activity and respiratory burst of leucocytes. Also Welker *et al.,* (2007) reported enhance survival rate in *O. niloticus* challenged with *S. iniae* when fish were feed 800 mg/ Kg dietary LF for 8 weeks. They concluded that the increased survival rate seemed to correspond with a decrease in plasma iron concentration and not an improvement of non-specific or specific immune functions. Also, Valenti and Antonini (2005) found that LF inhibits bacterial adhesion on host surface through ionic binding to biomaterials or specific binding to bacterial structures or both.

It could be concluded that LF is a safe immunostimulant protein when orally administrated as prophylactic measure, for long period. LF has been thought to powerful antibacterial in a variety of ways. Further, field trials are indicated for elucidation of the clinical efficacy before permitting the use of LF under intensive aquaculture system.

Table 1. Some Immunological and Hematological Parameters (Mean ± S.E) in Nile tilapia (*Oreochromis niloticus*) Treated with Lactoferrin for 2 week.

Fish Groups	Neutrophil Adhesion Cells/HPF	bactericidal activity/ unit	Lysozyme µg/ml	Macrophage oxidative burst index	TLC $10^3/\mu L$	Neutrophils $10^3/\mu L$	Eosinophils $10^3/\mu L$	Basophils $10^3/\mu L$	Lymphocyte $10^3/\mu L$	Monocyts $10^3/\mu L$
G1	8.32 ±0.41	1.31 ±0.11	8.74 ±0.51	4.01 ±0.31	36.51 ± 2.51	6.85 ± 0.42	0.76 ± 0.08[a]	0.18 ± 0.18	27.3 ± 1.62	1.42 ± 0.15
G2	8.18 ±0.36	1.28 ± 0.09	8.92 ± 0.44	4.21 ±0.23	38.1 ± 3.15	7.12 ± 0.51	0.98 ± 0.16[a b]	0.00	28.38 ± 1.92	1.61 ± 0.28
G3	8.29 ±0.25	1.38 ± 0.15	8.64 ± 0. 38	4.41 ±0.28	35.81 ± 3.85	6.72 ± 0.49	0.85 ± 0.12[a b]	0.00	26.7 ± 1.74	1.54 ± 0.21
G4	8.46 ±0.38	1.42 ± 0.18	8.81 ± 0. 49	4.52 ±0.35	39.1 ± 2.71	7.48 ± 0.61	1.16 ± 0.14[b]	0.00	28.72 ± 1.86	1.72 ± 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 for 4 week.

Fish Groups	Neutrophil Adhesion Cells/HPF	bactericidal activity/ unit	Lysozyme µg/ml	Macrophage oxidative burst index	TLC $10^3/\mu L$	Neutrophils $10^3/\mu L$	Eosinophils $10^3/\mu L$	Basophils $10^3/\mu L$	Lymphocytes $10^3/\mu L$	Monocyts $10^3/\mu L$	Mortality rate %
G1	8.15 ±0.32	1.29 ±0.13ab	8.94 ±0.21a	4.12 ±0.28a	36.32 ± 3.45	7.42 ± 0.56	0.96 ± 0.09	0.00 ± 0.00	26.3 ± 1.10a	1.64 ± 0.14	86.4 ±3.9a
G2	8.24 ±0.35	1.35 ± 0.12b	9.01 ± 0.32a	4.31 ±0.33a	39.41 ± 3.01	7.56 ± 0.41	1.02 ± 0.14	0.00	28.98 ± 2.45ab	1.58 ± 0.20	65.8 ±2.6b
G3	8.36 ±0.30	1.58 ± 0.10cb	9.12 ± 0. 48a	4.35 ±0.24a	39.15 ± 3.24	7.01 ± 0.32	1.08 ± 0.10	0.16 ± 0.10	29.15 ± 2.36ab	1.75 ± 0.25	31.7 ±4.5c
G4	8.48 ±0.44	1.62 ± 0.16cb	9.64 ± 0. 22b	5.14 ±0.21b	41.33 ± 3.81	7.76 ± 0.51	0.94 ± 0.12	0.00	30.85 ± 1.21b	1.78 ± 0.21	26.9 ±5.1c

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

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تقييم لاكتوفيرن البقرى فى تحسين الوظيفة المناعية ومقاومة المرض فى أسماك البلطى النيلي

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يلعب اللاكتوفيرن دورا هاما فى المناعة الغير متخصصة للأسماك والثدييات. وهو عبارة عن بروتين متحد مع الحديد. تهدف هذه الدراسة الى تقييم تأثير هذه المادة على مناعة ومقاومة أسماك البلطى النيلي ضد الأصابة بعدوى الأيروموناس هيدروفيلا. تم اجراء هذا البحث على أسماك البلطى النيلي والتى غذيت على عليقة تحتوى علـى لاكتـوفيرن بجرعـات مختلفـة (٢٠٠،٤٠٠ و ٦٠٠ ملجم/كجم علف) بالأضافة الى العليقة الضابطة (٠) وذلك لمدة ٤ أسابيع. كان لمستوى لاكتـوفيرن تأثير معنوى فى معدل الاعاشة بعد الأصابة بميكروب الأيروموناس هيدروفيلا وخاصة التى غذيت بجرعة ٦٠٠ ملجم/كجم علف.كان هناك زيادة فى القياسات المختلفة للمناعة المتخصصـة والغيـر متخصصة. بينما لم يكن هناك زيادة فى عدد الخلايا البيضاء وأختبار التصـاق الخلايـا البيضـاء المتعادلة. يخلص البحث الى ان خلط لاكتوفيرن بالعليقة قد أدى الى تحسن فى الوظائف المناعيـة ومقاومة أسماك البلطى النيلي ضد الأصابة بعدوى الأيروموناس هيدروفيلا.