Effect of dietary bovine lactoferrin on growth response, tolerance to air exposure and low salinity stress conditions in orange spotted grouper *Epinephelus coioides*

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Abstract

Lactoferrin (LF), which is known as a functional glycoprotein originated from mammalian fluid, was orally administrated to juvenile orange spotted grouper (initial wt = 3.0 g) to investigate its effects on growth response, mucus secretion, tolerance to air exposure and response in heat shock protein under low salinity condition. Test diets containing four different concentrations of LF (0, 400, 800 and 1200 mg LF / kg diet) were fed to the juvenile groupers for 30 days. At the termination of feeding trial, amounts of secreted mucus on body surface were measured, while fish were exposed to the air for 60 min to examine the recovery from respiratory distress condition. Furthermore, fish were exposed to low salinity (3.5 ppt) seawater for 0 and 6 h, respectively. After low salinity seawater exposure, the quantitative levels of heat shock protein 70 family (HSP70s) in branchial lamellae were measured. Dietary LF did not affect growth rate of this species under the condition applied in this study. Mucus secretion significantly increased in all LF supplemented groups compared to that in LF-free group. Recovery rate from air exposure stress test increased with increasing dietary LF level. Under the low salinity exposure, significantly higher levels of HSP70s were detected in 800 and 1200 mg LF supplemented groups than that in LF-free group in both 0 and 6 h exposure. These results demonstrated that dietary LF enhances tolerance to air exposure stress and low salinity stress together with increasing the level of HSP70s in branchial lamellae of orange spotted grouper juvenile.

keywords: Lactoferrin; Orange spotted grouper; *Epinephelus coioides*; Stress; Heat shock protein; Air exposure; Low salinity.

1. Introduction

Orange spotted grouper (*Epinephelus coioides*) is one of the important cultured species in the coastal area particularly in Southeast Asian countries due to its high market value for human consumption (Heemstra and Randall, 1993). Grouper fry transferred from wild or hatchery conditions to culture cage or pond, are occasionally exposed to physiological stressors such as handling, transportation and water quality change (Seng, 1997; Estudillo and Duray, 2003). For successful aquaculture it is important to minimize stress.

Recently (Sakai, 1999) reported that immunostimulants have the ability to reduce stress and enhance non-specific defense systems in finfish. Lactoferrin (LF) is known as iron-binding glycoprotein isolated from secreted fluid such as milk of mammals. Since LF was discovered, many biological functions of LF have been presented both *in vivo* and *in vitro* (Nemet and Simonovits, 1985; Hagiwara et al., 1997; Wakabayashi et al., 1999; Esteban et al., 2005). In finfish, orally administrated LF enhanced phagocytosis activity (Sakai et al., 1995) and chemiluminescent response (Sakai et al., 1993). Dietary LF also enhanced stress resistance against various stressors in rainbow trout (Kakuta, 1997), ayu (Kakuta et al., 1998), common carp (Kakuta, 1998) and Japanese flounder (Gallardo et al., 2000; Yokoyama et al., 2005). Therefore, it is likely that LF administration may improve the quality of seedlings of both freshwater and marine cultured species. However, there is no clear answer for the question on the basic mechanisms concerning the enhancement of stress resistance by LF administration since commercial feeds of unknown formulation, are used in most studies. Furthermore, the effectiveness of LF in tropical important cultured marine species such as orange spotted grouper has not been elucidated. This study was thus designed to investigate the effect of dietary LF on growth response, tolerability to emersion stress and low salinity in orange spotted grouper juvenile.

2. Materials and methods

2-1. *Test diet and LF analysis*

The basal composition of test diet (Table 1) was designed to satisfy the requirements of protein, lipid and other nutrients (Millamena and Golez. 2001). Bovine LF (97.2% purity, Lot No. 951110, Morinaga milk industry Co. Ltd., Tokyo, Japan) was supplemented at 4 levels (0, 400, 800 and 1200 mg LF / kg dry diet) in the test diet. Diets were pelleted with a meat grinder in 2.0 mm diameter, and dried below 39℃ for 3h to prevent loss of LF activity. Nutritional values of the test diet (Table 2) were measured according to AOAC (1990). The content of LF in the test diets were determined by sandwich enzyme-linked immunosorbent assay (ELISA) as described in a previous study (Yokoyama et al., 2005).

