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Effects of dietary supplementation of glycyrrhizic acid on growth performance, survival, innate immune response and parasite resistance in juvenile large yellow croaker, *Larimichthys crocea* (Richardson)

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Abstract

An 8-week feeding experiment was conducted to investigate the effects of dietary glycyrrhizic acid (GA) on growth, survival and immune response of juvenile large yellow croaker, in seawater floating net cages. GA was supplemented into the basal diet to formulate four isonitrogenous and isoenergetic practical diets containing 0.00% (the control diet), 0.01%, 0.02% and 0.04% GA of dry weight, respectively. Triplicate groups of 60 fish were fed to apparent satiation by hand twice daily. The results showed that the specific growth rate, survival rate and feed efficiency ratio revealed no significant differences among dietary treatments ($P > 0.05$). The phagocytic index of head kidney macrophage was significantly increased by the supplementation of 0.04% dietary GA compared to the control group ($P < 0.05$). Fish fed 0.04% dietary GA also showed significantly higher serum lysozyme activity than fish fed the control diet and diet with 0.01% GA ($P < 0.05$). The cumulative mortality rate after natural infestation of parasites (protozoan, *Cryptocaryon irritans* Brown) showed no significant differences among dietary treatments. These results suggested that dietary glycyrrhizic acid improved certain non-specific immunological parameters of juvenile large yellow croaker. However, GA was not able to protect juvenile large yellow croaker effectively from protozoan infection.

Keywords: *Larimichthys crocea*, glycyrrhizic acid, growth performance, immune response, disease resistance

Introduction

As intensive aquaculture expanded and culture density increased over the past decade, diseases occurred more frequently and with the application of antibiotics, many other problems arose such as spread of drug-resistant pathogens, negative impact on environment and food safety problems (Read & Fernandes 2003; Burridge, Weis, Cabello, Pizarro & Bostick 2010). Under this condition, there was a fast-growing interest in immunostimulants which boost immune systems and improve disease resistances with minor adverse side effects. β-glucan (Ai, Mai, Zhang, Tan, Zhang, Xu & Li 2007), chitin (Gopalakannan & Arul 2006), peptidoglycan (Zhou, Song, Huang & Wang 2006), lipopolysaccharides (Swain, Nayak, Nanda & Dash 2008), nucleotides (Li & Gatlin 2006), prebiotics (Merrifield, Dimitroglou, Foey, Davies, Baker, Bøgwald, Castex & Ringø 2010; Ringø, Olsen, Gifstad, Dalmo, Amlund, Hemre & Bakke 2010) and probiotics (Nayak 2010) have been widely investigated and commercially used in aquaculture around the world.

Glycyrrhizic acid (GA), an active component of liquorice, *Glycyrrhiza glabra*, roots, has been demonstrated to have many pharmacological properties in terrestrial animals and humans studies, such as anti-inflammation, antioxidation, antiviral activities and antimicrobial activities (Van Rossum, Vulto, De Man, Brouwer & Schalm 1998; Utsunomiya, Kobayashi, Herndon, Pollard & Suzuki 1999; Utsunomiya, Kobayashi, Ito, Pollard & Suzuki 2000; Cinatl, Morgenstern, Bauer, Chandra, Rabenau & Doerr 2003; Lin 2003; Mendes-Silva,

