AACL BIOFLUX

Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

Growth, plasma cortisol, liver and kidney histology, and resistance to vibriosis in brown-marbled grouper, *Epinephelus fuscoguttatus* fed onion and ginger

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Abstract. The health-promoting effects of dietary onion and ginger were studied in brown marbled grouper, *Epinephelus fuscoguttatus*. An eight-week feeding trial was conducted to evaluate the effects of dietary onion and ginger on growth, cortisol levels, histology and disease resistance in the fish. Five experimental diets were formulated to contain either onion (2%), ginger (2%), β-glucan (1%) or vitamin C (3%) and a control diet (without immunostimulants). Each diet was fed to triplicate groups of fish with an average weight of 10.85 ± 0.69g. Fish supplemented with either of the immunostimulants exhibited a significantly higher growth compared to the control group. Specific growth rate (SGR) and feed conversion efficiency (FCE) were significantly higher in the onion and ginger-supplemented fish than the control. Cortisol level was higher in the control compared to the immunostimulant-fed groups with onion and ginger-fed fish showing significantly lower cortisol levels. When experimentally infected with *Vibrio harveyi*, fish fed onion or ginger exhibited significantly reduced mortality when compared with the control and β-glucan, but not when compared with the vitamin C-fed group. Liver sections sampled 4 days post-infection showed no remarkable pathology except for the slight reduction in glycogen granules in the supplement-fed fish. The liver of non-supplemented infected fish showed necrosis, fatty globule deposition, vacuolation, and presence of short rod-shaped bacteria. Kidney sections in the supplemented groups, likewise, did not show significant pathology similar to the uninfected control, whereas those of infected control fish showed necrosis of the tubules and glomeruli resulting in severely altered morphology of the tissues and presence of bacteria in the necrotic areas. As a result of circumventing tissue damage, wound healing was faster in fish supplemented with ginger, onion, and vitamin C compared to the β-glucan and the control groups. This study showed the benefits of onion and ginger in promoting growth and alleviating stress and severity of vibriosis in grouper.
Key Words: immunostimulant, onion, ginger, grouper.

Introduction. Global demand for fish and other fishery products increases every year and intensification of farming practices to maximize production per unit area leads to overcrowding which adversely affects the health and growth of farmed fish. In aquaculture, the use of immunostimulants is being promoted since its role in increasing disease resistance of farmed species is well-recognized. Immunostimulants are said to be safer than chemotherapeutants, are more widely applicable to various species than vaccination, and might become a powerful tool in controlling fish diseases. Apart from modulating immune responses, they are also known for their growth-promoting activity. The utilization of natural sources of immunostimulants like onion and ginger has proven to boost the immune system in humans. Studies on their efficacy in fish, however, are very few and information on the quantitative dietary requirements for these natural substances is limited. At present, Vitamin C and β-glucan are the commonly used immunostimulants in animals and oral administration of these substances to stimulate the immune system is well-documented in some species of fish. In the Philippines, grouper is one of the promising species with high market value. Yet, production from aquaculture is inadequate to meet the market demand due to the difficulty in rearing the fish as they are highly susceptible to stress and diseases. Use of antibiotics and medicated feeds for grouper health management had been tried with some success. Nevertheless, these methods of treatment are costly, promote antimicrobial resistance and transfer among bacteria, contaminate aquatic ecosytems, and leave harmful residues in aquaculture products. As such, the role of nutrition as a crucial factor in host defense against pathogens to maintain better health status has been recognized. The addition of immunomodulatory substances in fish diets may help increase survival by supporting host immune functions that are necessary for protection against diseases. However, only a few studies on immunostimulation have been conducted in tropical marine finfishes such as the grouper.

