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Dietary onion and ginger enhance growth, hemato-immunological responses, and disease resistance in brown-marbled grouper, *Epinephelus fuscoguttatus*

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Abstract. A 12-week (September to December 2009) feeding trial was conducted to evaluate the immunostimulatory effects of different substances administered orally through the diet in the brown-marbled grouper, *Epinephelus fuscoguttatus.* Five experimental diets containing either onion, ginger, β-glucan, or vitamin C and a control diet (without immunostimulants) were fed to the fish weighing about 44 g for 12 weeks. Onion-fed fish showed significantly increased weight gain, hematocrit, and total Ig compared to the control group; however, leukocyte differential count and ROS production were unaffected. Ginger-fed fish likewise significantly increased total Ig, ROS production and lysozyme activity. However, it did not affect growth and hematocrit value. β-glucan significantly increased growth and total Ig but had no effect on the other parameters. Vitamin C significantly increased hematocrit, total Ig and ROS production but did not increase growth. Upon challenge with a bacterial pathogen *Vibrio harveyi*, mortality was significantly reduced in the onion, ginger and vitamin C-fed fish but not in the β–glucan-fed fish. This study demonstrated that onion and ginger could positively affect the innate immune responses and protect grouper against *Vibrio harveyi* infection.
Key Words: disease resistance, innate immunity, ginger, grouper, onion.

Introduction. Grouper (*Epinephelus fuscoguttatus* (Forsskål, 1775)) is one of the high-value aquaculture species with high economic potential in the Asia-Pacific region. However, the culture of groupers presents many challenges due to their sensitivity to stress and high susceptibility to infectious diseases. The use of antibiotics and chemotherapeutants to control diseases of grouper as practiced by some farmers have raised several issues including high operational cost, emergence of drug-resistant bacteria, suppression of immunity, and food and environmental contamination. In aquaculture, the use of probiotics and dietary enhancement have been recognized as alternative methods of health management. In particular, nutritional status has been increasingly acknowledged as a crucial factor in host defense against pathogens. As such, use of feed supplements aiming to improve not only the growth but also the health of aquaculture species has gained widespread interest and acceptance.

Supplementing immunomodulatory substances in fish diets may enhance disease resistance by reinforcing host innate immune functions that are necessary for protection against infectious diseases. The most commonly tested immunostimulants used to promote innate immunity in fish include glucan (Chen & Ainsworth 1992; Jorgensen & Robertsen 1995; Jeney et al 1997), levamisole (Siwicki 1987; Siwicki et al 1990; Jeney & Anderson 1993) and vitamin C (Liu et al 1989; Navarre & Halver 1989; Hardie et al

1991; Waagbo et al 1993; Adham et al 2000; Cuesta et al 2002; Sobhana et al 2002; Lin & Shiau 2005), but other less known substances have also been found effective. For instance, supplementation of *Sargassum fusiforme* polysaccharide extracts at 0.5% and 1% in shrimp diet resulted in enhanced immune response and improved resistance to vibriosis infection (Huang et al 2006). Likewise, shrimp fed with Indian herbs extract at 800 mg kg^{-1} diet exhibited high survival rate and reduced viral load after infection with WSSV (Citarasu et al 2006). Some plants including herbs and spices are also known for their immunomodulatory function, as they are rich sources of natural antioxidants. Onions and ginger have long been reported for their health benefits in humans and other animals but their immunomodulatory effects have scarcely been studied in fish. Onion (*Allium cepa*) and ginger (*Zingiber officinale*) are among the spices with reported antiplatelet, antibacterial, antifungal, antiviral, anti-inflammatory and antioxidant properties and have been confirmed to support the immune system in humans and other animal models (Akoachere et al 2002; Thomson et al 2002). The health-enhancing properties of onions have been attributed to the flavonoids particularly quercetin and the organosulfur compounds (Price & Rhodes 1997; Griffiths et al 2002; Coskun et al 2004). Ginger contains the antioxidants gingerols, shogaols and zingerone (Hori et al 2003). In rats, ginger favorably increased the activities of pancreatic lipase, chymotrypsin and amylase when consumed through the diet, whereas in fish, dietary intake of ginger significantly increased non-specific immune responses (Dugenci et al 2003). These plants are readily available but evidence of their effects especially in fish is scarce and information on the quantitative dietary requirements for these potential feed supplements is lacking. Hence, this study was carried out to determine the immunomodulatory effects of onion, ginger, β-glucan, and vitamin C and their influence on growth and disease resistance in the grouper, *E. fuscoguttatus*. β-glucan and vitamin C were used as positive controls being the well-studied and commonly used immunostimulants in fish.