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Assafim, Ruta, Monteiro, Guimarães & Zingali 2003; Rahman & Sultana 2006; Asl & Hosseinzadeh 2008; Kimura, Moro, Motegi, Maruyama, Sekine, Okamoto, Inoue, Sato & Ogihara 2008; Wolkerstorfer, Kurz, Bachhofner & Szolar 2009; Michaelis, Geiler, Naczk, Sithisarn, Ogbomo, Altenbrandt, Leutz, Doerr & Cinatl 2010). It was also reported that GA mediated most of these pharmacological properties via its immunomodulatory activities (Asl & Hosseinzadeh 2008), such as inducing T cells proliferation (Zhang, Isobe, Nagase, Lwin, Kato, Hamaguchi, Yokochi & Nakashima 1993; Utsunomiya *et al.* 1999, 2000), augmenting cytotoxic function mediated by hepatic lymphocytes and natural killer cell activity (Miyaji, Miyakawa, Watanabe, Kawamura & Abo 2002), and regulating cytokines release (Dai, Iwatani, Ishida, Terunuma, Kasai, Iwakula, Fujiwara & Ito 2001; Utsunomiya, Kobayashi, Ito, Herndon, Pollard & Suzuki 2001; Abe, Akbar, Hasebe, Horike & Onji 2003). In aquaculture, immunomodulatory effects of dietary GA on shrimp (*Litopenaeus vannamei*) and sea cucumber (*Apostichopus japonicus* Selenka) has been reported in several studies (Bai, Zhang, Mai, Wang, Xu & Ma 2010; Chang, Zhang, Mai, Ma & Xu 2010; Chen, Mai, Zhang, Ai, Xu, Liufu & Ma 2010). However, only few precedents exist for the use of GA with fish. An *in vitro* study with rainbow trout (*Oncorhynchus mykiss* W.) leucocytes showed treatment with GA induced the activation of leucocytes in number and function (Jang, Marsden, Kim, Choi & Secombes 1995). In another study with yellowtails (*Seriola quinqueradiata* Temminck & Schlegel), feeding GA for 4 weeks was observed to increase resistance to streptococcal infection (Edahiro, Hamaguchi & Kusuda 1991). Based on the current knowledge, it seems that the potential use of GA as an immunostimulant in fish feed is promising and further studies in this field are worthwhile.

Large yellow croaker (*Larimichthys crocea* Richardson) is a carnivorous species widely cultured in China. Over the past 5 years, the large yellow croaker aquaculture industry has been suffering from diseases, especially protozoan infection. Few studies on immunostimulant applications in large yellow croaker have been conducted (Jian & Wu 2003; Ai *et al.* 2007). The present study was undertaken to investigate the effects of dietary glycyrrhizic acid on the growth, survival, innate immune response and resistance to protozoan infection. This is the first study to test the efficacy of glycyrrhizic acid as dietary immunostimulant to improve the growth, immunity and pathogen resistance of fish.

Materials and methods

Experimental diets

The basal practical diet was formulated to contain approximately 44% crude protein and 11% lipid, which have been shown to be sufficient to support the optimal growth of large yellow croaker (Table 1). Glycyrrhizic acid (purity, >95.12%; Sigma, USA) was supplemented to the basal diet to obtain 0.00% (the control diet), 0.01%, 0.02% and 0.04% (of dry weight) glycyrrhizic acid, respectively. The wheat meal contents were adjusted to make the formulation 100%.

Table 1 Formulation and chemical proximate composition of the experimental diets (% dry matter)

Ingredient	Concentration (%)
Fish meal*	40.00
Soybean meal*	20.00
Wheat meal	26.90
Fish oil	5.00
Soybean oil	2.00
Lecithin	2.00
Mineral premix†	2.00
Vitamin premix‡	2.00
Mold inhibitor§	0.10
Proximate analysis	
Crude protein (%)	44.45
Crude lipid (%)	11.28
Ash (%)	10.59

*Fish meal: crude protein 74.3% dry matter, crude lipid 6.63% dry matter; soybean meal: crude protein 49.4% dry matter, crude lipid 0.95% dry matter.

†Mineral premix (mg or g kg^{-1} diet): $MgSO_4 \cdot 7H_2O$, 1200 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $FeSO_4 \cdot H_2O$, 80 mg; $MnSO_4 \cdot H_2O$, 45 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $NaSeSO_3 \cdot 5H_2O$ (1%), 20 mg; $Ca(IO_3)_2 \cdot 6H_2O$ (1%), 60 mg; $CaH_2PO_4 \cdot H_2O$, 10 g; zoelite, 8.485 g.

