The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (http://www.aquaculturehub.org) members and registered individuals and institutions. Please visit our website (http://siamb.org.il) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB),
University of Hawai'i at Mānoa Library
&
University of Hawai'i at Mānoa Aquaculture Program
in association with
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_67.2015.1202, 10 pages

Effects of Dietary Supplementation with Fermented *Ginkgo* Leaves on Innate Immunity, Antioxidant Capability, Lipid Metabolism, and Disease Resistance Against *Aeromonas hydrophila* Infection in Blunt Snout Bream (*Megalobrama amblycephala*)

Fuliang Cao[1,2]*, Yan Lu[1,2], Xuhui Zhang[1,2], Linguo Zhao[3], Juxian Yang[4], Xiaoshu Qin[1,2], Wenbin Liu[5], Wanwen Yu[1,2]

[1]Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, No. 159, Longpan Road, Nanjing 210037, PR China;
[2]College of Forestry, Nangjing Forestry University, No. 159, Longpan Road, Nanjing 210037, PR China;
[3]College of Chemical Engineering, Nanjing Forestry University, No. 159, Longpan Road, Nanjing 210037, PR China;
[4]College of Biology and the Environment, Nanjing Forestry University, No. 159, Longpan Road, Nanjing 210037, PR China;
[5]Key Laboratory of Aquatic Nutrition and Feed Science of Jiangsu Province, College of Animal Science and Technology, Nanjing Agricultural University, No. 1, Weigang Road, Nanjing 210095, PR China.

Key words: blunt snout bream; fermented *G. biloba* leaves; immunity; antioxidant; lipid metabolism; *Aeromonas hydrophila*

Abstract

This study was conducted to investigate the effect of *Candida utilis* and *Aspergillus niger,* combined with fermented *Ginkgo biloba* leaves (FGB) on immunity, antioxidant capability, lipid metabolism, and disease resistance against *Aeromonas hydrophila* infection in juvenile blunt snout bream (*Megalobrama amblycephala*). Fish were fed basal diets supplemented with fermented *Ginkgo biloba* leaves FGB1(0.125%), FGB2(0.25%), FGB3(0.5%), FGB4(1.0%), or Control (0.00%) respectively. At the end of the 60-day feeding trial, fish were challenged by *A. hydrophila* and mortality rate was recorded for the next 7 days. Results showed that, compared with the control, serum myeloperoxidase (MPO) activity of groups FGB1 and FGB2, alkaline phosphatase (AKP) and lysozyme (LZM) activity of group FGB2 increased significantly (P<0.05), while malondialdehyde (MDA) and low density lipoprotein cholesterol (LDL-C) contents of group FGB2, immunoglobulin M (IgM) activity of group FGB4 decreased significantly (P<0.05). Furthermore, Acid phosphatase (ACP), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH) and triglyceride (TG) activities were not affected (P>0.05). After *A. hydrophila* challenge, the relative mortality of group FGB1 and FGB2 were significantly lower than the control (P<0.05), however the highest rate was in group FGB4. Results of this study indicate that dietary supplementation of 0.125%~0.25% fermented *G. biloba* leaves can significantly enhance immunity, antioxidant capability, and lipid metabolism of blunt snout bream, as well as improve its disease resistance.

*Corresponding author. Fuliang Cao,Tex.: +86 25 85427099, +86 13913948118
E-mail address: fuliangcaonjfu@163.com

Introduction

In recent years, along with the promotion of large-scale and intensive aquaculture, global aquaculture has developed rapidly. Stress factors, however, such as overcrowding, poor water quality, grading, and handling tend to adversely affect the health of cultured fish (Li et al., 2004; Xia et al., 2015). These conditions leads to the suppression of the immune system, increasing the susceptibility of fish to infectious diseases (Harikrishnan et al., 2011), as well as the phenomenon of excessive body lipid deposition. The administration of antibiotics in aquaculture could reduce the demand for certain nutrients, promote growth, as well as prevent and treat diseases. However, continuous intensive use of antibiotics leads to immunosuppression and residue accumulation in tissues (Harikrishnan et al., 2009a, 2009b). It also leads to antibiotic resistance in bacteria. Furthermore, antibiotics resistance can be transferred to the aquaculture environment and to human pathogens (Wu et al., 2013). The intensive use of antibiotics can inhibit growth in aquatic animals. Therefore in order to control diseases it is imperative that safe and effective alternative feed additives be found.

