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Effects of small peptides, probiotics, prebiotics, and synbiotics on growth performance, digestive enzymes, and oxidative stress in orange-spotted grouper, *Epinephelus coioides*, juveniles reared in artificial seawater*

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Abstract Aquaculture production efficiency may increase by using feed additives. This study investigated the effects of different dietary additives [w/w: 2% small peptides, 0.01% probiotics (*Bacillus licheniformis*) and 0.2% prebiotics (inulin)] on growth performance, digestive enzyme activities, and oxidative stress in juvenile *Epinephelus coioides* reared in artificial seawater of two salt concentrations (13.5 vs. 28.5). Weight gain rate was significantly higher in fish fed the diet supplemented with small peptides, *B. licheniformis*, inulin, or synbiotics than that in fish fed the basal diet; the greatest weight gain rate was found in fish fed the small peptide treatment [56.0% higher than basal diet]. Higher feed efficiency was detected in fish fed the diet supplemented with small peptides than that of fish in the other dietary treatments. Total protease activity in the stomach and intestines was highest in fish fed the small peptide-treated diet, whereas lipase activity was highest in those fed synbiotics (combination of *Bacillus licheniformis* and inulin) than that in fish fed the other treatments. Antioxidant enzyme (total superoxide dismutase and catalase) activities and hepatic malondialdehyde content were higher in fish receiving the dietary supplements and maintained in artificial seawater containing 13.5 salinity compared with those in the control (28.5). Hepatic catalase activity in grouper fed the diets with small peptides or synbiotics decreased significantly compared with that in control fish. Overall, the three types of additives improved growth rate of juvenile grouper and digestive enzymes activities to varying degrees but did not effectively improve antioxidant capacity under low-salinity stress conditions.

Keyword: *Epinephelus coioides*; feed additives; growth performance; salinity stress; artificial seawater

1 INTRODUCTION

Epinephelus coioides (orange-spotted grouper) is one of the most economically important fish in China and Southeast Asian countries (Peng et al., 2008). Aquaculture of grouper and that of other species requires the application of biotechnology and microbiology to improve fish production. Diet and aquatic environment generally play the most important roles in aquaculture, and supplementing feed with various additives to improve growth is common in many cultured species (Farzanfar, 2006). Fish protein hydrolyzates are a potential fish meal replacement (Chalamaiah et al., 2012). Probiotics or prebiotics (as immunostimulants) have received much attention with the increased demand for environmentally friendly aquaculture. Probiotics can be administered either as a feed additive or added directly to the culture water (Moriarty, 1998).

Wide varieties of complex additives have been used to improve production of farmed animals. Small

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peptides, *Bacillus licheniformis*, and inulin have been used widely in aquaculture. Small peptides have a positive effect on growth of prawn and Atlantic salmon (*Salmo salar*) larvae (Teshima et al., 1993), and *B. licheniformis* improves growth performance of rainbow trout (*Oncorhynchus mykiss* Walbaum) (Raida et al., 2003). Inulin supplementation increases intestinal somatic indices of Atlantic salmon compared with those of fish fed a diet containing fish meal (Bakke-McKellep et al., 2007). According to Shan et al. (2008a), the digestive organs are sensitive to food components that cause immediate changes in digestive enzyme activities and influence fish health and growth (Mohapatra et al., 2013). Feed additives can also directly stimulate relevant digestive enzymes (Teshima et al., 1993; Bairagi et al., 2002). For example, small peptides can cross the epithelial barrier and exert their biological activity (i.e., stimulate allergen-specific effector cells (Espe et al., 1999).

Much research has concentrated on probiotics to control disease, stimulate the immune system, and promote growth (Sun et al., 2010). *Bacillus* produces quite a few probiotics, which have been considered one of the most promising preventative methods in aquaculture (Verschuere et al., 2000). In addition, *Bacillus* has nutritional and health benefits (Rengpipat et al., 2000).