2-2. *Feeding trial*

Feeding trial of orange spotted grouper juvenile (60 day old, initial body weight = 3.0g) was conducted at Aquaculture department, Southeast Asian Fisheries Development Center (AQD, SEAFDEC, Iloilo, Philippines). Fifteen fish were maintained in 60-l aquaria with 50-l natural temperature (31.0 ℃) seawater with triplicate tanks for each dietary treatment. Three or four round black-colored plastic mesh plates were placed in the rearing tank as a shelter. The test diet was fed 4 times (9:00, 11:00, 14:00 and 17:00) daily at a ration size of 10 % of body weight (wet weight basis) for day 1-15, and thereafter, ration size was adjusted to 8 % of body weight. Uneaten diet was removed by syphoning and weighed everyday. Fish were weighed

every 15 days. Feeding trial was terminated on day 30. Weight gain, feed intake, feed efficiency ratio (FER) and percent survival were observed as parameters to establish the effect of dietary LF on growth response of orange spotted grouper juvenile. During the trial, the survival rates were relatively high (more than 91%) and there was no significant difference in survival rates among test groups.

2-3. *Amount of secreted mucus*

After 30 day feeding trial. amounts of secreted mucus were quantitated by the method described in Kakuta et al. (1996). Secreted mucus from 5 fish in each test group with a constant area on the body surface (200 mm^2 on dorsal side) was collected by a small piece of sterilized cotton, and immediately suspended in 1ml of phosphate buffered saline (PBS, pH=7.2) and centrifuged (2000 × *g*, 10 min, room temperature). Total protein in the supernatant was determined by the method of Lowry (Lowry et al., 1951). Amounts of secreted mucus were expressed as mean relative values on the basis of total protein in the mucus sample of LF-free group.

2-4. *Air exposure test*

An air exposure test was carried out to evaluate the effect of LF administration on tolerance to respiratory distress condition described by Sakakura et al. (1998) with modification of exposure time. After feeding the test diets, 10 fish in each tank were randomly captured and removed water was removed from the body surface with a paper wiper then the fish were put on a black colored dry nylon net. After 60 min exposure, fish were put back into the tank with seawater under the same conditions as the feeding trial, and number of dead fish was recorded. Evidence of death of the test fish was judged by lacking of gill movement within 2h after return to the tank. The test was

repeated three times per test group. Recovery rate was calculated by following formula:

$$\text{Recovery rate (\%)} = \frac{Ni - Nd}{Ni} \times 100$$

Where N*i* = initial number of test fish, and N*d* = number of dead fish after air exposure. Data obtained from triplicated tanks were pooled and mean recovery rate was calculated.

2-5. *Low salinity seawater exposure and Analysis of heat shock protein*

After feeding trial, fish were exposed to low salinity sea water (3.5 ppt) to investigate the stress response through the measurement of heat shock protein 70 family (HSP70s) level in branchial lamella. Five fish from each test group were directly transferred into low salinity seawater as a test solution in the same aquaria as that in feeding trial with aeration. Test solution was prepared by blending natural seawater and dechlorinated tap water. Salinity of the test solution was confirmed with a reflect photometer. The gill samples were collected from the fish, which were exposed to test salinity for either 0 or 6 h, in all treatment groups. The fish without exposure to low salinity were the same ones as those used for mucus analysis. Branchial lamellae of the test fish were separated from gill arch and frozen immediately at -80℃ until analysis.

The level of HSP70s in branchial lamella, which cross-react with anti HSP70 mouse monoclonal antibody, was quantitated by ELISA with HSP70 quantitative kit (EKS-700. Stressgen Biotechnologies Corporation, Victoria, BC, Canada). Sample preparation was conducted with the method described in Lewis et al. (1999) with slight modification. The frozen branchial lamella was ground to a powder with liquid nitrogen using a cold pestle and mortar. Protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Rosch diagnosis Corporation, USA) and extraction buffer (attached in the kit)

were added into ground sample and homogenized with a Potter Elvehjem homogenizer (30 strokes) in ice. Homogenate was centrifuged (17,000 × *g*, 15 min, 4℃). Precipitate was discarded, and supernatant was subjected to HSP70s analysis performed with a microplate reader. Total protein content in the supernatant was determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin (Nakarai Tesque, Tokyo, Japan) as standard. The levels of HSP70s were expressed as the levels of HSP70s in the total protein content of the sample solution.