Ginkgo biloba L. (Family: Ginkgoaceae), a traditional herb in China, is used for treating inflammations, vascular, and cardiac diseases. Leaves of *G*. *biloba*, which are rich in protein, sugar, vitamin C, vitamin E, and beta carotene are well known for their high content of flavonoids and terpene lactones (Zhang et al., 2012; Cao et al., 2012; Zhao et al., 2013). There are a number of reports indicating that *Ginkgo* flavonoids and terpene lactones contain beneficial components, pharmacological and antioxidant activity of scavenging free radicals (Nie et al., 2004; Xie and Zhang, 2005; Mou et al., 2008). Fermentation is a useful tool to significantly increase the nutrients and active ingredients of *G*. *biloba* leaves (Zhang et al., 2012; Cao et al., 2012; Zhao et al., 2013). In addition, microbes are selectively screened for fermentation qualities as there are beneficial microbes which when fermented, which can effectively promote the body's growth and immunity. Screening is carried out for microorganisms which contribute to the fermentation of the leaves. Previous research concerning the biological effect of fermented *G*. *biloba* leaves was limited to livestock. *G. biloba* can improve the growth performance (Zhang et al., 2012; Cao et al.,2012; Zhao et al., 2013), enhance the immunity and antioxidant capacity (Cao et al.,2012), and ameliorate lipid metabolism (Cao et al.,2012; Zhao et al., 2013). however this has not been tested in aquaculture.

Blunt snout bream (*Megalobrama amblycephala*) is a herbivorous freshwater fish native to China. Aquaculture of this fish in China has expanded rapidly during the past decade because of its rapid growth, tender flesh, and high economic value. This species is also cultured in North America (from Canada to Mexico), Africa, Europe and other Asian countries (Li et al., 2012). It adapts well to different conditions, and thus is suitable for culture worldwide (Li et al., 2010). Compared to carp, it is not resistant to hypoxia and transportation, and is easily affected by external adverse factors, which can lead to injury and death. It is also prone to excessive fat deposition and metabolic disorders during its growth (Cai et al., 2014), seriously affecting development. Culture of blunt snout bream has been seriously inhibited by the high frequency of diseases caused by intensive fish farming. This study was conducted to investigate the effects of *C. utilis* and *A. niger,* combined fermented *G. biloba* leaves on immunity, antioxidant capability, lipid metabolism, and disease resistance of juvenile blunt snout bream (*Megalobrama amblycephala*). The data here may aid the use of FGB in this fish as well as other species sharing the same feeding habits.

Materials and methods

Culturing of C. utilis and A. niger. Fermented *G. biloba* leaves were produced using the methods described by Zhao et al. (2013). *C*. *utilis* (NFU-Y-186) and *A. niger* NL-1 used in this study was a laboratory strain isolate obtained from the College of Chemical Engineering, Nanjing Forest University, Nanjing, Jiangsu, PR China. The seed culture medium for *C. utilis*, contained (g/L): glucose 30, ammonium sulfate 8, KH_2PO_4 3 and anhydrous $MgSO_4$ 0.25. The final amount of *C. utilis* was 6-9$\times 10^9$ CFU/mL. *A. niger* was cultured with agar plating technique using sabouraud dextrose agar (Oxoid Ltd., Basingstoke, UK) and incubated at 24℃ for 7 days. *A. niger* spores were harvested by

tapping the top of the plate when turned upside down. Spore counts were determined using hematocytometer spores/mL, which were equivalent to 0.25g.

Preparation of fermented G. biloba leaves sample. Dried *Ginkgo* leaves picked during the last third September (*Ginkgo* garden for leaf use, Nanjing Forestry University, Jiangsu Province, PR China) were passed through a 2.0-mm sieve for use in this study. The solid-state fermentation medium contained 10 g solid medium (*Ginkgo* leaves: wheat bran: corncob=8:1.5:0.5) and 16 mL mineral salts (glucose: urea: $(NH_4)_2SO_4$: peptone: KH_2PO_4: $MgSO_4 \cdot 7H_2O$= 4:2:6:1:4:1) and was inoculated with 10% (mL/mL) of the *C. utilis* and *A. niger* suspensions for aerobiotic fermentation. In total, 0.1% of the *C. utilis* and *A. niger* suspensions were used to inoculate the fermentation medium for aerobic fermentation. Both samples were fermented for 48 h at 28-30℃. For the combined fermentation, medium was inoculated with 1 mL *C. utilis* suspension at 28-30℃ for a 24h aerobic fermentation in the first stage and then 2 mL *A. niger* suspension was added for an 84 h aerobic fermentation in the second stage.

Fermented samples were spread on a polythene sheet in a room at 30-40℃, dried for 6 days up to about 900 g/kg of the dry matter, and ground to pass through a 0.15-mm sieve. The changes of the ingredients before and after the fermentation are shown in Table 1.