Inulin oligosaccharides are new feed additives that have become widely used in aquaculture feed to promote fish growth and mineral absorption (Franck, 2005; Coudray et al., 2006). Inulin as a prebiotic is an indigestible food ingredient that benefits the host by selectively stimulating growth and/or activity of bacterial species in the colon (Cerezuela et al., 2013). In addition, dietary inulin supplementation influences gut morphology and microbiota of Arctic charr (*Salvelinus alpinus*) (Ringø et al., 2006), red drum (*Sciaenops ocellatus*) (Burr et al., 2009), and Atlantic salmon (Bakke-McKellep et al., 2007). However, a high level (15% w/w) of dietary inulin destroyed the organization of Arctic charr intestinal microvilli (Olsen et al., 2001).

The combined application of probiotics and prebiotics is known as synbiotics (Daniels et al., 2010). Synbiotics have been used in aquaculture to enhance immunity and feed efficiency (Rombout et al., 2010). Synbiotics and prebiotics also play roles stimulating growth performance and ameliorating stress by improving survival and colonization of live microbial dietary supplements (probiotics) in the gastrointestinal tract (Daniels et al., 2010).

The chemistry of the aquatic environment plays an important role in survival, growth, and regulation of fish reproduction (Shan et al., 2008b; Wang et al., 2014). Salinity is a key factor controlling egg fertilization and incubation, early embryogenesis, and larval growth in many fish species (Bœuf and Payan, 2001). Chronic low-salinity stress can cause significant physiological alterations in marine fish (Kulac et al., 2013), such as adverse effects on ion pumps and homeostasis (Hwang and Lee, 2007), changes in cell structure and energy metabolism (Avella et al., 2009), and oxidative stress. Oxidative metabolism continuously generates superoxide radicals and hydrogen peroxide, leading to serious cellular damage if produced in excess (Castex et al., 2010). Reactive oxygen species (ROS) are produced naturally and continuously in all animals during normal physiological processes (Livingstone, 2001). ROS have a protective effect at physiological concentrations, but excess ROS are harmful (Lushchak, 2011). Oxidative stress occurs when ROS overcome the antioxidant defenses (Kelly et al., 1998). Some reports have suggested that dietary additives can ameliorate oxidative stress (Mohapatra et al., 2013). For example, Varela et al. (2010) reported that adding a probiotic (PDP11, bacterial strain from gilthead seabream, *Sparus auratus*) to the gilthead seabream diet improves tolerance to high density stocking stress.

Grouper is a euryhaline species (Heemstra and Randall, 1993) that can survive salinities of 11–41 g salt/L (Sun et al., 2010). Optimal salinity for growth in artificial seawater is 28–30 g salt/L. However, fish may be exposed to oxidative stress, which impairs growth performance, because these artificial conditions differ from those encountered in the wild. In this situation, use of feed additives, such as small peptides, probiotics, or prebiotics, is considered a promising option in aquaculture, but little research is available on the topic. The aim of the present study was to investigate the nutritional effects of feed additives, particularly the effects of small peptides, *B. licheniformis*, inulin, and combined use of *B. licheniformis* and inulin on growth performance, digestive enzyme activities, and oxidative stress in grouper reared in artificial seawater containing two salt concentrations.

2 MATERIAL AND METHOD

2.1 Experimental diets

The basal diet was formulated to meet the

Table 1 Formulations and approximate compositions of the test diets (% w/w dry diet)

Material	Diet				
	I	II	III	IV	V
Fish meal	70.00	70.00	70.00	70.00	70.00
α-starch	21.70	21.70	21.70	21.70	21.70
Zeolite	2.20	2.20	2.20	2.20	2.20
Premix	1.30	1.30	1.30	1.30	1.30
Calcium biphosphate	1.80	1.80	1.80	1.80	1.80
Plant oil	1.50	1.50	1.50	1.50	1.50
Fish oil	1.50	1.50	1.50	1.50	1.50
Small peptides	0.00	2.00	0.00	0.00	0.00
Bacillus licheniformis	0.00	0.00	0.01	0.00	0.01
Inulin	0.00	0.00	0.00	0.20	0.20
Crude proteins	45.29	44.77	45.03	44.95	45.47
Crude lipids	10.43	10.25	10.81	10.02	11.07