‡Vitamin premix (mg or g kg^{-1} diet): thiamin 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B_{12} (1%), 10 mg; vitamin K_3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin (2%), 60 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; alpha-tocopherol (50%), 240 mg; ascorbic acid, 2000 mg; choline chloride (50%), 4000 mg; ethoxyquin 150 mg, wheat middling, 12.32 g.

§Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

Ingredients were ground into fine powder through 200 μm mesh. All ingredients were thoroughly mixed with fish oil and soybean oil, and water was added to produce stiff dough. The dough was then pelleted with an experimental feed mill and dried for about 12 h in a ventilated oven at 45°C. After drying, the diets were broken up and sieved into proper pellet size (1.5 × 5.0 mm, 2.5 × 5.0 mm), and were stored at −15°C until used.

Experimental procedure

Large yellow croaker was obtained from a commercial farm in Ningbo, China. Prior to the start of the experiment, the juveniles were reared in floating sea cages (3.0 × 3.0 × 3.0 m) and fed the control diet for 2 weeks to acclimate to the experimental diet and conditions.

At the onset of the feeding trial, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10 000) (Shanghai Reagent, Shanghai, China). Fish of similar sizes (with 9.79 ± 0.6 g mean initial body weight) were randomly distributed into 12 sea cages (1.0 × 1.0 × 1.5 m) and each cage was stocked with 60 fish. Each diet was randomly assigned to triplicate cages. Fish were hand-fed to apparent satiation twice daily (05:00 and 17:00). The feeding trial lasted for 8 weeks. During the experimental period, the temperature ranged from 22.5 to 31.5°C, salinity from 1018.8 to 1021.6 g L^{-1} and the dissolved oxygen was approximately 6 mg L^{-1}. At the end of the experiment, the fish were fasted for 24 h before harvest. Total number and body weight of fish in each cage were measured.

Sampling and functional immune assay

Collection of serum and assay of lysozyme and superoxide dismutase

Blood samples were collected from the caudal vein of five fish per cage with 27-gauge needles and 1-mL syringe, and allowed to clot at room temperature for 2 h and for 4–6 h at 4°C. Following centrifugation (836 × *g*, 10 min, 4°C), the serum was removed and frozen at −80°C for the determination of the activities of lysozyme and superoxide dismutase (SOD). The lysozyme activity was determined as described by Ellis (1990). Results were expressed in units of lysozyme mL^{-1} serum. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1} at 530 nm compared to the control (*Micrococcus lysodeikticus* suspension without serum). The SOD activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome c and 0.024 IU mL^{-1} xanthine oxidase. The reaction was triggered by the addition of the xanthine oxidase. One activity unit was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate measured at 550 nm (McCord & Fridovich 1969). Enzyme activity was expressed as units per mL serum (U mL^{-1}).

Isolation of macrophages and assay of their immune functions

Head kidney macrophages from five fish in each cage were isolated as described by Secombes (1990) with some modifications. Briefly, the head kidney was excised, cut into small fragments (around 1 mm^3) and transferred to RPMI-1640 (Gibco, Carlsbad, CA, USA) medium supplemented with 10 IU mL^{-1} heparin (Sigma, St. Louis, MO, USA), 100 IU mL^{-1} penicillin (Amresco, Solon, OH, USA), 100 IU mL^{-1} streptomycin (Amresco) and 2% foetal calf serum (FCS) (Gibco). Cell suspensions were prepared by forcing the head kidney through a 100 μm steel mesh. The resultant cell suspensions were enriched by centrifugation (836 × *g*, 25 min, 4°C) on 34% and 51% Percoll solutions (Pharmacia, Cambridge, England) (discontinuous density gradient centrifugation). The cells were collected at the 34–51% interface and washed twice. Cell viability was determined by the trypan blue exclusion method and the cell density was determined in a haemocytometer. Then additional RPMI 1640 medium was added to adjust the cell concentration (1 × 10^7 mL^{-1}) for analysis. Phagocytic activity was determined by a modified method of Pulsford, Crampe, Langston and Glynn (1995). The 100 μm cell suspensions of head kidney macrophages (1 × 10^7 cells mL^{-1}) were placed into a sterile test tube. 100 μL yeast suspension (Bakers yeast, Type II; Sigma, 1 × 10^8 cells mL^{-1}) was added to the tube and the tube was incubated for 45 min at 25°C. After incubation, the attached macrophages were resuspended. Then, the macrophage suspensions were examined by haemocytometer to determine the phagocytic index (100 cells were examined, triplicate examinations for