Materials and Method

Growth and immune response experiment

Experimental diets. Five experimental diets were formulated to contain either red onion (20 g kg^{-1}); ginger (20 g kg^{-1}); β-glucan (MacroGard, Biotec AHN AS, Norway, 10 g kg^{-1}); or vitamin C (Rovimix® Stay-C®, 3 g kg^{-1} L-ascorbyl-2-polyphosphate); and an unsupplemented control diet (Table 1). The addition of 20 g kg^{-1}onion and ginger was an estimated amount as there is no available information on the use of powder form in fish. In rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)), ginger was supplemented at 1% (10 g kg^{-1}) but in aqueous extract form (Dugenci et al 2003). The diets have an average protein and lipid levels of 45.98 ± 0.02% and 9.66 ± 0.06%, respectively. The experimental diets were prepared in pellet form and stored at -20°C until use.

Experimental conditions, fish, and feeding. Four hundred groupers obtained from SEAFDEC AQD hatchery weighing about 44 g on average were stocked at 40 fish in each of 10 duplicate 250-L capacity fiberglass tanks to which five dietary groups were assigned at random. Tanks were supplied with filtered sea water at a flow rate of 2 L min^{-1} and provided with sufficient aeration. The fish were acclimated to laboratory conditions for 2 weeks and weaned to the pelleted diet (control diet) for one week before the start of the feeding trial. Each diet was fed at 5% of the fish body weight (BW) per day and later adjusted to 3% BW given every 2 days. The feeding trial lasted for 12 weeks (September to December 2009).

Sampling. Average body weight was obtained by weighing each fish at the start of the feeding trial and every three weeks thereafter until termination of the feeding experiment. At the end of the trial, 10 fish from each tank were randomly sampled for the analysis of the immune indices. All analyses were done on the day of the sampling except for total Ig and lysozyme.

Table 1

Composition of the experimental diets (g kg^{-1})

Ingredients	*Control*	*Onion*	*Ginger*	*β-glucan*	*Vitamin C*
Fish Meal (Peruvian)	420	420	420	420	420
Soybean Meal	120	120	120	120	120
Acetes sp.	160	160	160	160	160
Squid Meal	60	60	60	60	60
Cod Liver Oil	30	30	30	30	30
Soybean Oil	30	30	30	30	30
Vitamin Mix	15	15	15	15	15
Mineral Mix	20	20	20	20	20
Bread Flour	100	100	100	100	100
Onion		20			
Ginger			20		
β-glucan[a]				10	
Vit. C[b]					8.57
Rice Bran	45	25	25	35	36.43
Proximate composition (%)					
Moisture	6.30	6.63	6.44	6.81	6.48
Crude Protein[c]	46.01	46.03	45.94	45.96	45.98
Crude Lipid[c]	9.58	9.53	9.67	9.65	9.89
Crude Fiber[c]	2.60	2.05	2.10	2.85	2.72
Ash[c]	14.29	13.98	14.13	14.04	14.40

[a] MacroGard; [b] Rovimix Stay-C 35 (L-ascorbyl-2-polyphosphate); [c] Dry matter basis.