The premix was supplemented with the following vitamins and minerals (mg/kg): vitamin E 150, vitamin K 10, vitamin B1 15, vitamin B2 25, vitamin B6 20, vitamin B12 0.05, pantothenic acid 50, niacin 100, biotin 0.5, folic acid 5, inositol 200, vitamin C 500, Mg 800, Fe 170, Zn 50, Cu 15, Mn 15, Co 7, I 0.5, Se 0.2; vitamin A 15 000 IU/kg, vitamin D 3 000 IU/kg, choline 1 000 IU/kg. Dietary treatments I, II, III, IV, and V were the basal diet (control), small peptides, *B. licheniformis*, inulin, and synbiotics, respectively.

nutritional requirements of grouper during the experimental period (Espe et al., 1999; Sun et al., 2010). Five diets containing different crude protein and crude lipid contents were analyzed according to the AOAC method (AOAC, 1990); the formulations and approximate compositions of the experimental diets are listed in Table 1. The control (I) treatment was the basal diet, and the other treatments included the basal diet supplemented with additives as follows: treatment II: 2% (w/w) small peptides (Peptiva® Aquaculture; Vitech Bio-Chem Corp., Glendale, CA, USA); treatment III: 0.01% (w/w) *B. licheniformis* (Lishengsu; effective bacteria 10^9 cfu/g, Xinhai Lisheng Biological Corp., Suzhou, China); treatment IV: 0.2% (w/w) inulin (Beizhuo Bio-Technology Corp., Shanghai, China); and treatment V: 0.01% (w/w) *B. licheniformis* and 0.2% (w/w) inulin (synbiotic). All diets were prepared in the laboratory. The dry ingredients were ground through 60-mm mesh. The fine powder was weighed, and mixed thoroughly with oil (plant and fish oil). An appropriate volume of water was added to produce stiff dough, and the dough was pelleted using a 2-mm-diameter mill. The experimental feed was air-dried overnight and stored at 4°C until use.

2.2 Feeding experiment

Juvenile grouper were purchased from Qingdao Universal Aquaculture Co. (Qingdao, Jiaonan City, China) in March 2009. They were transported to the laboratory and acclimated in three 300-L fiberglass tanks containing artificial sea water (28.5±1.0; prepared by adding sea salt to aerated tap water) for 2 weeks. The grouper were fed once daily (at 8 a.m.) with the control diet pellets during the acclimation period (Wang et al., 2013). After acclimation, healthy grouper (mean weight, 7.12±0.57 g) were divided randomly into 15 tanks (five treatments in triplicate) with 20 fish in each replicate tank. All grouper were hand-fed the experimental diets at the same fixed rate (2%–2.5% wet body mass) twice daily (8 a.m. and 4 p.m.) (Li et al., 2006) for 60 days. In a pre-experiment, fish did not feed actively when fed in excess of 2.5% wet body mass under artificial seawater conditions. Fish from each tank were weighed individually every 2 weeks until the end of the trial.

The feeding experiment was conducted in a laboratory recirculating-aquaculture system. Water quality parameters during the experiment were: water temperature, 25±0.5°C; salinity, 28.5±1.0; pH, 8.1±0.1 (temperature, salinity, and pH were tested using the YSI 556MPS meter; YSI Inc., Yellow Springs, OH, USA); dissolved oxygen, $\geq 5\times10^{-6}$ with continuous aeration; total ammonium <0.1 mg/L; and a 12-h light:12-h dark photoperiod.

2.3 Low-salinity stress experiment

The water quality parameters and the dietary treatments during this experiment were the same as those during the feeding experiment described above, except that fish were exposed to low-salt artificial seawater (13.5) for 1 week after the control treatment in full salinity (28.5). The purpose of this experiment was to measure the effects of the different diets on oxidative stress in juvenile grouper reared in low-salinity artificial seawater.