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each tube). Production of intracellular superoxide anion (O_2^-) was evaluated using nitroblue tetrazolium (NBT) (Sigma) reduction following the method of Secombes (1990) with some modifications. A 100 μL cell suspension was stained with 100 μL 0.3% NBT and 100 μL Phorbol 12-myristate 13-acetate (PMA) (Sigma) (1 mg mL^{-1}) for 40 min. Absolute methanol was added to terminate the staining. Each tube was washed three times with 70% methanol and air-dried. Then 120 μL 2 M KOH and 140 μL dimethyl sulfoxide (DMSO, Sigma) were added and the color was subsequently measured at 630 nm with a spectrophotometer using KOH/DMSO as a blank.

Natural infestation of parasites

At the day 56 of feeding experiment, experimental fish in present study showed significantly decreased appetite with visible white spots scattered on the body. Same events were reflected by many local farmers in this area almost at the same time. Experimental fish were confirmed to be infected with *C. irritans*, which was the main threat to large yellow croaker culture and caused serious damage to the large yellow croaker culture industry in the past 5 years, according to morphological and molecular identification following the methods of Sun, Zhu, Xie, Wu, Li, Lin and Song (2006). Thus, the feeding experiment had to be ended which lasted for 56 days. Experimental fish were weighed and live fish number in each cage was counted. Phagocytic index and respiratory burst activity of head kidney macrophage were determined immediately and serum samples were taken, flash frozen in liquid nitrogen and then stored at −80°C for the later analysis of serum lysozyme and SOD activities. After that, 40 experimental fish were left in each cage to determine the cumulative mortality during the natural infestation of parasites in the following days. Fifteen days later, the average cumulative mortality reached nearly 50%. Thus, remaining live fish number in each cage was counted to calculate the actual cumulative mortality in 15 days following natural parasite infestation.

Calculations and statistical methods

The following variables were calculated:

$$\text{Specific growth rate (SGR) (\% d}^{-1}\text{)} = (\text{Ln } W_t - \text{Ln } W_O) \times 100/t.$$

$$\text{Feed efficiency ratio (FER)} = \text{wet weight gain in g/dry feed fed in g.}$$

$$\text{Survival rate (\%)} = N_t \times 100/N_O.$$

$$\text{Hepatosomatic index (HSI) (\%)} = \text{liver wet weight} \times 100/\text{body wet weight.}$$

$$\text{Viscerosomatic index (VSI) (\%)} = \text{visceral wet weight} \times 100/\text{body wet weight.}$$

$$\text{Phagocytic index} = \text{number of yeast ingested/number of macrophages observed.}$$

where W_t and W_O were final and initial fish weight, respectively; N_t and N_O were final and initial number of fish, respectively; t is duration of the experiment (day).

All data were subjected to one-way analysis of variance in SPSS 15.0 for Windows. Differences among the means were tested by Tukey's multiple range test. The level of significance was chosen at $P < 0.05$ and the results were presented as means with pooled SEM. (standard error of the mean).

Results

Growth performance and survival

The dietary GA supplementation increased the final weight and specific growth rate (SGR) slightly compared with the control group, and fish fed diets with 0.02% GA showed the highest final weight and SGR. However, no significant differences in the SGR and final weight were observed among GA supplemented groups and the control group ($P > 0.05$) (Table 2).

There were also no significant differences observed in survival rate, feed efficiency ratio, hepatosomatic index and viscerosomatic index among GA supplemented groups and the control group ($P > 0.05$) (Table 2).