2-6. *Statistical analysis*

Percent survival and recovery data were normalized by arcsine transformation before analysis. All data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test to detect significant ($P< 0.05$) differences among test groups using a program Kaleida Graph 3.6 for Macintosh (Synergy Software, PA, USA).

3. Results

3-1. *Feeding trial*

Result of feeding trial is shown in Table 3. All LF supplemented groups did not show significant differences in weight gain, feed intake and FER compared to those of LF-free group.

3-2. *Amounts of secreted mucus*

Higher amount of secreted mucus was observed (Fig.1) in all LF supplemented group than that in LF-free group. The highest concentration of LF (1200 mg LF) supplemented group showed significantly lower amount of secreted mucus secretion compare to those of the other LF supplemented groups.

3-2. *Recovery rate of Air exposure test*

In the air exposure test, fish fed the diet supplemented with the highest concentration of LF (1200 mg LF) showed significantly higher recovery rate compare to that of fish fed the diet without LF. The recovery rate of fish increased with increasing LF supplemented level in the diet (Fig. 2).

3-3. *HSP70s level*

In the present ELISA, HSP70s were detected in all test groups (Fig. 3). Fish exposed for 6 h to low salinity seawater showed higher levels of HSP70s than those of fish before exposure to low salinity in each LF supplemented groups. The levels of HSP70s in branchial lamella before or after exposure to the low salinity seawater increased with increasing dietary LF supplement level. Significantly higher levels of HSP70s were detected in fish fed the diet with 800 and 1200 mg LF supplemented groups compare to those of 0 and 400 mg LF supplemented groups before or after exposure to the low salinity seawater.

4. Discussion

Under the conditions of our study LF had no effect on growth. This confirms

earlier studies by Lygren (Lygren et al., 1999). Moreover, Yamauchi et al. (2000) reported long-term oral administrated LF did not affect on growth response of rats. However, significantly higher growth was observed in goldfish fed the diet containing LF (0.001, 0.01 and 0.1%) for 28 days compared with LF-free group (Kakuta, 1996a), in which the commercial feeds are applied to the fish. Therefore, unknown ingredients and LF might synergetically affect the growth performance.

In the present study, higher amounts of mucus secretion were observed in all LF supplemented groups compared to that of LF-free group. This was also observed in red seabream (Kakuta et al., 1996) and gold fish (Kakukta, 1996b). Kakuta et al. (1998) reported that the number of mucilage cells increased with oral LF administration in juvenile ayu. Mucus secretion on body surface is important phenomenon regarded as a part of non-specific defense mechanisms against parasite and bacterial infection with its enzymatic systems (Hjelmeland et al., 1983), and prevents fish body from being directly exposed to pollutant and stressor through environmental water. Therefore, oral LF administration will be an effective method to improve natural barriers of finfish.

In the air exposure test, increasing LF level in the test diet increased recovery rate of test fish. The recovery rate of test fish reached above 80 % at the highest rate after 60 min exposure to air. Sakakura et al. (1998) reported that yellowtail, which was exposed to air for 4 min, showed less than 40 % at the highest survival rate. On the other hand, Gallardo et al. (2000) reported that Japanese flounder juveniles when fed the diet containing LF showed high recovery rates to 20 min air exposure, and high survival rates to low oxygen stress. Higher tolerance to high density stress was also reported in rainbow trout treated with dietary LF (Kakuta, 1997). These results demonstrate that grouper juvenile may have potently strong tolerance to hypoxic condition than the other species, and dietary LF contributes tolerance for various fish species against respiratory distress condition.

We did not identify the molecular weight of HSP detected in the present study,

therefore, results may indicate HSP detected consists not only HSP70 but also other HSP families, which have around 70 kDa of molecular mass. However this method could detect target antigen more quantitatively compared to the other assay e.g., immuno blotting. A monoclonal antibody to mammalian HSP70 indicated cross reactivity with fish HSP70s (Burkhardt et al., 1998; DuBeau et al., 1998). Crustacean HSP86 and green alga HSP70 was quantitated by ELISA with mouse monoclonal antibody cross-react with human HSP (Cimino et al., 2002; Bierkens et al., 1998). In addition, the amino acid sequence of fish HSP has been highly conserved among rat, bovine and human (Yokoyama et al., 1998; Pan et al., 2000). Those suggest that anti mammalian HSP70 monoclonal antibody may cross-react with HSP70 across species.