Table1. Changes in the main nutritional ingredients in *Ginkgo* leaves before and after fermentation

Nutritional ingredients	*Before fermentation*	*After fermentation*
Total flavonoids (mg of quercetin equivalents/g)	26.45	26.82
Polysaccharides (g/kg)	4.37	6.43
Protein (g/kg)	136.4	269.34
Total ginkgolic acid (g/kg)	1.665	0.052
Total amino acid (g/kg)	95.98	172.45
Indispensable amino acid (g/kg)	36.30	61.98
Ferment cells number (g)	0	2.50×109
Cellulase (IU/g)	0	1500
Hemicellulase (IU/g)	0	16000
Glucosidase (IU/g)	0	4500

Experimental diets. Prior to use, all ingredients were analyzed for proximate composition and the data obtained was used as a basis for feed formulation. Fish meal, soybean meal, cotton seed meal, and rapeseed meal were used as protein sources. Equal portions of fish oil and soybean oil were used as lipid sources. Wheat flour was used as carbohydrate source (Table 2). Five experimental diets were formulated to contain fermented G. *biloba* leaves (0.0%, 0.125%, 0.25%, 0.5%, 1.0%) levels following a single factorial design. Feed ingredients were ground into fine powder then thoroughly mixed and blended in oil and sufficient water to form soft dough. The dough was then pelleted (without injected steam) using a pellet mill with a 2 mm diameter die. The experimental feed was air-dried at 33℃ overnight and stored in sealed plastic bags at -20℃ until use. The ingredients and composition of the basal diet are given in Table 1.

Fish and experimental design. Juvenile blunt snout bream were obtained from the Fish Hatchery of Yangzhou (Yangzhou, Jiangsu Province, China). Prior to the experiment, fish were acclimated in laboratory conditions by feeding a commercial diet for 1 week. After the acclimation, 600 healthy fish of similar size (average weight, 9.31±0.09g) were randomly divided into 5 experimental groups, distributed into 20 plastic tanks (0.9×0.8 m, D×H) with 4 replicates each following a completely randomized design (CRD). The experimental trial was conducted with the various levels of FGB leaves and a control (cont. 0.0% FGB), FGB1 (0.125%), FGB2 (0.25%), FGB3 (0.5%), FGB4 (1.0%) for 60 days. During the experimental period, fish were fed three times daily at 8:00, 12:00, 17:00 h, respectively. Fish were hand-fed to apparent satiation with utmost care to minimize feed waste, and held under natural photoperiod conditions throughout the feeding trial. Water temperature, pH, and dissolved oxygen, were monitored using a YSI

556 MPS multi-probe field meter (Geotech,USA). Water temperature was maintained at 25 ± 0.3 ℃. pH fluctuated between 7.0-7.5, and dissolved oxygen was maintained approximately at 5.0 mg/L during the feeding trial.

Table2. The basic diets and nutrition levels

Ingredients	*Content（%）*
Fish meal	8.00
Soybean meal	30.00
Rapeseed meal	16
Cotton meal	16
Soybean oil	2
Fish oil	2
Bran	13
Flour	10
Calcium dihydrogen phosphate	1.8
Premix[1]	1
Salt	0.20
Total	100
Moisture(%)	6.62
Crude protein（%）	32.49
Crude fat（%）	10.19
Ash（%）	10.48
Gross energy（MJ/Kg）	18.17

Note: Premix supplied the following minerals (per kilogram diet): $CuSO_4 \cdot 5H_2O$ 2.0g, $FeSO_4 \cdot 7H_2O$ 25 g, $ZnSO_4 \cdot 7H_2O$ 22 g, KI, 0.026 g, $MnSO_4 \cdot 4H_2O$ 7 g, Na_2SeO_3 0.04 g, $CoCl_2 \cdot 6H_2O$ 0.1 g. Premix supplied the following vitamins (per kilogram diet): VA 900000IU, VD 200 000 IU, VE 4 500 mg, VK_3 220 mg, VB_1 320 mg, $VB_2$1 090 mg, VB_5 2 000 mg, VB_6 500 mg, VB_{12} 1.6 mg, VC 5 000 mg, pantothenate 1 000 mg, folic acid 165 mg, choline 60 000 mg.

Sampling and analysis.

Sampling. At the end of feeding trial, fish were starved for 24h before sampling. Five fish from each replicate were anesthetized in diluted MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 100 mg/L. Each fish was weighed and blood samples were taken from the caudal vein using heparinized plastic syringes. The blood sample was separated by centrifugation, and the supernatant was pooled and stored at -20℃ for subsequent analysis. Individual livers were washed thoroughly with chilled saline (0.9g NaCl/L), dried quickly over a piece of filter paper, and stored at -20℃ for subsequent analysis. The abdominal fat was separated and weighed to determine the abdominal fat percentage.

Proximate analysis of the experimental diets. The proximate composition of the experimental diets was determined according to the standard AOAC method (AOAC, 1995). Moisture was determined by oven drying at 105℃ till constant weight. Crude protein was analyzed by micro-Kjeldahl method and multiplied by a factor of 6.25 after acid digestion using an Auto Kjeldahl System (1030-Auto-analyzer, Tecator, Hoganas, Sweden); ash was analyzed by combustion at 550℃ for 4h. Gross energy in diets was measured by a Bomb Calorimeter (Parr 1281. Parr Instrument Company, Moline, IL, USA).