Immune parameters

The phagocytic index of head kidney macrophage increased significantly with the increasing dietary GA levels. The phagocytic index value in fish fed diets with 0.04% GA was significantly higher compared to the control group ($P < 0.05$) (Fig. 1a). However, there were no significant differences in phagocytic index among the 0.01% GA group, 0.02% GA group and the control group ($P > 0.05$).

The serum lysozyme activity was also significantly influenced by the GA supplementation ($P < 0.05$) (Fig. 1b). Fish fed diets with 0.04% GA

Table 2 Growth performance of large yellow croaker (*Larimichthys crocea*) fed the diets with graded levels of glycyrrhizic acid (Means with pooled SEM)

	Glycyrrhizic acid supplementation level (%)				ANOVA		
Growth response	**0.00**	**0.01**	**0.02**	**0.04**	**Pooled SEM**	***F*-value**	***P*-value**
Final weight (g)	34.85	34.96	36.94	36.09	0.55	0.762	0.547
Specific growth rate (% d^{-1})	2.11	2.12	2.21	2.17	0.03	0.747	0.554
Survival rate (%)	96.24	93.55	98.39	96.24	1.29	0.508	0.689
Feed efficiency ratio	0.89	0.84	0.87	0.86	0.01	0.423	0.742
Hepatosomatic index(%)	1.38	1.54	1.76	1.72	0.08	1.113	0.358
Viscerosomatic index(%)	7.37	7.59	8.91	8.16	0.31	1.318	0.334

Data expressed as mean of triplicate ± SEM. Means in the same row sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).
SEM, Standard error of means.

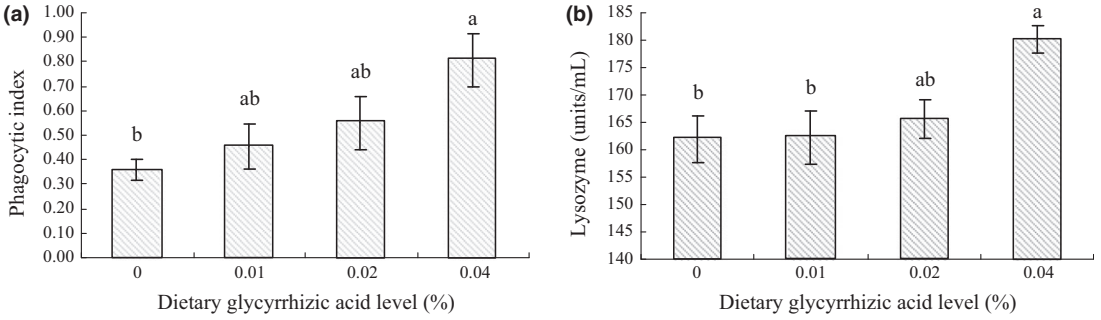

Figure 1 Phagocytic index of head kidney macrophage (a) and serum lysozyme activity (b) of large yellow croaker (*Larimichthys crocea*) fed with graded doses of dietary glycyrrhizic acid. Values (means ± SEM) in bars that do not have the same letter are significantly different ($P < 0.05$) among treatments.