Besides being a mediator of denatured functional protein caused by various stressors (Iwama et al., 1999), HSP plays a role in novel protein synthesis as molecular chaperone (Elis, 1999). In the HSP analysis, HSP70s was detected not only from fish exposed to low salinity seawater for 6 hours but also from those without exposure with lower level of HSP70s. HSP would be detected as chaperone when fish was under the non-stress condition. Branchial HSP response in fish has been well investigated. However, most research indicated HSP level in whole gill or branchial lamella may include red blood cell (RBC). Delaney and Klesius (2004) indicated that level of HSP70 of blood cell increased under the hypoxic condition in Nile tilapia juvenile. Currie et al. (1999) also reported that induction of HSP gene transcription was observed in rainbow trout RBC by heat shock treatment. It is likely that the level of HSP in RBC changes with various stressors. In the present HSP analysis, detected HSP70s might have originated from branchial cells together with that from RBC.

Little is known about HSP response in finfish with water salinity change. Deane et al. (2002) demonstrated that both hypersaline and hypoosmotic salinities affect expression of different classes of hepatic HSP in black sea bream in terms of salinity adaptation. In this study, significantly higher levels of HSP70s were observed in

branchial lamella from fish fed the diet containing 800 and 1200 mg LF than those of 400 and 0 mg LF supplemented groups under low salinity exposure. Induction of HSP70s level in branchial tissue with low salinity exposure was first confirmed in orange spotted grouper in the present study. Furthermore, dietary LF could stimulate HSP70s synthesis under low salinity condition over the same time interval, and inducible HSP70s may maintain gill functions of test fish.

Although there is very limited information on the linkage between dietary LF and HSP, Yokoyama et al. (2005) recently demonstrated that LF administration enhanced synthesis of HSP in Japanese flounders when exposed to continuous high temperature condition. Bagni et al. (2005) showed dietary β-glucan or alginic acid enhances gill and hepatic HSP in sea bass. The authors suggested that this elevated level of HSP attributed to phagocyte stimulation with dietary factors. In vitro effect of LF in fish has been showed to stimulate phagocyte activity (Sakai et al., 1995, Kamilya et al., 2005). These suggest that tissue HSP levels are concomitantly increased by enhancement of phagocytic cell in tissue with dietary LF treatment. Further studies are required to clarify whether inducible HSP is originated from phagocytic cell. Other reports also showed synthesized HSP family contribute to apply tolerance and survival to stress in finfish (Dilorio et al., 1996; Brown et al., 1995) and finfish cell line (Mosser et al., 1998). DuBeau et al. (1998) demonstrated that Atlantic salmon fry pretreated with mild heat shock showed tolerance to osmotic shock with increasing HSP70 level in branchial tissue. Thus, inducible HSP may prevent mortality caused by various stressors. In this study, branchial HSP70s increased with dietary LF treatment, therefore, it is expected that dietary LF contributes tolerance to stress with induction of HSP70s for the grouper juvenile.

Results showed that dietary LF enhanced tolerance to air exposure and low salinity stress by the stimulating of nonspecific defense mechanism such as HSP and mucus secretion. In conclusion, LF supplementation could be regarded as one of the effective

means to give tolerance to physiological stressor and improve the quality of juvenile orange spotted grouper. However, further studies comparing in vivo and in vitro effects of LF in response to heat shock protein are needed to investigate specific biological action of dietary LF on contribution of stress tolerance against various stressors in finfish.

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Table 1.

Basal diet composition

Ingredient	g / kg dry diet
Brown fish meal	650
Dextrin	50
α-starch	50
Squid liver oil	50
Soybean lecithin	30
Vitamin mixture[*1]	30
Mineral mixture[*2]	30
Activated gluten	50
α-Cellulose + Bovine lactoferrin[*3]	60

[*1]Vitamin mixture (mg/kg dry diet): β-Carotene 96.3; Vitamin D_3 9.7; Vitamin K_3 45.8; α-Tocopherol 384.9; Thiamin-HCl 57.8; Riboflavin 192.4; Biotin 5.8; Inositol 3849.1; Niacin 769.7; Ca-Panthothenate 269.5; Folic acid 14.4; Choline chloride 7869.3; p-Aminobenzoic acid 383.3; L-Ascorbyl-2-Mono phosphate-Sodium-Calcium salt 100.0.