Chemical analysis. In this experiment, protein content in the liver was determined using Coomassie Brilliant Blue. Serum acid phosphatase (ACP), alkaline phosphatase (AKP) and lysozyme (LZM), myeloperoxidase (MPO), immunoglobulin M (IgM), liver superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione (GSH), catalase (CAT), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) were measured using a kit which was purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China) following manufacturer's instructions.

Abdominal fat percent (IPF, %)= 100× abdominal fat weight (g)/body weight (g)

Challenge test. A. hydrophila (Ah, BSK-10) was provided by Freshwater Fisheries Research Center, Chinese Acadamy of Fishery Sciences (Wuxi, Jiangsu Province, China) and was activated following the methods described by Christybapita et al. (2007). The 7-day LD50 (Ah, BSK-10 dose that killed 50% of the test fish) was determined by intraperitoneal injection of 50 fish with graded doses of (10^5, 10^6, 10^7,5×10^7) and the result showed that the LD50 on day 7 was 1×10^6 cfu/mL.

At the termination of the feeding experiment, 10 fish from each replicate were injected intraperitoneally using medical syringes with 1 mL/kg body weight of *A. hydrophila*. Fish continued to receive their assigned diets after the injection. Fish were carefully monitored and mortality was recorded three times daily for the next 7 days. Mortality rate was determined on the 8th day using the following formula.

Mortality rate (%)= $N_t\times 100/N_0$

Where N_t and N_0 were the dead and initial number of fish in the challenge test respectively.

Statistical analysis. Data were analyzed using the SPSS General Linear Models (GLM) procedure (SPSS7.5, Michigan Avenue Chicago, IL, USA) for significant differences among treatment means. If significant (P<0.05) differences were found in factors, Duncan's multiple range test (Duncan, 1995) was used to rank the means. All data were presented as means$\pm$ S.E.M (standard error of the mean) of four replications.

Results

Immune responses. Parameters indicating the immune responses of juvenile blunt snout bream are presented in Table 3. The plasma MPO content of group FGB1 and FGB2 was significantly higher than that in control group (P<0.05), meanwhile, plasma AKP and LZM contents of group FGB2 were significantly greater than in control group (P<0.05). While little difference (P>0.05) was observed in plasma ACP compared with control group the plasma IgM content of group FGB4 was significantly lower than that in control group (P<0.05), whereas the other treatment group showed little difference (P>0.05) compared to the control group.

Table 3. Effects of fermented *Ginkgo* leaves on the serum immune index of juvenile blunt snout bream (*Megalobrama amblycephala*)

Group[1]	*MPO(U L-1)*	*IgM(g L-1)*	*AKP(U L-1)*	*ACP(U L-1)*	*LZM(U mL-1)*
Cont.	6129.79±182.26^{b}	0.759±0.005^{a}	53.0±2.0^{b}	2343.3±190.5^{a}	91.2±8.0^{b}
FGB1	8304.16±189.79^{a}	0.773±0.004^{a}	47.7±3.8^{b}	2476.3±146.7^{a}	112.0±16.8^{ab}
FGB2	7613.08±234.01^{a}	0.772±0.006^{a}	64.5±4.7^{a}	1912.9±236.4^{a}	128.8±8.0^{a}
FGB3	6362.01±347.54^{b}	0.766±0.005^{a}	48.8±1.4^{b}	2360.0±180.0^{a}	105.6±6.4^{ab}
FGB4	6424.12±173.43^{b}	0.576±0.0003^{b}	52.0±1.3^{b}	2197.5±30.5^{a}	97.6±13.6^{ab}

Notes: [1]Cont.= basal diet; FGB1= basal diet with 0.125% fermented *Ginkgo* leaves; FGB2= basal diet with 0.25% fermented *Ginkgo* leaves; FGB3= basal diet with 0.5% fermented *Ginkgo* leaves; FGB4= basal diet with 1.0% fermented *Ginkgo* leaves.

[2] different superscripts indicate statistical significance(P<0.05) of values in the same column.

Antioxidant capabilities. Liver antioxidant capabilities of juvenile blunt snout bream are presented in Table 4. No significant differences (P>0.05) were observed in the liver SOD, GSH, CAT activities, whereas the opposite was true for liver MDA activity. MDA activity of group FGB2 was significantly (P<0.05) lower than that in the control group.