2.4 Sample collection and analytical methods

Fish from each tank were weighed at the beginning and end of the feeding trial. The fish were fasted for 24 h at the end of the experimental period. Then, three fish from each tank were selected randomly, sacrificed with an overdose of anesthetic (10 mg/L MS-222; Argent Laboratories, Redmond, WA, USA), and dissected on an ice tray. The stomach, liver, and intestines were removed, cleaned of fat, blotted on

filter paper, frozen in liquid nitrogen, and stored at -70°C. Portions (0.1 g) of the liver, stomach, and intestinal samples were homogenized separately (Pro 200 Hand-Held; PRO Scientific Inc., Oxford, CT, USA) using nine volumes of normal saline (8.6 g/L, 4°C), centrifuged at 3 500 r/min for 10 min, and the supernatant was stored at -70°C until activities of total protease (PRO), amylase (AMY), lipase (LPS), total superoxide dismutase (T-SOD), and catalase (CAT), and malondialdehyde (MDA) content (Wang et al., 2013) were measured.

PRO activity was assayed according to Lowry et al. (1951) using Folin-phenol reagent. PRO activity was defined as the amount of enzyme needed to catalyze the formation of 1 μg of tyrosine/min at 37°C. AMY activity was determined using a kit purchased from Nanjing Jiancheng Bioengineering Research Institute (NJJCBIO, Nanjing, China). One unit of AMY activity was defined as the amount of enzyme required to hydrolyze 10 mg of soluble starch (Sigma-Aldrich, Shanghai, China) in 30 min at 37°C. LPS activity was determined using triglyceride (Sigma-Aldrich) as the substrate according to the method of McKellar and Cholettle (1986). One unit of LPS activity was estimated as the amount of enzyme required to breakdown 1 μmol of triglyceride substrate/min at 37°C/mg tissue protein.

T-SOD and CAT activities and MDA content were measured using kits purchased from NJJCBIO (Wang et al., 2002; Lu et al., 2009). The T-SOD assay was based on SOD-mediated inhibition of nitrite formation from hydroxylammonium in the presence of an O_2^- generator (Elstner and Heupel, 1976). SOD activity was expressed as units/mg protein and determined by measuring the decrease in optical density of the reaction solution at 550 nm. One unit of T-SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. One unit of CAT activity was defined as the amount of enzyme required to transform 1 μmol of H_2O_2/min at 25°C and pH 7.0. MDA concentration was determined based on the chemical reaction between MDA and 2-thiobarbituric acid (Ohkawa et al., 1979) and was expressed as nmol MDA/mg protein. Total soluble protein content of the supernatant was measured using the Bradford method (Bradford, 1976).

2.5 Statistical analysis

All data were examined by one-way analysis of variance and Duncan's comparison of means using SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, USA) to detect differences ($P \leq 0.05$) among treatments. Data are presented as mean±standard error.

Table 2 Effect of the different diets on growth performance of juvenile *Epinephelus coioides*

Diet	Initial fresh weight (g/fish)	Final fresh weight (g/fish)	WG (%)	FE (%)
I	7.06±0.28	14.71±0.65^{b}	108.32±0.95^{d}	40.41±0.99 ^{c}
II	7.13±0.54	19.18±1.46^{a}	169.00±0.10^{a}	61.23±0.10 ^{a}
III	6.97±0.37	14.96±0.57^{b}	114.81±3.22^{c}	41.58±0.67 ^{c}
IV	7.19±0.39	16.51±1.12^{ab}	129.45±3.13^{b}	46.92±0.66 ^{b}
V	6.93±0.46	15.76±0.80^{b}	127.65±3.57^{b}	46.22±0.75 ^{b}

Note: Data are mean±standard error (n=3). Significant differences ($P \leq 0.05$) among treatments are indicated by different letters in each column. Weight gain rate (WG, %)=$[(w_t-w_0)\times100]/w_0$. Feed efficiency (FE, %)=$[(w_t-w_0)\times100]/C$, where w_t is mean final weight (g), w_0 is mean initial weight (g), and C is feed intake of fish in each experimental treatment. Dietary treatments I, II, III, IV and V were the basal diet (control), small peptides, *B. licheniformis*, inulin, and synbiotics, respectively.