Immunostimulants can enhance the activities of leukocytes such as the macrophages and natural killer cells. The activation of these cellular responses is associated with increased protection against infectious diseases. Several types of immunostimulants may include synthetic chemicals, bacterial derivatives, animals and plant components, and some dietary additives. Immunostimulating herbs have been reported to protect humans and other animals against chronic diseases. Among them are onion (*Allium cepa*) and ginger (*Zingiber officinale*) which contain compounds with anticarcinogenic properties, antiplatelet activity, antithrombotic activity, antiasthmathic and antibiotic effects that provide significant host protection against diseases (Craig 1999; Akoachere et al 2002; Griffiths et al 2002; Rahman et al 2011). Ginger has shown antimicrobial and immunostimulant effects in grouper (Punitha et al 2008). Also, dietary intake of ginger significantly enhanced the non-specific immune responses in rainbow trout (Dugenci et al 2003). Nutritional factors such as vitamin C which mainly aid the function of phagocytic cells and increase their bactericidal activities are known immunostimulants in many species. High levels of vitamin C are found to increase resistance against infection in channel catfish (*Ictalurus punctatus*), rainbow trout (*Onchorhynchus mykiss*), and Atlantic salmon (*Salmo salar*) (Li & Lovell 1985; Hardie et al 1991; Wahli et al 1995). Likewise, β-glucans - microbial cell wall derivatives - have been successfully utilized for their ability to activate host defense mechanisms against viral, parasitic, and bacterial infections. In vitro and in vivo studies in rats demonstrated that β-glucans induce the activation of macrophages (Seljelid et al 1987; Reichner et al 2001). Atlantic salmon and catfish injected with yeast glucan exhibited increased resistance to bacteria (Robertsen et al 1990; Chen & Ainsworth 1992), though such an effect was not shown in rainbow trout (Thompson et al 1995). Improved non-specific immune responses was likewise obtained in β-glucan-injected grouper (Tavarro 2002). The present study evaluated onion and ginger vis-a-vis vitamin C and β-glucan as dietary immunostimulants and as adaptogenic agents to enhance disease resistance and survival against vibriosis in grouper, *Epinephelus fuscoguttatus* (Forsskål, 1775).

β-glucan and vitamin C served as control immunostimulants being the most commonly used substances in fish.

Material and Method

Experimental diets and rearing conditions. Five experimental diets were formulated to contain either onion (2%, red onion), ginger (2%), β-glucan (1%, MacroGard) or vitamin C (3%, Stay-C), and a diet without any of the immunostimulants serving as the control (Apines-Amar et al 2012). The experimental diets were prepared in pellet form and stored at -20°C until use.

E. fuscoguttatus weighing about 10.85 ± 0.69 g were randomly stocked in fifteen 250 L capacity tanks at 55 fish each. The fish were fed the diets at 5% of the body weight (BW) per day and later adjusted to 3% BW given every other day. Tanks were supplied with filtered sea water with a flow rate of 2.0 liters min^{-1} and provided with sufficient aeration. The fish were acclimated under laboratory conditions and weaned to the test diet (control diet) for one (1) week before the start of the experiment. The feeding trial lasted for 8 weeks (February to March 2010 at SEAFDEC AQD).

Sampling. The growth of fish was measured every 2 weeks. All fish in each tank were weighed individually. After the feeding trial, 4 fish per tank (12 fish/treatment) were randomly sampled for cortisol analysis using a kit (Oxford Biomedical Research).

Preparation of inoculum. *Vibrio harveyi* (Caipang et al 2011, 2012) from the frozen stock was passed twice in live grouper to recover its virulence. After bacteria had been reisolated from the liver of the moribund fish, they were plated in Brain-Heart Infusion Agar with 1.5% NaCl (BHIA+) for 20 hours at room temperature. The colonies were re-suspended in PBS and the cell concentration was determined by measuring the OD at 600 nm and plate-counting on BHIA+.

Challenge test. The fish that had been fed the experimental diets were stocked in triplicate tanks at 10 fish/tank. Each fish was injected (IM) with 2.138×10^6 CFU *Vibrio harveyi*/fish (LD_{50} result). Mortality was monitored daily for 10 days.

Histopathology. Fish were sampled 4 days post infection and tissues were excised and fixed in 10% buffered formalin. The fixed samples were submitted to the histopathology laboratory for processing.