Hemato-immunological parameters

Hematocrit. Blood was drawn into a capillary hematocrit tube with one end sealed with cha-seal tube sealing compound (Medline), and centrifuged for 5 min using a microhematocrit centrifuge. Hematocrit values were obtained using the microhematocrit tube reader (Hawkley and Sons Ltd.).

Differential count. Blood smears were prepared by placing a small drop of blood near one end of a clean glass slide and spread evenly with a spreader slide oriented at an angle of about 30°. The smears were air dried, fixed in a methanol based fixative solution, and stained using the Diff-Quik Staining Technique. Each blood smear was dipped in a solution containing buffered eosin Y (Solution I) followed by dipping in buffered methylene blue and azure A dye (Solution II). After staining, the smears were rinsed in distilled water to remove excess dye and allowed to air dry. The blood cells were examined and counted under the microscope (40X) using a differential counter and expressed as percentage of total cells counted.

Total immunoglobulin (Ig). Total Ig from serum samples was assayed using the Biuret method for protein determination (Sigma Chemicals, Diagnostic method 690). The absorbance of the samples was measured before and after precipitation of the immunoglobulin component with polyethylene glycol (Siwicki & Anderson 1993). The difference in the protein content is considered as the total immunoglobulin.

Lysozyme activity. Lysozyme activity was determined turbidimetrically (Ellis 1990). Serum samples (25 μL) were placed into triplicate wells of a 96-well microtiter plate. Then, 0.075% *Micrococcus lysodeikticus* suspension was added at 175 μL into each well and mixed properly. The change in turbidity was measured at 450 nm at 5 min interval in a microplate reader (Bio Tek Instruments).

Respiratory burst assay. Production of superoxide anion was measured following the method of Siwicki & Anderson (1993) with some modifications. Blood sample was drawn in a capillary tube and centrifuged at 1500g for 5 min. The peripheral blood leukocytes

(PBL) together with the serum were collected by breaking the tube at the point and washed with 50 μL HBSS. The cell suspension was mixed properly using a pipette. Fifteen (15) μL of the mixture was transferred into each microtube containing 15 μL of either HBSS or NBT and incubated at room temperature for 1 hour. Thereafter, 400 μL of DMSO was added into each tube, mixed properly and centrifuged at 4ºC for 5 min at 1500g. The supernatant (250 μL) was transferred into the wells of a 96-well microtiter plate and absorbance was read at 540 nm in a microplate reader (Bio Tek Instruments).

Challenge trial. Vibrio harveyi were inoculated in Brain Heart Infusion Agar + 1.5% NaCl (BHIA+) and were grown at room temperature for 20 hours. Cultures were suspended in PBS and the bacterial concentration was adjusted to$10^{6.33}$CFU mL^{-1} based on the result of the preliminary study on the determination of the LD_{50} (dose that can cause 50% mortality to the experimental fish). The fish previously fed with the experimental diets were stocked at 10 fish per tank, with 3 replicates per treatment. Each fish was injected intramuscularly (IM) with 100 μL of the prepared bacteria. Mortality was recorded for 10 days.

Statistical analysis. Results were analyzed by one-way ANOVA using SYSTAT version 8.0. Data for the cumulative mortality were arc sine transformed before statistical analysis was performed. Differences between treatments were compared by Least Significance Difference test. Significance level was set at $p<0.05$.

Results and Discussion. Dietary intake of the immunostimulants resulted not only in enhanced immune responses but also in improved growth and disease resistance of *E. fuscoguttatus*. Growth was significantly higher in fish fed onion, ginger, β-glucan, and vitamin C as reflected significant increases in weight gains in these groups compared to the control (Table 2). Also, feed conversion efficiency (FCE) was statistically higher in the immunostimulant-fed fish indicating better feed performance compared to the control group. Herbal extracts containing ginger also improved the survival, growth and immune responses of *Epinephelus tauvina* (Forsskål, 1775) (Punitha et al 2008). Likewise, vitamin C supplementation has been reported to enhance growth in Atlantic salmon (*Salmo salar* Linnaeus, 1758) and grouper (Sandnes et al 1990; Lin & Shiau 2005), while onion had no effect on growth and feed intake in pigs (Ostrowska et al 2004).