[*2]Mineral mixture (mg/kg dry diet): NaCl 1077.9; $MgSO_4 \cdot 7H_2O$ 3800.2; $NaHPO_4$ 2419.1; KH_2PO_4 6651.9; $Ca(H_2PO4)_2 \cdot 2H_2O$ 3766.9; Fe Citrate 823.8; Ca Lactate 9071.0; $Al(OH)_3$ 5.2; $ZnSO_4 \cdot 7H_2O$ 99.0; $CuSO_4$ 2.8; $MnSO_4 \cdot 5H_2O$ 22.2; $Ca(IO_3)_2$ 5.4; $CoSO_4 \cdot 7H_2O$ 27.7.

Table 2.

Nutritional values of test diets

LF supplemented (mg / kg)	Protein* (%)	Fat* (%)	Ash* (%)	LF content* (mg / kg)	Moisture (%)
0	48.2	13.0	13.0	0.0	9.0
400	49.6	13.7	12.9	376.6	9.0
800	49.8	14.1	12.8	770.9	9.6
1200	48.7	14.1	12.5	1131.9	9.9

*Dry weight basis

Table 3.

Results of 30-day feeding trial[1]

LF supplemented (mg / kg)	Weight gain[2] (%)	Feed intake (g)	Feed efficiency ratio[3]
0	376.5 ± 16.4	11.1 ± 0.66	1.0 ± 0.11
400	324.1 ± 34.7	9.8 ± 1.36	1.0 ± 0.06
800	362.8 ± 51.9	10.8 ± 1.06	0.9 ± 0.09
1200	354.8 ± 23.3	10.8 ± 0.83	0.9 ± 0.04

[1]Values are expressed as mean ± standard deviation (n=3).

[2]Weight gain (%) = {(Mean Final BW- Mean Initial BW)/Mean Initial BW}×100

[3]Feed efficiency ratio = Wet weight gain (g) / Dry Feed Intake (g)

Figure legends

Fig. 1. Relative amounts of mucus secretion on body surface of orange spotted grouper juvenile. Values are expressed as mean relative amount plus standard deviation on the basis of amount of mucus in 0 mg LF supplemented group (n=5 fish). Values with the same letter are not significantly different ($P<0.05$).

Fig. 2. Recovery rate of orange spotted grouper juveniles fed diets supplemented with different levels of LF from air exposure (60 min) stress. Values are expressed as mean recovery rate plus standard deviation (n=3). Values with the same letter are not significantly different ($P<0.05$).

Fig. 3. Levels of HSP70s detected by ELISA from branchial lamella in orange spotted grouper juvenile exposed to low salinity seawater for 0 (gray column) and 6 (open column). Values are given as mean values with standard deviations (n=5 fish). Asterisks indicates significant difference from 0 mg LF supplemented group in each sampling time ($P<0.05$).