3 RESULT

Juvenile grouper fed the diets supplemented with small peptides, *B. licheniformis*, inulin, or synbiotics tended to have better growth performance than those on the basal diet during the 60-day feeding trial (Table 2). Weight gain rates (WG, %) of fish fed the diets with small peptides, *B. licheniformis*, inulin, or synbiotics were significantly higher than those of fish fed the basal diet, and the highest weight gain rate was found in fish fed the small peptide-supplemented diet [56.0% ($P \leq 0.05$) higher than control]. Feed efficiency (FE, %) was influenced by the dietary treatments throughout the entire feeding period; the highest FE was found in fish fed the diet supplemented with small peptides (Table 2).

The feed additives had a significant effect on digestive enzyme activities (Table 3). PRO activity in fish fed the small peptide treatment increased significantly by 24.2% in the stomach and by 18% in the intestines ($P \leq 0.05$), whereas the other treatments were slightly (non-significantly) higher than that of the basal diet treatment. Furthermore, the greatest increase in LPS activity was detected in the stomach (by 12.0%) and intestines (by 9.0%) in fish treated with synbiotics compared with those in fish fed the basal diet. Intestinal AMY activity was not different among the fish fed the different diets and was below the detection limit in the stomach (Table 3).

T-SOD and CAT activities and MDA content in the liver increased significantly in fish fed any of the

Table 3 Effect of different diets on digestive enzymes in the stomach and intestines of juvenile *Epinephelus coioides*

Diet	Stomach		Intestines		
	PRO (U/g)	LPS (U/g)	PRO (U/g)	LPS (U/g)	AMY (U/mg)
I	807.39±62.67^{b}	367.40±7.54^{b}	413.47±17.79^{b}	306.10±27.56	3.90±0.66
II	1003.00±96.48^{a}	397.44±7.46^{a}	487.87±45.41^{a}	306.96±16.31	4.17±0.32
III	904.93±68.90^{ab}	366.49±9.13^{b}	440.43±19.14^{ab}	299.14±27.82	3.87±0.47
IV	820.21±71.37^{b}	371.30±2.88^{b}	428.40±56.09^{b}	310.92±12.81	3.85±0.46
V	875.87±51.37^{ab}	411.36±28.90^{a}	447.17±23.46^{ab}	333.58±16.61	4.18±0.68

Note: Data are mean±standard error (n=3). Significant differences (P≤0.05) among treatments are indicated by different letters in each column. AMY activity in stomach was below the detection limit. Dietary treatments I, II, III, IV, and V were the basal diet (control), small peptides, *B. licheniformis*, inulin, and synbiotics, respectively.

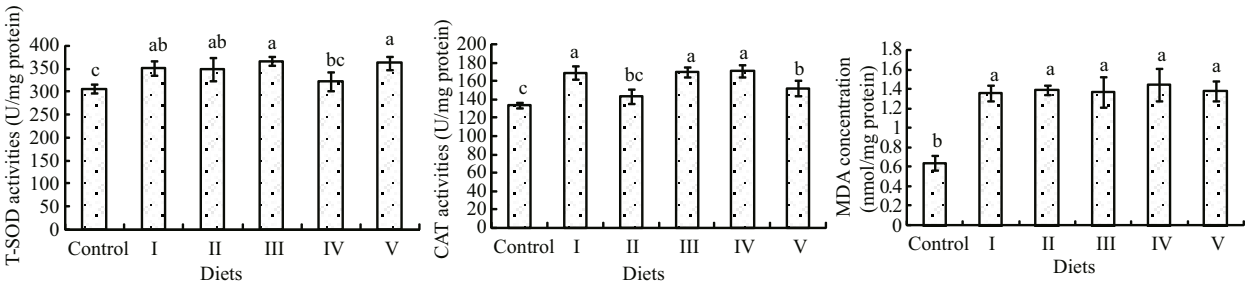

Fig.1 Effect of different diets on hepatic oxidative stress parameters in juvenile *Epinephelus coioides* reared in low salinity (13.5) artificial seawater for 1 week

Data are mean±standard error (n=3). Significant differences (P≤0.05) among treatments are indicated by different letters in each panel. Control treatment was the basal diet with fish maintained in full-salinity (28.5) artificial seawater. Dietary treatments I, II, III, IV, and V were the basal diet (control), small peptides, *B. licheniformis*, inulin, and synbiotics, respectively, with fish maintained in low-salinity (13.5) artificial seawater for 1 week.