Table 2

Growth and feed efficiency of *E. fuscoguttatus* fed the different immunostimulants for 12 weeks[1]

Dietary treatment	*Initial weight (g)*	*Final weight (g)*	*Weight gain (g)*	*Feed consumed (g/fish)*	*SGR (%/day)*[2]	*Feed conversion efficiency*[3]
Control	45.53	89.45	43.92[a]	95.91	0.75[a]	0.46[a]
Onion	43.06	100.92	57.86[b]	92.81	0.95[b]	0.62[c]
Ginger	43.51	93.13	49.62[b]	92.73	0.85[b]	0.53[b]
β-glucan	44.48	100.17	55.69[b]	93.15	0.90[b]	0.60[bc]
Vitamin C	43.24	92.58	49.34[b]	93.52	0.85[b]	0.53[b]

[1]Values in a column not sharing the same superscript are significantly different ($p<0.05$);
[2]Specific Growth Rate = $[(\ln Wt_{final} - \ln Wt_{initial})/\text{no.of.days}] \times 100$;
[3]Feed Conversion Efficiency = Weight gain (g)/Feed consumed (g).

Hematological parameters are important in evaluating health and physiological conditions in fish (Sandnes et al 1988). The results of the present study indicated that addition of immunostimulants in the diets slightly increased the hematocrit level in grouper. Hematocrit was statistically higher in the onion- and vitamin C-fed groups than the control but was not markedly different from other supplemented groups (Table 3). Polyphenols and flavonoids in onions and ginger affect erythrocyte membrane fragility (Sivonova et al 2004) by protecting cells from possible damage against oxidative radicals. This is in contrast to pig fed onions, which exhibited lower PCV, hemoglobin and erythrocytes in a dose-dependent manner (Ostrowska et al 2004) which may be induced

by high intakes of onion disulfides (Munday et al 2003). Absence of adverse effect on the hematocrit level in the present study may indicate that at such feeding level and duration no toxic reactions detrimental to the fish were elicited. Among the leukocytes, lymphocyte percentage was higher in the onion- followed by ginger-fed fish compared to the control (Table 3). Increased lymphocyte concentration was also noted in pigs that consumed onions (Ostrowska et al 2004), whereas purified allicin from garlic (*Allium sativum*) stimulated mouse splenocytes and enhanced cell-mediated immunity in human peripheral blood mononuclear cells (Patya et al 2004). Most of the effects of onions have been attributed to cysteine sulfoxide (CSO) with S-propenyl-CSO as the predominant S compound (Keusgen et al 2002; Ostrowska et al 2004). Sulfur-containing compounds such as methyl sulfonate methane (MSM) have immunomodulating properties (Amar & Faisan 2011) which are attributed to S being a component of the antioxidant enzyme Glutathione peroxidase. However, the varied components of onion and ginger may exert further biological effects through different mechanisms either separately or synergistically. Generally, higher percentages of lymphocytes and lower percentages of neutrophils were observed in the supplemented groups compared with the control, although these differences were not statistically significant (data not shown). This could indicate that the supplements were able to stimulate lymphocyte proliferation while concomitantly reducing the proliferation of inflammatory cells.

Innate humoral immune defenses such as lysozyme serve as the first line of defense when a pathogen enters the body of a host animal. Lysozyme can kill bacteria by breaking the β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of the bacterial cell wall (Paulsen et al 2001). The ginger-fed fish showed a significant increase in lysozyme activity compared to control fish (Table 3). Oral administration of fresh ginger also improved the activity of serum lysozyme in mice (Wang et al 2001). However, enhanced lysozyme activity was reported in fish fed vitamin C (Waagbo et al 1993; Roberts et al 1995; Cuesta et al 2002; Lin & Shiau 2005) or injected with β-glucan (Tavarro 2002).