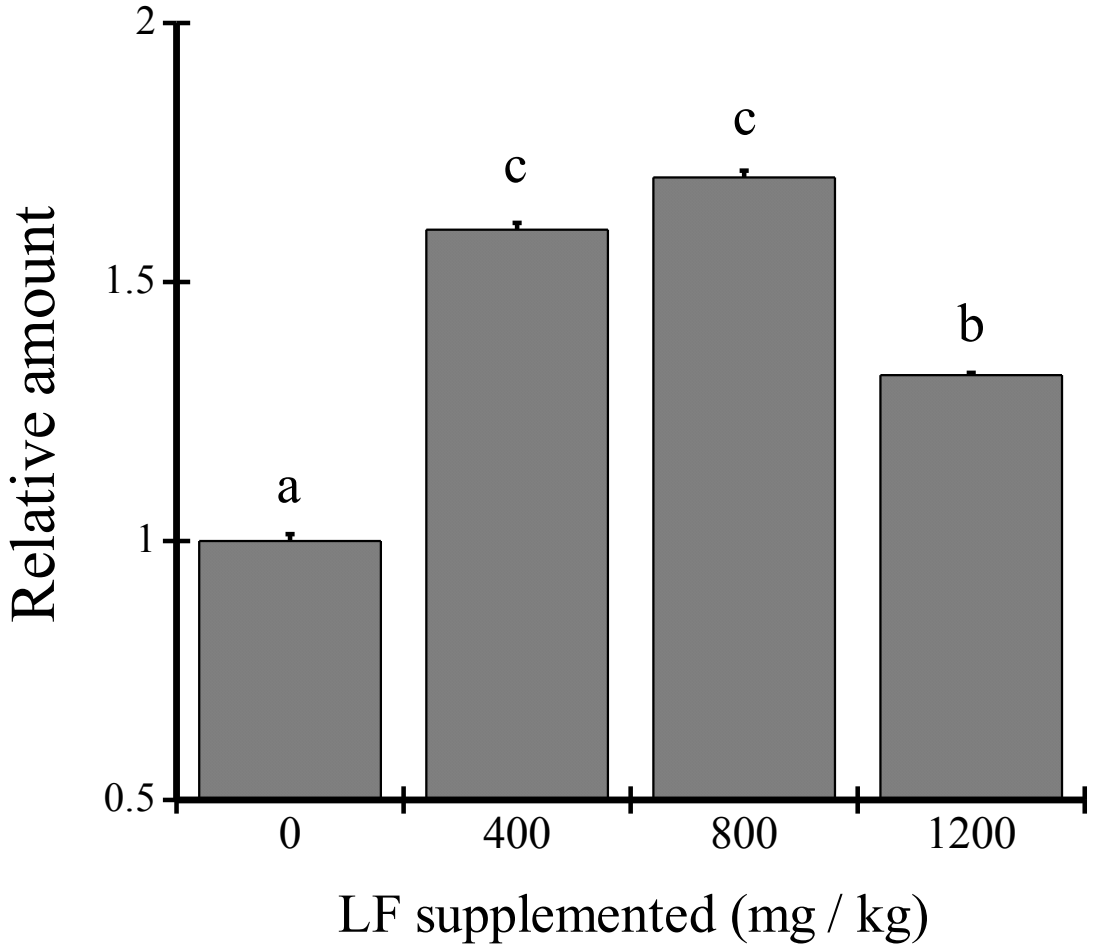
2
1.5
1
0.5
Relative amount
a
c
c
b
0
400
800
1200
LF supplemented (mg / kg)

Fig. 1.

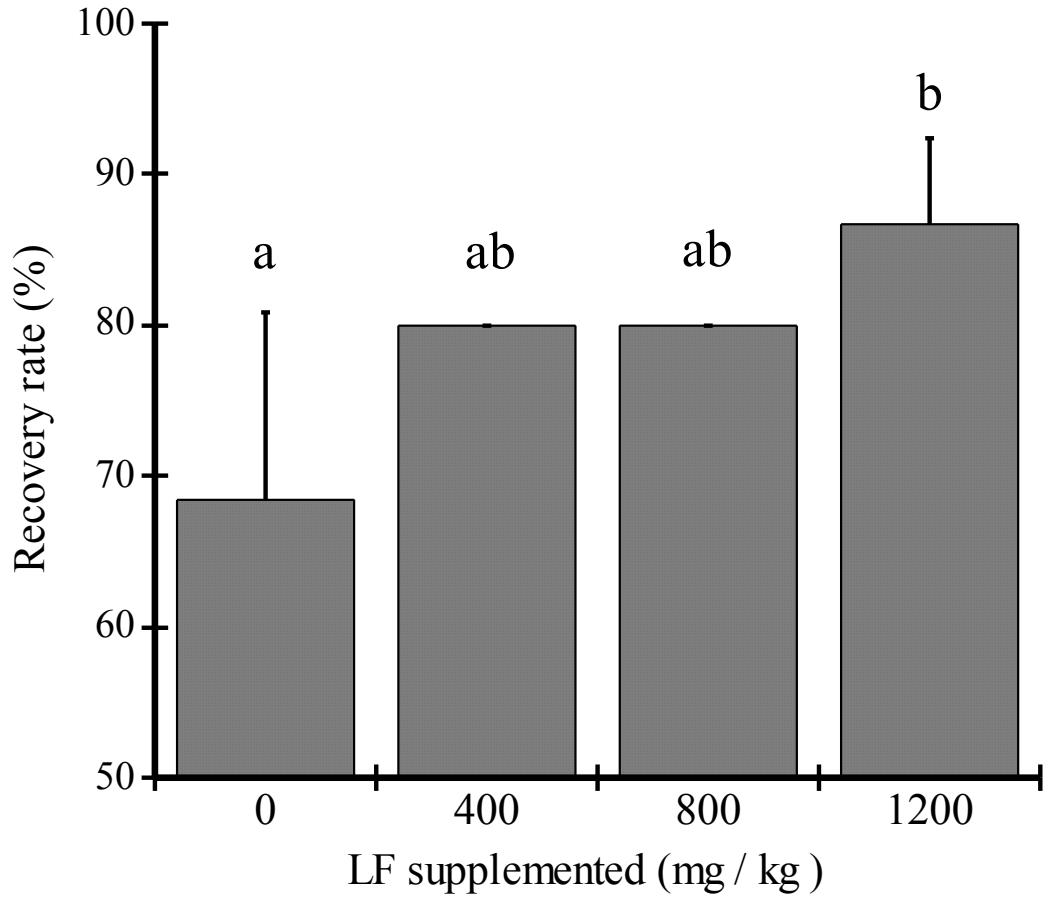
100
90
80
70
60
50
Recovery rate (%)
a
ab
ab
b
0
400
800
1200
LF supplemented (mg / kg)