Statistical analysis. Data were analyzed by one-way ANOVA using SYSTAT version 8.0 (SPSS, Chicago, USA). Differences between treatments were compared by Tukey's test. Values were considered significant at $p < 0.05$.

Results and Discussion. This study demonstrated the growth and health-promoting effects of onion and ginger as dietary supplements in grouper, *E. fuscoguttatus*. Weight gain was significantly improved in all the immunostimulant-supplemented fish compared to the control (Figure 1). Specific growth rate (SGR) and feed conversion efficiency (FCE) were statistically higher in the onion- and ginger-fed but not in β-glucan- and vitamin C-fed fish compared to the control (Table 1). Citarasu (2010) reported that ginger also exhibited growth-promoting effects in addition to immunostimulation. Low levels of plasma cortisol were obtained in all the supplemented groups but significantly reduced levels were attained only in the onion and ginger-supplemented fish (Figure 2). Inverse relationships between cortisol level and SGR and cortisol level and FCE were observed with the use of various immunostimulants in this study (Figure 3). SGR and FCE increased as cortisol levels decreased as a consequence of supplementation. Lower cortisol levels and higher weight gains indicate lesser stress and better growth performance in the onion and ginger-fed fish than the control, β-glucan and vitamin C-fed groups. However, Ostrowska et al (2004) reported that onion had no effect on growth in pigs. Control of stress as shown by reduced cortisol levels in the immunostimulant-supplemented fish in the present study is correlated not only with increased growth and FCE but also with protection of tissues with immune and metabolic functions, and with significantly higher survival upon bacterial challenge. This effect could be attributed the high antioxidant and antimicrobial activities in ginger (Halvorsen et al 2002; Punitha et al 2008). Cumulative mortality in the onion-, ginger-, and vitamin C-supplemented fish but not the β-glucan-fed fish was significantly lower than the control (Figure 4). Previously, innate immunity indices and survival in *E. fuscoguttatus* were improved by onion and ginger supplementation (Apines-Amar et al 2012). Diets supplemented with ginger stimulated lysozyme and total Ig production better than the other groups and this was associated with low mortality. Other natural sources of immunostimulants such as combination of the Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) extract and the mineral boron in the diet of Nile tilapia (*Oreochromis niloticus*) also resulted in reduced mortality after *Aeromonas hydrophila* infection (Ardó et al 2008). Likewise, Punitha et al (2008) reported that a mixture of all-plant extracts including ginger fed to the grouper, *E. tauvina* also resulted in improved survival, growth, and immune responses. The immunostimulating effects and enhanced disease resistance shown by onion and ginger in *E. fuscoguttatus* in this study were also observed in grouper, *E. malabaricus* fed higher levels of ascorbic acid (Lin & Shiau 2005). On the other hand, continuous feeding of β-glucan for 8 weeks in this study did not protect the fish against infection as previously observed in grouper, *E. fuscoguttatu*s (Apines-Amar et al 2012) and in catfish (Duncan & Klesius 1996). This could be attributed to overstimulation of the immune system requiring added energy costs, and the absence of antioxidant effect in β-glucan. Although evidence concerning this effect of β-glucan is equivocal, recommendations for pulsed feeding of β-glucan supplemented diets should be followed.