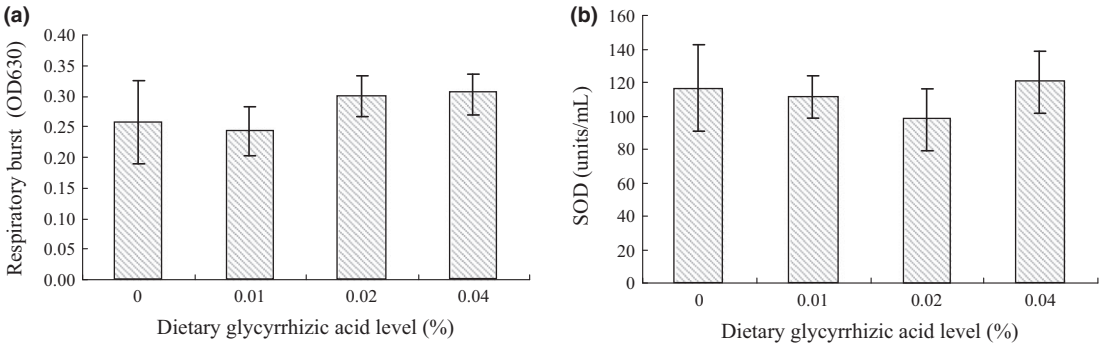

Figure 2 Respiratory burst activity of head kidney macrophage (a) and serum superoxide dismutase (SOD) (b) activity of large yellow croaker (*Larimichthys crocea*) fed with graded doses of dietary glycyrrhizic acid. Values (means ± SEM) in bars that do not have the same letter are significantly different ($P < 0.05$) among treatments.

showed significantly higher serum lysozyme activity than fish fed diets with 0.01% GA and the control diet. The serum lysozyme activity of 0.02% GA group was slightly higher than the control group ($P > 0.05$).

No significant differences were observed in respiratory burst activity of head kidney macrophage (Fig. 2a) and serum superoxide dismutase activity (Fig. 2b) ($P > 0.05$) among GA supplemented groups and the control group. Fish showed higher

respiratory burst activity of head kidney macrophage in 0.02% and 0.04% GA group compared to the control group ($P > 0.05$).

Cumulative mortality rate

No significant differences were observed in the cumulative mortality rate after the natural protozoan infection among GA supplemented groups and the control group ($P > 0.05$) (Fig. 3).

Discussion

Results of the present study showed incorporation of glycyrrhizic acid in diets at the concentration of 0–0.04% d.w. did not significantly affect the growth performance and survival rate of juvenile large yellow croaker, *L. crocea*. To date, no previous studies concerning effects of dietary GA on growth performance of fish and mammal have been reported. A study with white shrimp *L. vannamei* showed dietary GA at 0.005%–0.02% d.w. significantly improved the specific growth rate after 8 weeks of feeding trial (Chen *et al.* 2010). Bai *et al.* (2010) also reported feeding 0.06% d.w. dietary GA for 41 days significantly enhanced the specific growth rate of white shrimp *L. vannamei*. It is still not clear that how dietary GA affects growth performances of aquatic animals and further investigations are needed.

Unlike the deficient effects of GA on growth performance of experiment fish, the immunomodulatory activity of glycyrrhizic acid in large yellow croaker diet (0.04% d.w.) was demonstrated by the assay of certain non-specific immunological parameters in the present study. The modulatory effects of GA on fish innate immune response were also observed in an *in vitro* study with rainbow trout (*O. mykiss* W.) leucocytes, which showed treatment with GA enhanced the macrophage respiratory burst activity, increased lymphocyte proliferation responses to the mitogen phytohaemagglutinin, and elicited the release of a macrophage activating factor from head kidney leukocytes (Jang *et al.* 1995).

Figure 3 Cumulative mortality following natural infection of *Cryptocaryon irritans* Brown of large yellow croaker (*Larimichthys crocea*) fed with graded doses of dietary glycyrrhizic acid. Values (means ± SEM) in bars that do not have the same letter are significantly different ($P < 0.05$) among treatments.

The respiratory burst activity of head kidney macrophages and serum superoxide dismutase, however, were not significantly influenced by the inclusion of GA in diets in the present study. This may be related to that different immunological parameters might respond differently to dietary GA supplement. Akamatsu, Komura, Asada and Niwa (1991) reported that GA significantly decreased neutrophil-generated O_2^-, H_2O_2 and OH in human, while any of the ROS generated in a cell-free, xanthine-xanthine oxidase system as well as the neutrophil chemotaxis or phagocytosis was not affected. Moreover, considering that the immunoregulatory actions of GA were reported to be displayed in a dose-dependent manner (Akamatsu *et al.* 1991; Sasaki, Takei, Kobayashi, Pollard & Suzuki 2002-2003; Matsui, Matsumoto, Sonoda, Ando, Aizu-Yokota, Sato & Kasahara 2004), the GA doses causing significant influences might be inconsistent with different immunological parameters. The immunity fatigue due to long-term continuous administration of a same immunostimulant might also partially account for the absence of significant influence of GA on certain immune parameters in this study (Bai *et al.* 2010).