Table 4. Effects of fermented *Ginkgo* leaves on antioxidant enzyme activites of juvenile blunt snout bream (*Megalobrama amblycephala*)

Group[1]	*SOD (U mgprot-1)*	*MDA (nmol mgprot-1)*	*GSH (umol mgprot-1)*	*CAT (U mgprot-1)*
Cont.	1.63±0.53^{a}	0.93±0.11^{a}	48.00±1.56^{a}	4.65±0.23^{a}
FGB1	2.19±0.22^{a}	0.87±0.08^{ab}	40.47±0.68^{a}	4.34±0.22^{a}
FGB2	1.81±0.12^{a}	0.69±0.02^{b}	47.99±4.89^{a}	4.36±0.08^{a}
FGB3	1.95±0.04^{a}	0.97±0.07^{a}	46.87±6.12^{a}	4.76±0.17^{a}
FGB4	1.91±0.24^{a}	1.03±0.09^{a}	42.27±1.78^{a}	4.89±0.45^{a}

Notes: [1]Cont.= basal diet; FGB1= basal diet with 0.125% fermented *Ginkgo* leaves; FGB2= basal diet with 0.25% fermented *Ginkgo* leaves; FGB3= basal diet with 0.5% fermented *Ginkgo* leaves; FGB4= basal diet with 1.0% fermented *Ginkgo* leaves.

different superscripts indicate statistical significance(P<0.05) of values in the same column.

Lipid metabolism. Parameters indicating the lipid metabolism of juvenile blunt snout bream are shown in Table 5. Both IPF and TG content were not affected (P>0.05) by fermented *G. biloba* leaves. HDL-C content of group FGB2 was not significantly higher than that in control group (P>0.05), and was significantly higher than that in group FGB1 and FGB3 (P<0.05) whereas TC content of group FGB4 was lower than that in control group (P>0.05), and was significantly lower than that in group FGB3 (P<0.05). When compared with control group, LDL-C content of group FGB2 was significantly decreased (P<0.05). LDL-C content of group FGB3, however, was significantly enhanced (P<0.05).

Table 5. Effects of fermented *Ginkgo* leaves on lipid metabolism of juvenile blunt snout bream (*Megalobrama amblycephala*)

Group[1]	*IPF(%)*	*HDL-C (mmol L-1)*	*LDL-C (mmol L-1)*	*TG (mmol L-1)*	*TC (mmol L-1)*
Cont.	5.30±0.29^{a}	3.24±0.15^{ab}	1.81±0.08^{b}	4.23±0.34^{a}	5.14±0.14^{ab}
FGB1	5.34±0.26^{a}	2.99±0.06^{b}	1.78±0.10^{b}	4.11±0.32^{a}	5.15±0.26^{ab}
FGB2	5.28±0.26^{a}	3.69±0.17^{a}	1.34±0.01^{c}	4.08±0.46^{a}	5.08±0.15^{ab}
FGB3	5.67±0.45^{a}	3.09±0.22^{b}	2.77±0.11^{a}	4.48±0.41^{a}	5.66±0.59^{a}
FGB4	5.71±0.11^{a}	3.35±0.09^{ab}	1.77±0.08^{b}	3.85±0.33^{a}	4.56±0.36^{b}

Notes: [1]Cont.= basal diet; FGB1= basal diet with 0.125% fermented *Ginkgo* leaves; FGB2= basal diet with 0.25% fermented *Ginkgo* leaves; FGB3= basal diet with 0.5% fermented *Ginkgo* leaves; FGB4= basal diet with 1.0% fermented *Ginkgo* leaves.
different superscripts indicate statistical significance(P<0.05) of values in the same column.

Mortality rate after A. hydrophila challenge. After challenge with *A. hydrophila*, the first death was recorded after 24h. Cumulative mortality was recorded up to 7 days after injection and determined at the 8th day (Fig.1). The mortality rate of both groups FGB1 and FGB2 was significantly lower (P<0.05) than that of control group, and mortality rate of group FGB2 was significantly lowest. Mortality rate of group FGB4 was significantly higher than the control group (P<0.05).

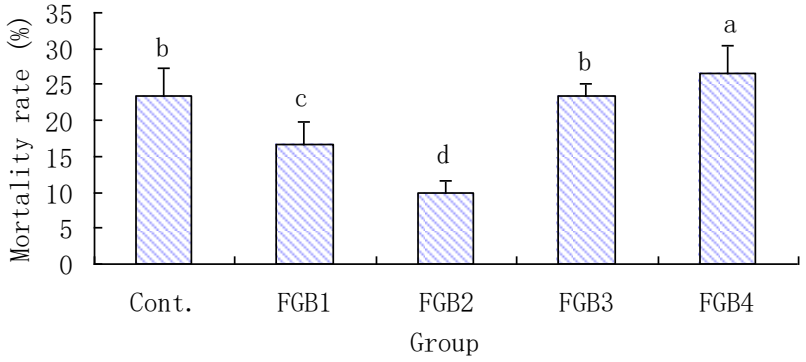

Fig.1. Mortality rate (%) of juvenile blunt snout bream (*Megalobrama amblycephala*) after 7 days of *A. hydrophila* challenge.
Note: Cont.= basal diet 0.0%FGB; FGB1= basal diet with 0.125% ; FGB2= 0.25% ; FGB3= 0.5% ; FGB4= 1.0% . FGB respectively.