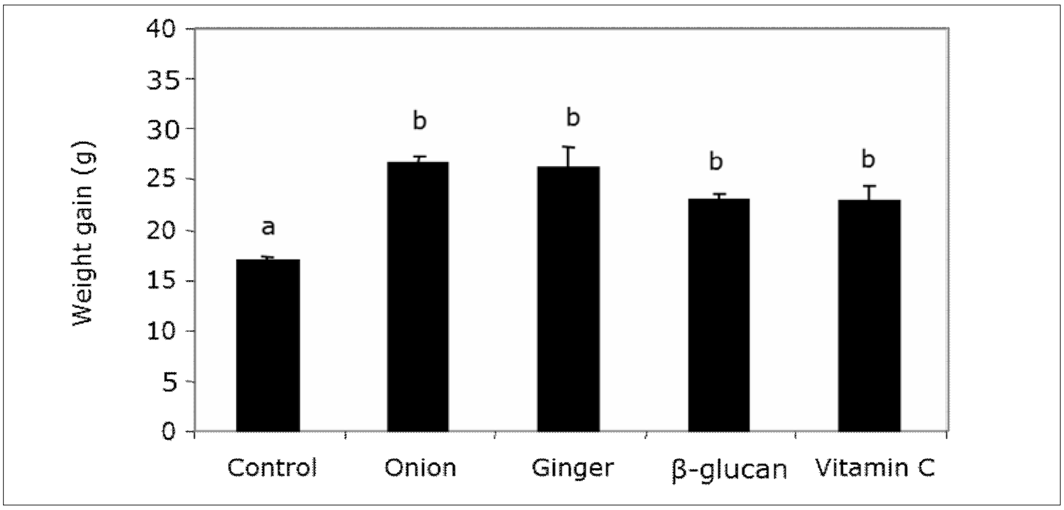

Figure 1. Weight gain in *E. fuscoguttatus* fed the different immunostimulants for 8 weeks. Bars are means of three groups of fish (n=3), with 55 fish per group + SD.

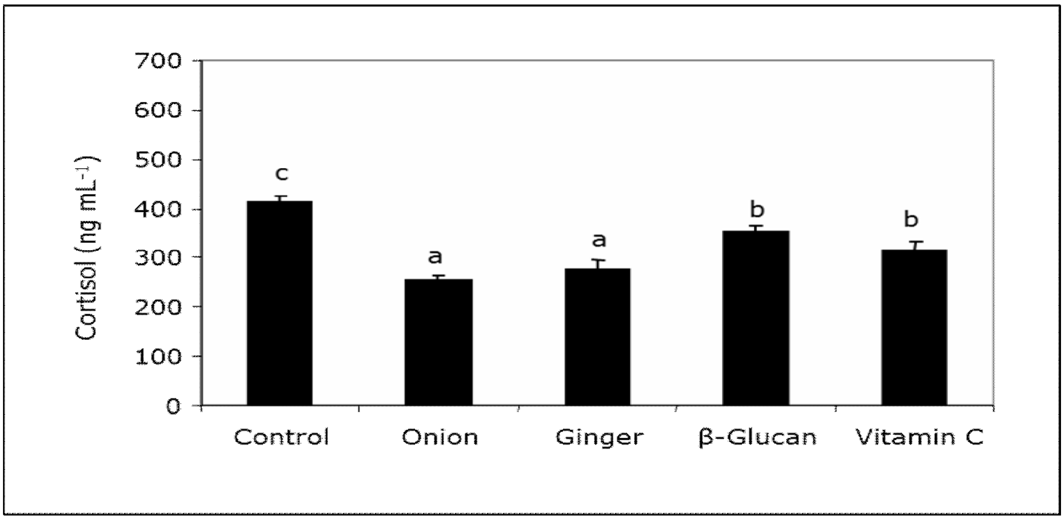

Figure 2. Cortisol level in *E. fuscoguttatus* fed the different immunostimulants for 8 weeks. Bar are means of three groups of fish (n=3), with 4 fish per group + SD.

