EFFECT OF DIETS CONTAINING FISH PROTEIN HYDROLISATES ON GROWTH AND IMMUNE PERFORMANCE OF ASIAN SEABASS (*Lates calcarifer* Bloch) WHEN REARED IN FARM CONDITIONS

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A B S T R A C T

We investigated the effects of fish protein hydrolysate (FPH) on zootechnical performance and immune response of the Asian Seabass *Lates calcarifer* Bloch. Experimental fish were fed with 3 diets: a local commercial diet (control), coated or not, with 2 and 3% FPH (w/w). Twelve thousand Asian Seabass juveniles (5.88±0.56 g) were divided into three groups and two replicates reared in nursery tanks (2000 L). The remaining fish were then used for grow-out experiment in floating net cages (1m x 1 m x 3 m). Zootechnical performances were assessed at both stages with following indicators: total weight gain (TWG), % relative weight gain (% RWG), % specific growth rate (% SGR), final weight (g) and final length (cm). At the end of each trial period, fish immune status was assessed through blood sampling and the measurement of Neutrophile (%), Monocyte (%), Lymphocyte (%), Macrophage (10^5 cell/mL), Leukocyte (10^3 cell/mL) and Phagocytes activity (%). At the end of the nursery trial, an immersion bacterial challenge with *Vibrio parahaemolyticus* (10^5 cells mL^{-1}) was implemented. The results showed that dietary FPH supplementation significantly influenced the growth and immune status of Asian Seabass when compared to the control group. Fish fed FPH supplemented diet yielded higher growth rates and survival rates than non supplemented group. Fish phagocytic activity and resistance to a bacterial challenge were also improved by dietary FPH supplementation. These results may be related to the significant changes observed in fish leukocyte profiles, when fed FPH supplemented diets. Altogether, these results show the positive contribution of FPH to the sustainability of Asian seabass farming.

Keywords: *Functional hydrolysates, Asian Seabass, Zootechnical performance, Immune response, Phagocytic activity, Nursery, Grow out*

INTRODUCTION

In Asian Seabass culture, disease outbreaks are being increasingly reported as a major constraint to the sustainable growth of production (Chong et al*.,* 1987; Bloch and Larsen, 1993; Chou et al*.,* 1998). Many diseases are linked to the stress conditions associated with the intensification systems of farming and the degradation of the environmental quality. Under poor conditions, live food and larvi are often opportunistically infected by fungi, bacteria and viruses. Conventional treatment of aquatic diseases through the use of disinfectants and antibiotics to overcome the bacterial infection problem are having limited value and has stimulated the development of bacterial resistance (Defoirdt et al., 2007; Subasinghe, 1997; Cabello, 2006). Another problem created by the unrestricted use of antibiotics is the presence of residual antibiotics in commercialized aquaculture products (Saitanu et al., 1994; Grave et al., 1996; 1999; Goldburg et al., 2001; European Commission, 2001a,b). This problem has led to allergy and toxicity in humans (Alderman and Hastings, 1998; Cabello, 2006) and resulted in shifts in the diversity of the microbiota due to the permanent existence of large amounts of antibiotics in the environment (Cabello, 2003). Consequently, novel preventive approaches in aquaculture are urgently needed, *e.g*. vaccines, immunostimulants and probiotics (Marques et al., 2006). Currently, the industry goes towards much more holistic approach consisting in protecting aquatic animals from diseases without the use of antibiotics by enhancing the resistance of

cultured fish to diseases (Defoirdt et al., 2007; Smith, V.J et al., 2003) and the use of enzymatic hydrolysates processed from fish by-products and showing immunostimulating properties is one of this promising holistic approach (Cook et al., 2003).

Over recent years, fish protein hydrolysates (FPH) have been used as ingredients for marine fish diets (Ouellet *et al*., 1997; Aguila *et al*., 2007), as a naturally high source of digestible nutrients, low molecular weight compounds such as nucleotides, amino acid and derivatives and bioactive peptides showing antioxidative, hormone like, antistress or antimicrobial activities (Klompong et al., 2007; Thiansilakul et al., 2007; Liaset and Espe, 2008). Several studies have investigated the effects of FPH on growth performance and nonspecific immunity of several fish species (Berge and Storebakken, 1996; Bøgwald et al., 1996; Carvalho et al., 1997; Liang et al., 2006). However, to our knowledge, no similar studies have been done on the Asian Seabass *Lates calcarifer,* especially at two different rearing periods, namely: the nursery phase and grow-out phase. Therefore, the aim of the present study was to evaluate the effects of dietary supplementation with functional hydrolysates on zootechnical and immune performance in *L. calcarifer* when reared in farm conditions.

MATERIALS AND METHODS

Fish protein hydrolysates (FPH)

Fish protein hydrolysate (FPH) obtained from fishery co-products (AQUATIV, SPF Diana, Batch No. 203140305, ACTIPAL HL1, krill and tuna hydrolysate liquid for aquafeed) was used in the experiments and stored at 4^0C until the end of experiment.

Experimental Animals

1-2 g of *L. calcarifer*) obtained from Batam Mariculture Development Center were used as the test animals. The culture density at the nursery phase was 2000 fish/m^3 and 500 fish at 1 x 1 x 3 m floating net cages. Acclimatization process was conducted for 2 weeks preceding the experiment and 80 – 100 % of water renewal was performed daily.