Natural Igs are considered as components of the innate immune system since they are produced without any apparent antigenic stimulation, are found in the serum of healthy vertebrates and are polyreactive showing reactivity for non-self associated molecular patterns like LPS, viral and parasitic products (Whyte 2007). Natural antibodies are a well-known phenomenon in fish and they take part in both viral and bacterial defense in rainbow trout and goldfish (*Carassius auratus* (Linnaeus, 1758)), whereas in cod (*Gadus morhua* Linnaeus, 1758) high level of IgM and the natural antibody response may contribute to the poor specific response (Magnadottir et al 1999). In this study, all the supplemented groups especially the ginger-fed fish exhibited significantly increased total Ig levels (Table 3) suggesting better immunocompetence. On the whole, it appears that the diets supplemented with ginger stimulated lysozyme and total Ig production better than the other groups.

Increased superoxide anion production was obtained in fish supplemented with vitamin C, ginger, onion, and β-glucan in descending order (Table 3). In rainbow trout, high intracellular superoxide release was observed when fish were given dietary immunostimulants such as ginger (Siwicki et al 1994; Dugenci et al 2003). Improved production of superoxide anion was likewise observed in *Epinephelus coioides* (Hamilton, 1822) after injection with β-glucan (Tavarro 2002). The superoxide anion produced during the respiratory burst is important in the bactericidal activity of macrophages (Secombes & Olivier 1997). The higher respiratory burst activity in the ginger- and vitamin C-fed fish in this study suggests higher production of microbicidal reactive oxygen (ROS) and free radicals without adverse effects on host cells and tissues. Effective scavenging or neutralization of excess free radicals to maintain the integrity of the host's immune cells may be attributed to the synergy between dietary antioxidants and endogenous antioxidant enzymes. The results indicated that ginger and vitamin C seemed to be the more effective stimulator of ROS while at the same time promoters of antioxidant defenses than control and β–glucan. The immunomodulatory effects of onion and ginger in grouper can presumably be attributed to a better coordination of their stimulatory and antioxidant scavenging properties. Significantly better survival rate in

this study was also observed in the onion and ginger-fed groups compared with the control and even with the β-glucan-supplemented fish (Figure 1)

Table 3

Immune parameters measured after 12 weeks of feeding the different immunostimulants*

Dietary treatment	*Hematocrit*[a] *(%)*	*Monocyte*[b] *(%)*	*Total Ig*[c] (mg mL^{-1})	*Lysozyme*[d] μg mL^{-1}	*ROS*[e] *Abs (540nm)*
Control	36.65[a]	1.50[a]	13.76[a]	11.39[a]	0.032[a]
Onion	38.43[b]	2.54[b]	19.05[b]	11.60[a]	0.037[abc]
Ginger	37.73[ab]	3.23[c]	21.80[c]	13.94[b]	0.041[bc]
β-glucan	37.53[ab]	2.58[b]	19.91[bc]	12.26[ab]	0.035[ab]
Vitamin C	38.58[b]	2.63[b]	18.73[b]	12.99[ab]	0.042[c]

*Values in a column not sharing the same superscript letters are significantly different ($p<0.05$);
[a] Values represent means of 16 fish ± SD; [b] Values represent means of 16 fish ± SD;
[c] Total immunoglobulin; Values represent means of 20 fish ± SD; [d] Values represent means of 12 fish ± SD;
[e] Reactive Oxygen Species values represent means of 14 fish ± SD.