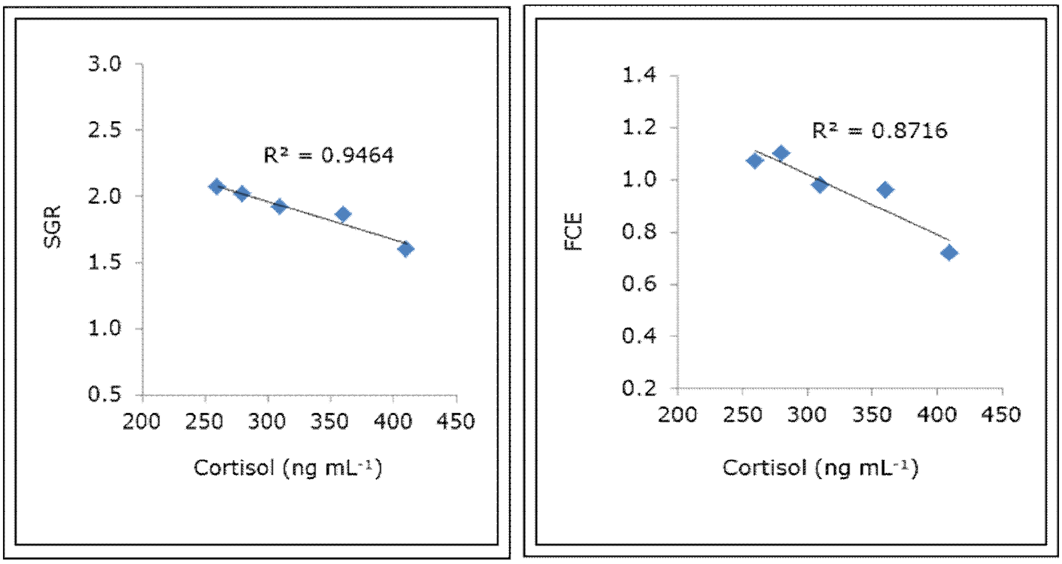

Figure 3. Influence of cortisol level on specific growth rate (SGR) and feed conversion efficiency (FCE) in *E. fuscoguttatus* fed the different immunostimulants for 8 weeks.

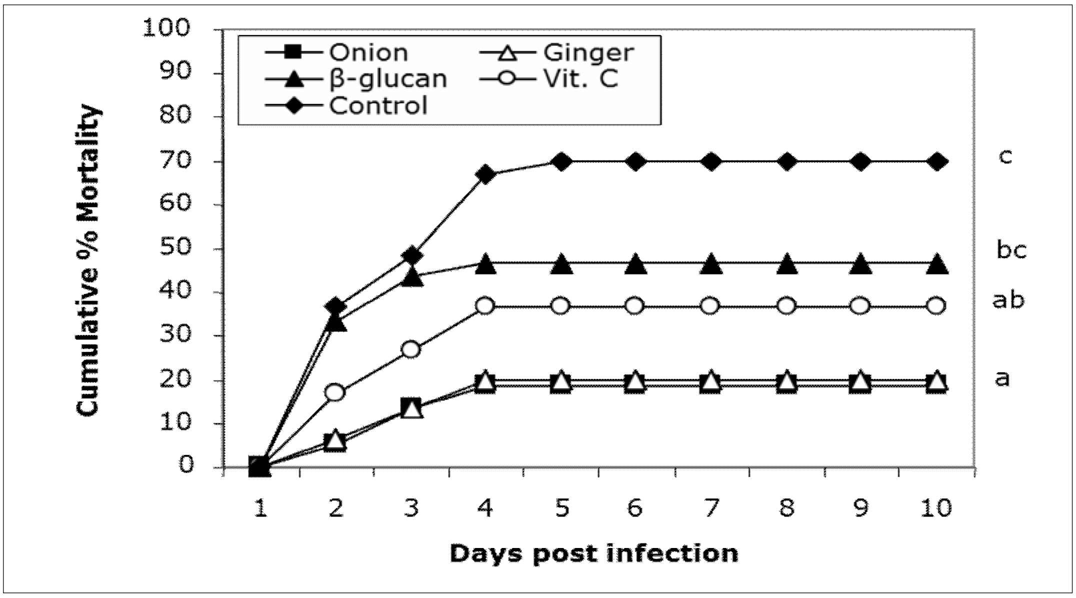

Figure 4. Mortality in *E. fuscoguttatus* fed the different immunostimulants for 8 weeks. Values are means of three groups of fish (n=3), with 10 fish per group.