Discussion

Chinese herbal medicine can improve the specific and nonspecific immunity of fish, thereby improving resistance to infectious diseases (Harikrishnan et al., 2011). For thousands of years, *G. biloba* has been one of the important Chinese herbs. China has been a large producer, producing about forty thousand tons of *Ginkgo* leaves a year. It is important to find a way to utilize this herbal resource as a feed ingredient and demonstrate its potential economic value for the feed industry.

Fermentation is a useful tool for the production of biological materials. It has health promoting properties and is an optimal way to mediate the overproduction of various fruits and vegetables (Zhang et al., 2012). Microbial fermentation of Chinese herbs is a

process which has long been used. They can be enriched with vitamins, enzymes, other growth factors, and other antioxidants (Ng et al., 2011). A crucial advantage in the production of FGB is that the required technology is affordable and feasible even for small farmers (Zhao et al., 2013). In this experiment the combination of *C. utilis* and *A. niger* were used to ferment *Ginkgo* leaves. They not only have bioactive elements such as flavonoids and terpene lactones but also contain beneficial bacteria as well as highly active composite enzymes that absorb nutrients for the intestines after fermentation (Table 1).

Effects of fermented Ginkgo leaves on the serum immune index of juvenile blunt snout bream (Megalobrama amblycephala). In this study, the application of 0.125~0.25% FGB significantly improved the innate immunity of blunt snout bream. This was supported by the fact that fish fed 0.125~0.25% experimental diets obtained significantly higher serum MPO, AKP, and lysozyme activities when compared with fish fed the control diet. Similar to our findings, the application of dietary Chinese herbal medicine and its products (*Sorpha flavescens*, *Tinospora cordifolia* and *Eclipta albab*) has also been reported by others to improve the immunity of some fish species (Wu et al, 2013; Alexander et al., 2010; Christybapita et al., 2007).

According to previous reports, the immunostimulatory nature of FGB may be attributed to its flavonoids and terpenoid lactones (Zhou et al., 2015; Xie and Zhang, 2005; Nie et al., 2004). Quercetin of *G. biloba* flavonoids is structurally similar to natural estrogens and is known to exert several estrogen-like biological effects in animals. It can improve immunity in animals by acting on estrogen receptors of the immune organs and cells directly or regulating the secretion pituitary growth hormone (GH), prolactin (PRL), and somatostatin (SS) (Xie and Zhang, 2005). Ginkgolide can also regulate the immune system. Its mechanism may serve as ginkgolides that have some effect on macrophages and neutrophils (Nie et al., 2004). However, further studies in fish are still needed to elucidate this issue.

IgM is the main immunoglobulin present in teleosts. It could provide instant protection for fish against pathogens of broad specificity (Zhang et al., 2013). Our study showed little difference in the plasma IgM content, in fact it was significantly reduced when adding 1.0% FGB. This indicated that there was no positive correlation between the effect of immunostimulants and the dosage. It has been reported that a high dosage inhibits immune responses in rainbow trout (Kajita et al., 1990).

Effects of fermented ginkgo leaves on antioxidant enzyme activites of juvenile blunt snout bream (Megalobrama amblycephala). Malondaldehyde (MDA) is considered an indicator of oxidative stress in fish (Modesto and Maritinez, 2010). Generally, the level of MDA indirectly reflects the severity of harm done to cellsafter exposure to free radicals. In the present study, blunt snout bream fed 0.25% FGB demonstrated lower liver MDA levels, suggesting improved resistance to antioxidants in this species. A similar pattern was also found in Wuchang bream fed with emodin (Ming et al., 2012) and *Penaeus monodon* supplemented with honeysuckle (Chen et al., 2013). This may be connected with flavonoids in FGB. Nine types of flavonoids separated from *G. biloba* leaves may eliminate the O^{2-} and inhibit the respiratory burst of polymorphonuclear (Mou et al. 2008). The antioxidant index obtained here was consistent with the results observed in immunity diagnosis. Together, they reflect a consistent improvement in the health status of diets supplemented with fermented *G. biloba* leaves.

Effects of fermented Ginkgo leaves on lipid metabolism of juvenile blunt snout bream (Megalobrama amblycephala). Flavonoids have received considerable attention for their lipolytic activity in vitro and in mammals (Zarrouki et al., 2010). Some flavonoids form insoluble complexes with cholesterol in the digesta and inhibit the intestinal absorption of endogenous and exogenous cholesterol (Rao and Gurfinkel, 2000). This effect was confirmed in the present study. We found that fish fed 0.25% FGB showed lower serum LDL-C ($P<0.05$), higher serum HDL-C ($P>0.05$) and lower serum TC ($P>0.05$) levels. These results are in agreement with previous results observed in laying hens (Zhao et al., 2013) and broilers (Cao et al., 2012), which probably suggests lipid metabolism improvement of this species.