dietary treatments after they were transferred from 28.5 to 13.5 artificial seawater, compared with those in fish in the control treatment (P≤0.05) (Fig.1). Hepatic T-SOD activity of grouper fed the diet with *B. licheniformis* and synbiotics increased greater than that in the other treatments when compared with the basal diet treatment. Hepatic CAT activity in fish fed the diet with small peptides or synbiotics decreased significantly (by 15.4 and 10.0%, respectively; P≤0.05), compared with that in fish fed the basal diet. No differences in CAT activity were detected in grouper fed the basal, *B. licheniformis*, or inulin diets. In addition, changing salinity did not alter MDA content in fish fed any of the dietary treatments (Fig.1).

4 DISCUSSION

In this study, we investigated the effect of small peptides, *B. licheniformis*, and inulin on feed intake, growth performance, digestive enzyme activities, and oxidative stress in grouper. Small peptide additives have many functions in aquaculture, such as promoting growth (Ghosh et al., 2008), enhancing immunity, and excluding pathogens (Qi et al., 2009). Teshima et al. (1993) and Berge and Storebakken (1996) reported that including a moderate amount of small peptides in the diet has a positive effect on growth of prawn and Atlantic salmon larvae. In the present study, small peptide supplementation significantly promoted growth of grouper. Comparable results were reported by Espe et al. (1999), who showed a positive effect of low-molecular-weight peptides in a protein hydrolyzate on fish growth. The improved growth performance due to small peptide feed supplements may be attributed to factors such as increased protein digestibility or an attractant effect of the free amino acids liberated during production of the small peptides (Berge and Storebakken, 1996). Kotzamanis et al. (2007) indicated that different molecular-weight fractions and concentrations of feed-soluble peptides may affect growth performance and immunological status of sea bass larvae. The small peptide transport and absorption system is rapid and not easily saturated, so different free amino acids can easily be taken up in the intestines, and small peptides can be absorbed as free amino acids to promote growth (Fu et al., 2001).

The *B. licheniformis* supplement has been used widely in fish aquaculture (Nayak, 2010). In our study, *B. licheniformis* improved growth performance

compared with that of fish fed the basal diet. Arena et al. (2006) reported that *B. licheniformis* acts as an antiviral agent and improves growth performance in rainbow trout when administered with *Bacillus subtilis* (Raida et al., 2003).

Manning and Gibson (2004) discovered that non-digestible dietary ingredients (e.g., inulin and fructo-oligosaccharides) may act as prebiotics by beneficially affecting growth of the host or by activating the metabolism of health-promoting bacteria and modulating microbiota in the gastrointestinal tract. Inulin has been demonstrated to be an effective prebiotic (Kolida et al., 2002). Coudray et al. (2006) observed that inulin selectively promotes growth and activity of some probiotic bacteria (e.g., lactobacilli and bifidobacteria). Similar results were observed here, as inulin and the synbiotic treatments significantly improved growth of grouper compared with that of fish fed the basal diet.

Many reports have shown that fish growth and FE improve depending on the diet, but the exact mechanisms remain unclear. Some possible explanations are that feed additives directly stimulate relevant digestive enzymes (e.g., PRO, AMY, and LPS) (Teshima et al., 1993; Bairagi et al., 2002) and/or that feed additives modulate colonic microbiota and indirectly stimulate digestive enzymes. Mahious et al. (2006) observed that animal feed supplemented with small peptides promotes intestinal development, accelerates growth of villi, and improves digestive enzyme activities. In the present study, PRO and LPS activities increased significantly in the stomach and intestines of fish treated with 2% w/w small peptides, confirming that small peptides can stimulate secretion of digestive enzymes to promote growth of grouper. Comparable findings were reported in other species; for example, Berge and Storebakken (1996) observed that fish protein hydrolysate in the starter diet increases protein digestibility in Atlantic salmon fry.