Fig. 2.

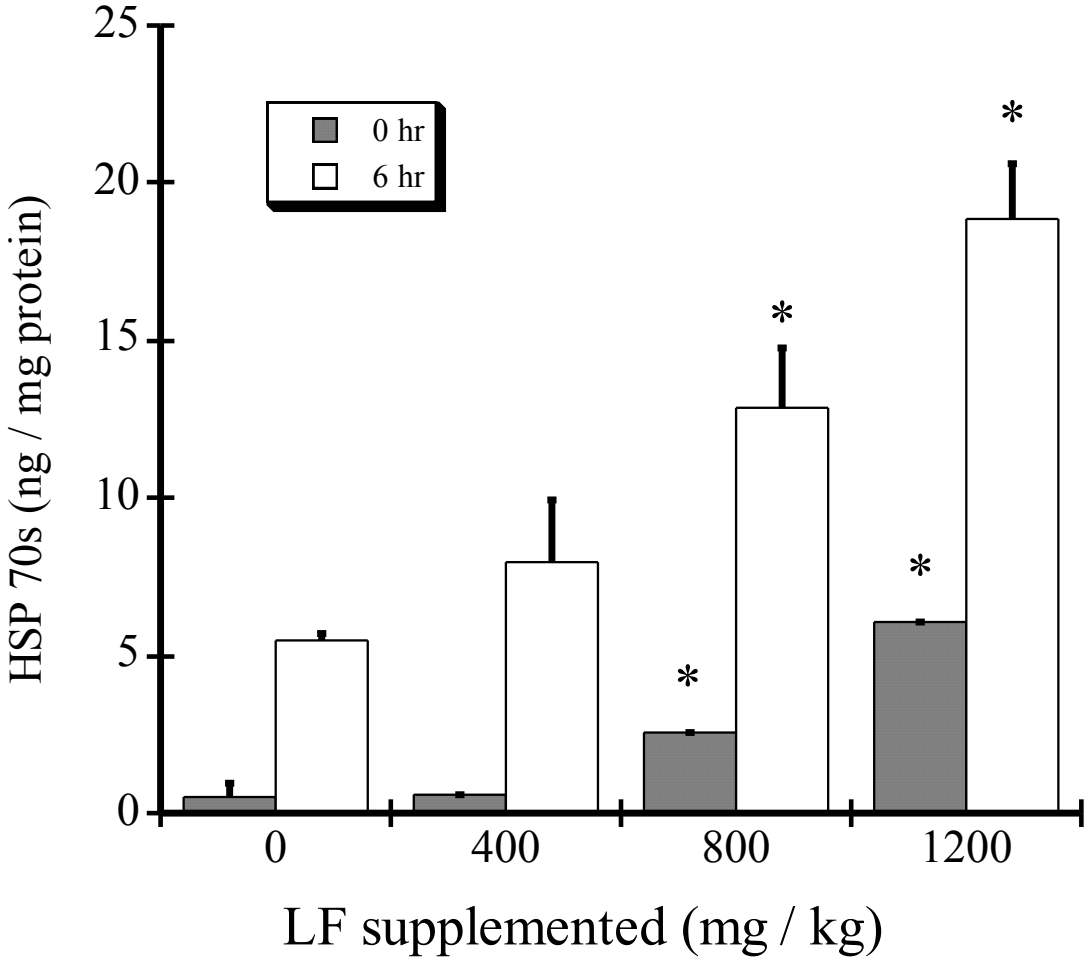
25
20
15
10
5
0
HSP 70s (ng / mg protein)
0 hr
6 hr
*
*
*
*
0
400
800
1200
LF supplemented (mg / kg)

Fig. 3.