Mortality rate after A. hydrophila challenge. In this study, the treatment groups FGB1, FGB2 fed with fermented *G. biloba* leaves showed significantly lower mortality rates,

mortality rate of group FGB2 was the lowest. However, mortality rate of group FGB4 was significantly higher than that of the control group. This might be explained by the fact that the serum IgM of group FGB4 was lower than that of control group. When blunt snout bream are infected with *A. hydrophila*, a specific immune response occurs, producing IgM to scavenge harmful bacteria invading the body and thus reducing the mortality rate. These results suggest that 0.25% FGB sufficiently induces the highest inflammatory response. Immunostimulants do not usually show a linear dose-effect relationship. Maximum effect has been induced with an intermediate dose and no effects and even toxicity have been reported at high doses (Bliznakov and Adler, 1972).In conclusion, the present study indicated that dietary supplementation of 0.125%~0.25% FGB can significantly enhance the immunity, antioxidant capacity, and lipid metabolism of blunt snout bream, as well as improve disease resistance. This may be due to flavonoids and terpenoid lactones contained in FGB. In fact, from a practical point of view, these findings may provide us with an affordable and effective alternative to antibiotics for this species. However, in vitro efficacy of FGB of various sources should be further investigated in vivo in order to ascertain their molecular activity.

Acknowledgments

This research was supported financially by a Special Program of National Science and Technology: Public welfare Industry Project (Project no. 201004015). We would like to thank the Key Laboratory of Aquatic Nutrition and Feed Science of Jiangsu Province, Nanjing Agricultural University, for use of their facilities and donation of the bream. We would like to express our appreciation to the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

Alexander C. P., Kirubakaran C. J. W. and Michael R. D., 2010. Water soluble fraction of *Tinospora cordifolia* leaves enhanced the non-specific immune mechanisms and disease resistance in *Oreochromis mossambicus*. *Fish Shellfish Immunol.*, 29(5): 765-772.

AOAC., 1995. Official methods of analysis of official analytical chemists international. 16th ed. Arlington VA: *Association of Official Analytical Chemists*.

Bliznakov E., and Adler A., 1972. Nonlinear response of the reticuloendothelial system upon stimulation. *Pathol Microbiol*., 38: 393-410.

Cai D. S., Jiang G. Z., Wang L. N., Lu K. L., Qian Y., Liu W. B., Li X. F., 2014. Effects of dietary glycyrrhetinic acid levels on growth performance, lipid deposition and antioxidant capacity of blunt snout bream *(Megalobrama amblycephala*). *J Fisheries China*, 38(9): 1514-1521.

Cao F. L., Zhang X. H., Yu W. W., Zhao L. G., Wang T. , 2012. Effect of feeding fermented *Ginkgo biloba* leaves on growth performance, meat quality, and lipid metabolism in broilers. *Poultry Sci*., 91(5): 1210-1221.

Chen X., Lin H. Z., Jiang S. G., Wu K. C., Liu Y. J., Tian L. X., Zhang Y. Q., Niu J., 2013. Dietary supplementation of *honeysuckle* improves the growth, survival and immunity of *Penaeus monodon*. *Fish Shellfish Immunol.*, 35(1): 161-169.

Christybapita D., Divyagnaneswari M. and Michael R. D., 2007. Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*. *Fish Shellfish Immunol*., 23(4): 840-852.

Harikrishnan R., Balasundaram C., Dharaneedharan S., Moon Y. G., Kim M. C., Kim J. S., Heo M.S., 2009a. Effect of plant active compounds on immune response and disease resistance in *Cirrhina mrigala* infected with fungal fish pathogen, *Aphanomyces invadans*. *Aquac. Res*., 40(10): 1170-1181.

Harikrishnan R., Balasundaram C., Kim M. C., Kim J. S., Han Y.J., Heo M. S., 2009b. Innate immune response and disease resistance in *Carassius auratus* by triherbal solvent extracts. *Fish Shellfish Immunol.*, 27(3): 508-515.

Harikrishnan R., Balasundaram C. and Heo M. S., 2011. Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture*, 317(1): 1-15.

Kajita Y., Sakai M., Atsuta S., Kobayashi M., 1990. The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. *Fish Pathol.*, 25(2): 93-98.

Li P., Lewis D. H. and Gatlin D. M., 2004. Dietary oligonucleotides from yeast RNA influence immune responses and resistance of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) to *Streptococcus* iniae infection. *Fish Shellfish Immunol*., 16(5): 561-569.

Li X. F., Liu W. B., Jiang Y. Y., Zhu H., Ge X. P., 2010. Effects of dietary protein and lipid levels in practical diets on growth performance and body composition of blunt snout bream (*Megalobrama amblycephala*) fingerlings. *Aquaculture*, 303(1): 65-70.