Table 1

Growth and feed performance in *E. fuscoguttatus* fed the different immunostimulants for 8 weeks*

Dietary treatment	*SGR*[1] *(%/day)*	*Feed consumed (g/fish)*	*FCE*[2]
Control	1.60 ± 0.12^{a}	23.65 ± 0.77^{a}	0.72 ± 0.04^{a}
Onion	2.07 ± 0.17^{b}	24.22 ± 0.98^{a}	1.07 ± 0.08^{b}
Ginger	2.02 ± 0.14^{b}	23.94 ± 0.50^{a}	1.10 ± 0.02^{b}
β-glucan	1.92 ± 0.17^{ab}	23.41 ± 0.74^{a}	0.98 ± 0.02^{ab}
Vitamin C	1.86 ± 0.18^{ab}	23.79 ± 0.89^{a}	0.96 ± 0.09^{ab}

[*]Values are mean ± SD. Values in a column nor sharing the same superscript are significantly different ($p < 0.05$); [1]Specific Growth Rate = 100 x [(ln Wt final – ln Wt initial)/(no. of days)]; [2]Feed Conversion Efficiency = Weight gain (g) / Feed consumed (g).

Hematoxilin & Eosin stained liver sections sampled at the peak of mortality (day 4) showed the liver to have no significant pathology, except for the reduction and apparent disappearance of glycogen granules in the supplement-fed fish (Figure 5). Liver of the vitamin C-fed fish showed near-normal histological morphology. The liver of non-supplemented infected fish showed necrosis and fatty globule deposition, vacuolation, and the presence of short rod-shaped bacteria. Kidney sections likewise did not show significant pathology in the supplemented groups compared to the uninfected control (Figure 6). Except for minimal infiltration of inflammatory cells, the kidney tubules and glomeruli were intact. The kidney of infected controls showed necrosis of the tubules and glomeruli resulting in severely altered morphology of the tissues. Bacteria can be seen in the necrotic areas. This implies that the immunostimulants imparted protection on liver and kidney tissues upon bacterial infection.

Most of the bioactivity of onions have been attributed to the flavonoids (quercetin and kaempferol) and cysteine sulfoxide (CSO) with S-propenyl-CSO as the predominant S compound (Keusgen et al 2002; Ostrowska et al 2004; Santas et al 2010), whereas ginger contains compounds such as the terpenes gingerol, zingerone and its derivatives (Griffiths et al 2002; Rahman et al 2011). The varied components of onions and ginger may exert their biological effects through different mechanisms either separately or synergistically, resulting in the observed overall beneficial effect as determined in the present study. It appears that onion and ginger supplements could be provided to grouper to reduce stress, improve growth and prevent occurrence of high mortalities.