Sun et al. (2011) reported that probiotic treatment may increase digestive enzyme activities in *E. coioides*. Wang and Xu (2006) showed increased digestive enzymes activities in carp (*Cyprinus carpio*) in response to *Bacillus* sp. Moreover, Tovar et al. (2002) noted an increase in digestive enzyme (AMY and PRO) secretion in sea bass (*Dicentrarchus labrax*) larvae when fish were also fed live yeast (*Debaryomyces hansenii*). Although the feed containing *B. licheniformis* in the present study tended to improve PRO intestinal activity, a greater quantity of probiotic could improve fish digestive enzyme activities.

A few studies have reported that dietary prebiotics increase digestive enzyme activities by promoting specific beneficial bacteria in the intestine and modulating local cytokine concentrations (Seifert and Watzl, 2007; Ringø et al., 2010). Mahious et al. (2006) reported that a dietary inulin (2% w/w of diet) supplement changes the gut microbiota in turbot (*Psetta maxima*) larvae (*Vibrio* spp. was dominant but *Bacillus* spp. was not detected). The present results indicate that inulin alone did not enhance fish digestive enzyme activities, whereas supplementing with *B. licheniformis* and inulin together tended to increase digestive enzyme activities in the stomach and intestines. These findings suggest that inulin may not be an optimal prebiotic for grouper reared in artificial seawater. However, the growth rate of grouper fed the symbiotic-supplemented diet increased significantly compared with that of fish fed the basal diet. Therefore, further studies are needed to elucidate the relationship between inulin and *B. licheniformis* in various diets.

Most studies have reported the growth-promoting (Espe et al., 1999; Coudray et al., 2006; Ghosh et al., 2008) and immune-enhancing (Arena et al., 2006; Cerezuela et al., 2012) effects of feed additives; however, little information is available on antioxidative stress. The metabolic responses of grouper to salinity have been reported (Wang et al., 2011; Tsui et al., 2012; García et al., 2013). These studies suggested that grouper are vulnerable to changes in salinity, possibly leading to a variety of physiological responses, such as stimulation of energy metabolism and changes in antioxidative enzyme activities (Lushchak, 2011).

Liu et al. (2007) reported that changes in salinity are associated with generating ROS and oxidative damage, including lipid peroxidation (i.e., MDA generation) and disruption in proteins and nucleic acids (Di Giulio et al., 1989). Similar studies have reported that small peptides, *B. licheniformis*, and inulin have anti-stress properties, improve stress resistance, and boost immunity (Daniels et al., 2010; Ringø et al., 2010). SOD and CAT are the two main enzymes that detoxify ROS (Di Giulio et al., 1993).

Rengpipat et al. (1998) used pepsin-digested casein to obtain small peptides that minimized ROS activity, and their antioxidant activity was higher than that of glutathione. Dietary supplementation with *B. licheniformis* diminishes oxidative stress in juvenile shrimp (*Litopenaeus vannamei*) and is associated with a significant increase in SOD activity

compared with that in control shrimp (Li et al., 2007). Wang and Chen (2005) reported that eliminating superoxide anions may decrease antioxidant enzyme activities. In the present study, the diet supplements decreased T-SOD activity in grouper, and grouper fed the small peptide- or symbiotic-treated diets had decreased CAT activities. However, no significant change in MDA content was noted in fish fed any of the dietary treatments under low-salinity stress, but MDA content was higher than that of fish in the control (high-salinity). These results suggest that oxidative stress was not eliminated. Thus, dietary supplements did not improve antioxidant capacity under low-salinity stress conditions. However, direct comparisons among various supplements are difficult because of their different functions and sites of action. Therefore, further study is needed to illustrate the effects of different dietary supplements on antioxidant enzymes in fish.

5 CONCLUSION

Three kinds of feed additives (small peptides, *B. licheniformis*, and inulin) significantly improved growth rates of grouper under artificial seawater conditions, compared with that of fish fed a basal diet. Digestive enzyme activities (total PRO, AMY, and LPS) were significantly enhanced by the small peptide supplement. However, none of the dietary supplements improved antioxidant capacity under low-salinity stress conditions. Overall, the best supplement was the small peptides; however, *B. licheniformis*, inulin, and synbiotics also had positive effects.

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