Li X. F., Liu W. B., Lu K. L., Xu W. N., Wang Y., 2012. Dietary carbohydrate/lipid ratio affects stress, oxidative status and non-specific immune responses of fingerling blunt snout bream, *Megalobrama amblycephala*. *Fish Shellfish Immunol*., 33: 316-323.

Martinez-Alvarez R. M., Hidalgo M. C., Domezain A., Morales A. E. Garcia-Gallego. M., Sanz. A., 2002. Physiological changes of sturgeon *Acipenser naccarii* caused by increasing environmental salinity. *J. Exp. Biol.*, 205(23): 3699-3706.

Ming J., Xie J., Xu P., Ge X. P., Liu W. B., Ye J. Y., 2012. Effects of emodin and vitamin C on growth performance, biochemical parameters and two HSP70s mRNA expression of Wuchang bream (*Megalobrama amblycephala*) under high temperature stress. *Fish Shellfish Immunol.*, 32(5): 651-661.

Modesto K. A. and Martinez C. B. R., 2010. Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. *Chemosphere*, 78(3): 294-299.

Mou L. L. , KOU J. P., ZHU D. N., YU B. Y., 2008. Antioxidant activities of the Chemical Constituents Isolated from the Leaves of *Ginkgo biloba*. *Chin. J. Nat. Med.*, 6(1): 26-29.

Ng C. C., Wang C. Y., Wang Y. P., Tzeng W. S., Shyu Y. T., 2011. Lactic acid bacterial fermentation on the production of functional antioxidant herbal Anoectochilus formosanus. *Hayata. J. Biosci. Bioeng.*, 111(3): 289-293.

Nie Z. G., Peng S. Y. and Wang W. J., 2004. Effects of ginkgolide B on lipopolysaccharide-induced TNFα production in mouse peritoneal macrophages and NF-κB activation in rat pleural polymorphonuclear leukocytes. *Acta Pharmaceutica Sinica*, 39(6): 415-418.

Rao A. V. and Gurfinkel D. M., 2000. The bioactivity of saponins: triterpenoid and steroidal glycosides. *Drug Metabolism and drug interactions*, 17(1-4): 211-236.

Wu Y. R., Gong Q. F., Fang H., Liang W. W., Ming C., He R. J., 2013. Effect of *Sophora flavescens* on non-specific immune response of tilapia (*Oreochromis niloticus*) and disease resistance against *Streptococcus agalactiae*. *Fish Shellfish Immunol*., 34(1): 220-227.

Xia S. L., Ge X. P., Liu B., Xie J., Miao L. H., Ren Q. L., Zhang W. X., Jiang X. J., Chen R. L., Pan L. K., 2015. Effects of supplemented dietary curcumin on growth and non-specific immune responses in juvenile wuchang bream (*Megalobrama amblycephala*). *Isr. J. Aquacult. - Bamidgeh,* [IJA_67.2015.1174].

Xie P. and Zhang M. H., 2005. The effect of flavonoids on immune function in animals. *Acta Zoonutrimenta Sinica*, 17(3): 17-20.

Zarrouki B., Pillon N. J., Kalbacher E., Soula H. A., NJomen G. N., Grand L., Chambert S. Geloen A., Soulage C. O., 2010. Cirsimarin, a potent antilipogenic flavonoid, decreases fat deposition in mice intra-abdominal adipose tissue. *Int. J. Obesity*, 34(11): 1566-1575.

Zhang C. N., Li X. F., Xu W. N., Jiang G. Z., Lu K. L., Wang L. N., Liu W. B., 2013. Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on innate immunity, antioxidant capability and disease resistance of triangular bteam (*Megalobrama terminalis*). *Fish Shellfish Immunol*., 35(5): 1380-1386.

Zhang X., Cao F., Sun Z., Yu W. W., Zhao L. G., Wang G. B., Wang T., 2012. Effect of feeding *Aspergillus niger*-fermented *Ginkgo biloba*-leaves on growth, small intestinal structure and function of broiler chicks. *Livest. Sci.*, 147(1): 170-180.

Zhao L., Zhang X., Cao F., Sun D. F., Wang T., Wang G. B., 2013. Effect of dietary supplementation with fermented *Ginkgo*-leaves on performance, egg quality, lipid metabolism and egg-yolk fatty acids composition in laying hens. *Livest. Sci.*, 155(1): 77-85.

Zhou C. P., Lin H. Z., Ge X. P., Niu J., Wang J., Wang Y., Chen L. X., Huang Z., Yu W., Tan X. H., 2015. The effects of dietary soybean isoflavones on growth, innate immune responses, hepatic antioxidant abilities and disease resistance of juvenile golden pompano *Trachinotus ovatus*. *Fish Shellfish Immunol*., 43: 158-166.