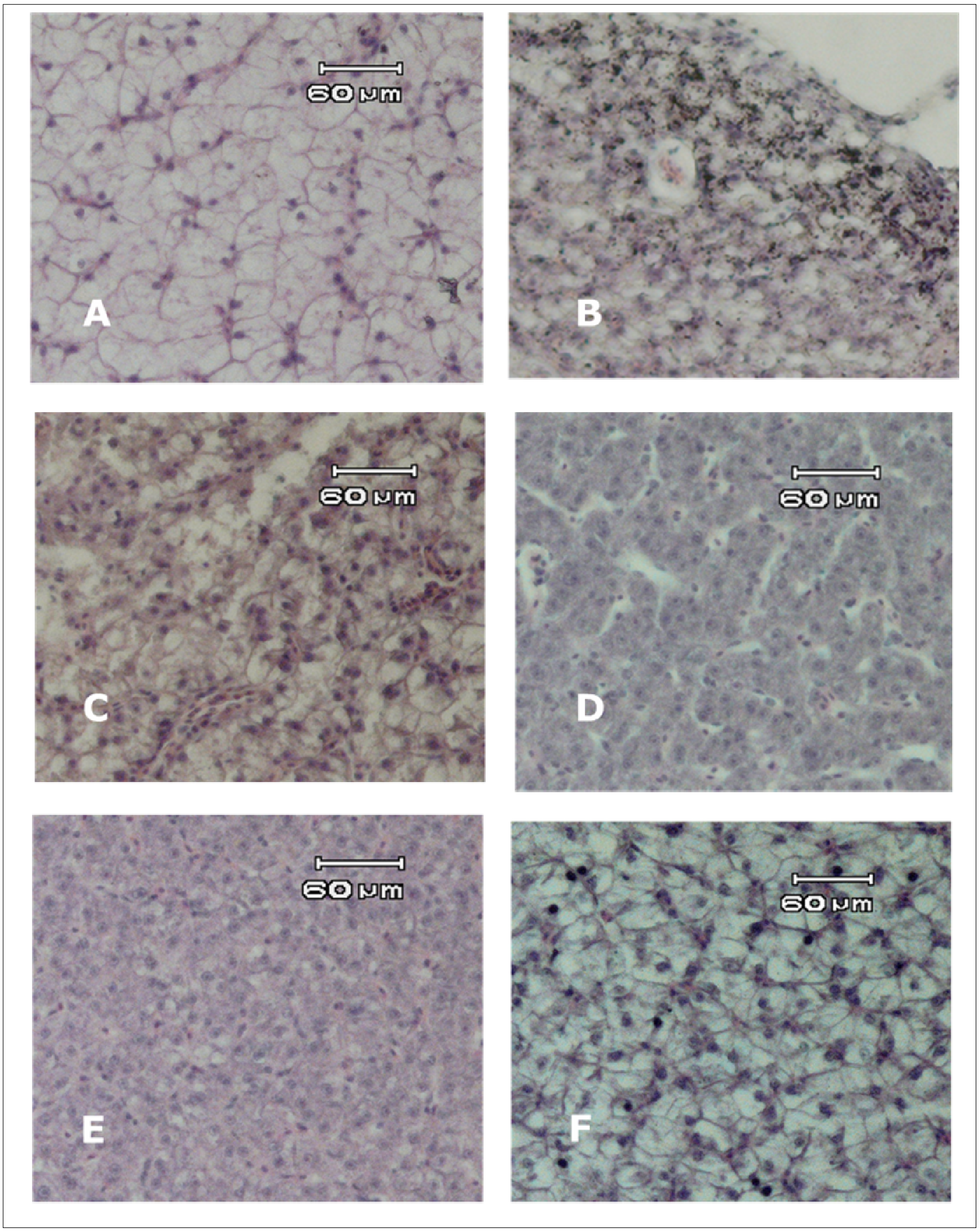

Figure 5. Histopathology of the liver of *E. fuscoguttatus*. *Vibrio harveyi*-infected tissues were sampled 4 days post infection: A) uninfected control; B) infected control; C) onion infected; D) ginger infected; E) β-glucan infected; F) vitamin C infected.