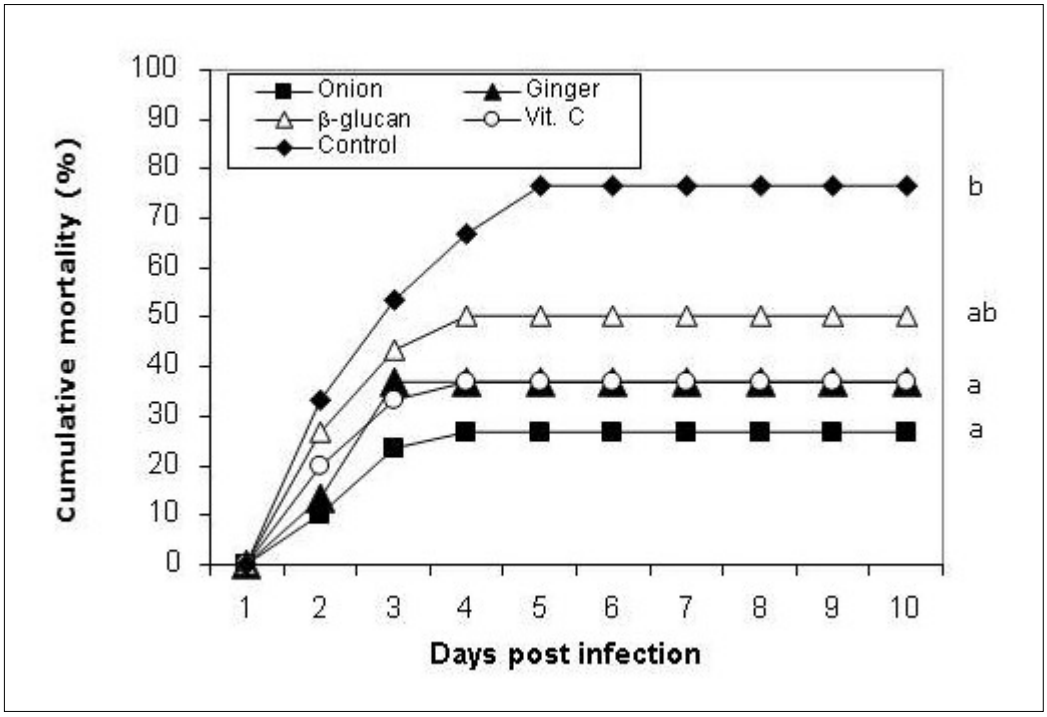

Figure 1. Cumulative (%) mortality in *E. fuscogutattus* fed the different immunostimulants. Values represent means of three groups of fish (n=3 tanks). Means not sharing the same superscript letters are significantly different ($p<0.05$).

The results of the present study showed that oral administration of onion and ginger in grouper for 12 weeks resulted in either improved growth, or enhanced innate immune defenses or both and improved resistance against *V. harveyi* infection. Herbs such as ginger have been reported for their biological effects such as growth promotion and immunostimulation (Citarasu 2010). β-glucan, in the present study, improved growth and increased total Ig, whereas vitamin C increased hematocrit, total Ig and ROS production but without effect on growth. Both immunostimulants did not significantly confer resistance to *V. harveyi* infection (Figure 1). It has been reported that the efficacy of glucan could vary depending on the route and duration of administration (Ogier de Baulny et al 1996; Sakai 1999). In the present study, oral administration of β-glucan in grouper for 12 weeks improved growth and total Ig production but not other immune parameters measured and did not protect the fish from bacterial infection. Matsuo & Miyazano (1993) reported that rainbow trout fed with peptidoglycan for 56 days were not protected from *V. anguillarum* infection, however, the fish fed for 28 days exhibited

enhanced protection. Feeding β-glucan for a shorter period in the present study might have resulted in better performance. This study provides new information on the use of herbs such as onion and ginger as immunostimulants in grouper.

Conclusions. The immunomodulatory effects of onion and ginger as previously reported in humans and other animal models were likewise demonstrated in grouper in this study. As immunostimulants for grouper, the benefits of onion and ginger appeared to be comparable to vitamin C and even better than β–glucan when administered orally for 12 weeks. High mortalities might be avoided if onion and ginger could be provided to fish before the onset of diseases.

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