The mechanisms of the immunoregulatory functions of GA are being elucidated in animal and human studies. GA has been reported to inhibit the production of interleukin (IL)-10 and CC-chemokine ligand (CCL) 2 by macrophages infected with HIV (Takei, Kobayashi, Herndon, Pollard & Suzuki 2006). It has also been reported to inhibit inflammation (Van Rossum *et al.* 1998), augment natural killer cell activity (Itoh & Kumagai 1983) and induce the production of interferon (IFN)-γ (Abe, Ebina & Ishida 1982; Shinada, Azuma, Kawai, Sazaki, Yoshida, Yoshida, Suzutani & Sakuma 1986), IL-2 (Zhang *et al.* 1993; Zhang, Kato, Isobe, Hamaguchi, Yokochi & Nakashima 1995), IL-10 (Abe *et al.* 2003), IL-12 (Dai *et al.*

2001; Utsunomiya *et al.* 2001), CCL4 and CCL5 (Sasaki *et al.* 2002-2003). Studies with thermal injured mice and MAIDS mice (mice infected with LP-BM5 murine leukemia virus) suggested that by inducing CD4 + T cells which suppress type 2 cytokine production by burn-associated/MAIDS-associated type 2 T cells, GA improves the resistance of mice to *Candida albicans* infection (Utsunomiya *et al.* 1999, 2000). However, no information is available regarding the specific mechanisms of glycyrrhizic acid on the immune system of fish. More research effort in this field is needed.

Besides the immunomodulatory activities, the anti-pathogen properties (mainly antiviral and antimicrobial activities) of GA have also been wildly recorded in terrestrial animals and human (Utsunomiya *et al.* 1999, 2000; Cinatl *et al.* 2003; Lin 2003; Harada 2005; Hoever, Baltina, Michaelis, Kondratenko, Baltina, Tolstikov, Doerr & Cinatl 2005; Wolkerstorfer *et al.* 2009; Michaelis *et al.* 2010). In fish, feeding yellowtails (*S. quinqueradiata* Temminck & Schlegel) with GA for 4 weeks increased resistance to streptococcal infection (Edahiro *et al.* 1991). However, in the present study, feeding 0–0.04% dietary GA did not enhance the resistance to parasite (protozoan, *C. irritans* Brown) infection. The antiprotozoal activity of Chinese licorice roots and its chalcone compounds was demonstrated in *in vitro*/*in vivo* pharmaceutic studies with animals (Chen, Theander, Christensen, Hviid, Zhai & Kharazmi 1994; Jenett-Siems, Mockenhaupt, Bienzle, Gupta & Eich 1999; Christensen & Kharazmi 2001). It was observed that chalcones altered the ultrastructure of the parasite mitochondria and inhibited their function by selectively inhibiting fumarate reductase in the respiratory chain of the parasite (Zhai, Blom, Chen, Christensen & Kharazmi 1995; Chen, Zhai, Christensen, Theander & Kharazmi 2001). The lack of antiprotozoal activities of GA in the present study may be due to the GA concentration and the experiment animal used (considering the differences between the parasitic modes of parasites in fish and terrestrial animals due to their enormously different living environments), or that the anti-parasitic activity of GA is not as efficient as its antiviral and antimicrobial activities.

In conclusion, dietary GA supplementation (0.04% d.w.) enhanced certain non-specific immunological parameters after 8 weeks of feeding trial, but at the concentration of 0–0.04% d.w., dietary GA was not able to significantly affect the growth performance and protect juvenile large yellow croaker effectively from the natural infestation of protozoan *C. irritans* Brown. Further studies are needed to elucidate the efficacy of glycyrrhizic acid as an immunostimulant in fish feed and the underlying mechanisms.

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