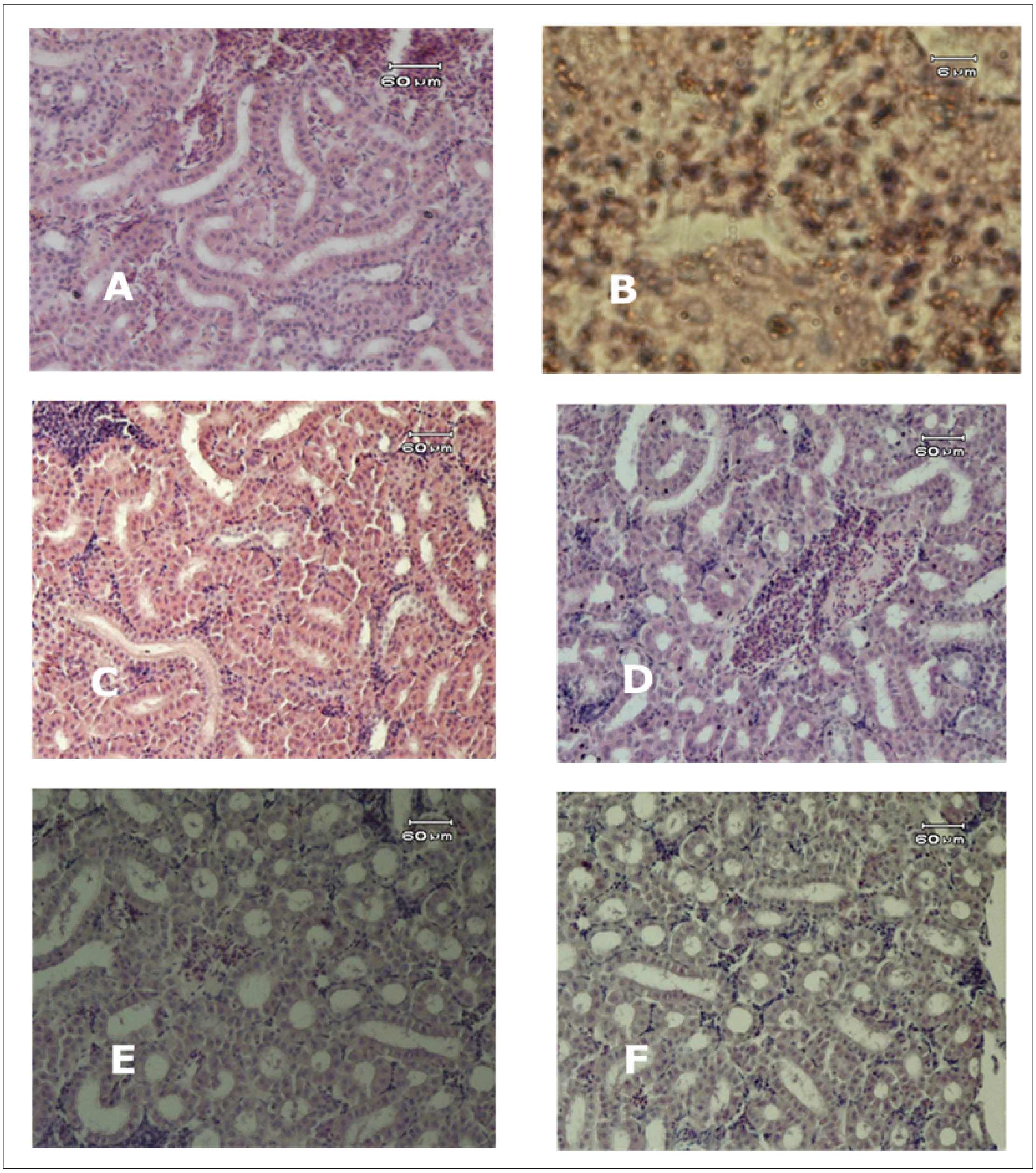

Figure 6. Histopathology of the kidney of *E. fuscoguttatus*. *Vibrio harveyi*-infected infected tissues were sampled 4 days post infection: A) uninfected control; B) infected control; C) onion infected; D) ginger infected; E) β–glucan infected; F) vitamin C infected.

Conclusions. It is evident from the present study that dietary supplementation of onion and ginger improved growth, reduced stress, imparted protective effects on kidney and liver tissues, and enhanced the resistance from *Vibrio harveyi* infection in brown marbled-grouper with effects similar to vitamin C and better than β-glucan.

Acknowledgements. The University of the Philippines Visayas is gratefully acknowledged for providing the research fund and the Southeast Asian Fisheries Development Center, Aquaculture Department (SEAFDEC/AQD) for the use of their research facilities.

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Received: 18 September 2013. Accepted: 01 October 2013. Published online: 15 October 2013.
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How to cite this article:
Apines-Amar M. J. S., Amar E. C., Faisan Jr. J. P., 2013 Growth, plasma cortisol, liver and kidney histology, and resistance to vibriosis in brown-marbled grouper, *Epinephelus fuscoguttatus* fed onion and ginger. AACL Bioflux 6(6):530-538.