Experimental Design and Diet Administration

Both nursery and grow-out trials were implemented at the Batam Mariculture Development Center for 6 weeks and 9 weeks respectively. Experimental design consisted in graded levels of supplemented hydrolysate (0, 2 and 3% w/w) coated on a local extruded commercial diet using a cement mixer. Each experimental diet was randomly allocated to 2 replicate tanks (2000 L capacity) or cages (1 x 1 x 3 m) for nursery and grow-out rearing respectively. Feeding rates were fixed to 7% of the biomass equally distributed in 3 meals over 12 hours, with daily adjustments based on mortality rates and growth model assumptions. The experimental tanks were provided with continuous aeration and water was changed daily before feeding. Tanks were cleaned and uneaten feed was collected 2h after the feeding. Water quality parameters such as temperature, pH, salinity and dissolved oxygen were daily monitored using portable instruments, while critical parameters such as total ammonia (NH_3) and nitrite (NO_2) were measured on alternate days following standard methods. The trials were terminated when fish reached 20 g and 50 to 60 g during the nursery and grow-out periods respectively.

Zootechnical Performances

Growth performance was expressed as the total weight gain (TWG), relative weight gain (RWG) and specific growth rate (SGR). The calculation formulas were as follows:

TWG (g) = Wt − Wi,
RWG (%) = (Wt – Wi) × 100 / Wi,
SGR (%) = (ln$W$t – ln$W$i)× 100 / d,
Survival (%) = (number of fish harvested / number of fish stocked) × 100.

Feed conversion ratio (FCR) = total amount of feed consumed (kg) / biomass increase (kg).

where Wi and Wt are the initial and final mean weights (g), respectively, and d represents the number of feeding days (Mojjada et al., 2013).

Blood sampling and analysis

At the end of the trial, ten fish per group (five fish randomly captured from each cage) were sampled. Blood was sampled from the caudal vein of the individual fish after anaesthetization. The whole blood was collected in a syringe,

allowed to clot for 1 h in microtubes at room temperature and followed by 5 h at 4 °C, and then serum was harvested by centrifuging at 1500×*g* for 5 min at 4 °C. All serum samples were preserved at −20 °C prior to analysis. The number and percentage of leukocyte count, neutrophile, monocyte and phagocytic was determined based on Anderson and Sewicki (1993); Blaxhall PC (1972) and Wedemeyer and Yasutake (1977).

Water Quality Analysis

Ammonia and Posphate were determined spectrophotometrically at 560 and 640 nm, respectively (Palkin Elmer© Lambda XLS), Nitrite and Nitrate were determined colorimetrically (HACH DR 890), Turbidity, pH, dissolved oxygen, salinity and temperature were determined by using turbidimeter (Thermo Scientific), pH meter (oakton), DO meter (oakton), refractometer (ATAGO) and thermometer (Oakton), respectively.

Total number of bacteria and *Vibrio* Analysis

Every week, approximately 150 mL of water from each rearing tank were sampled to count bacteria, according to the methods previously described (Gatesoupe, 1995). Under sterile conditions, 1 mL of sample was put in to three different dilutions, namely 10^3, 10^2 and 10^1. Dilution was performed by using sterile *Trisalt solution*. After homogenization, appropriate dilutions were inoculated on Plate Count Agar (PCA, AES Laboratoire, enriched with 18 g L^{-1} NaCl, pH adjusted to 7.8) for total number of bacteria analysis and TCBS agar (thiosulfate-citrate-bile-salt agar, AES Laboratoire, dissolved in half-strength seawater) for rough estimation of bacteria and *Vibrio* spp. counts. The plates were incubated for 24 hours at 28º - 30º C and counting was performed by using colony counter.

Challenge test

Challenge test was performed by using "bath challenge" with *V. parahaemolyticus* at a density of 10^5 cells/mL after termination of the feeding trial of the nursery. Twenty fish from each supplemented and control group replicate (±20 g) were immersed in *V. parahaemolyticus* suspension for six hours. Fish were then transferred to aquaria and observed for five days for any clinical abnormalities and mortalities.

Bacterial Culture

Isolates of the bacterial strains *V. parahaemolyticus* obtained from Brackishwater Research Centre, Jepara that previously stored in 30 % glycerol at -80 ^{0}C, were aseptically inoculated in 30 mL marine broth by incubation overnight at 25-28 ^{0}C with constant agitation. 150 µL was subsequently transferred and grown to stationery phase in 30 mL marine broth six hours before challenge. The bacterial densities were determined spectrophotometrically at an optical density of 550 nm. The bacterial densities were calculated using the equation: Concentration (CFU/mL) = [1200*10^6*OD] according to McFarland standard, assuming that an OD_{550} = 1.000 corresponds to 1.2×10^9 cells/mL

Bacterial Stock

1 ml of the bacterial colony was transferred and grown to stationery phase in 5 mL of *Difco™ Marine Broth 2216* by incubation overnight at 25-28 ^{0}C with constant agitation. Bacterial suspensions were then transferred to centrifugation tubes and centifugated at 4000 g for 5 minutes. The supernatant was discarded and pellets were resuspended in 7 mL filtered autoclaved sea water (FASW). The solution was homogenized and 3 ml of 30% Glycerol solution was added. 150 µL of each colony was distributed to the sterilized eppendorf tube and stored at -80 ^{0}C.

Statistical analysis

Data for growth and immune performance of *L. calcarifer* are presented as mean values followed by the standard deviation. Survival data of *L. calcarifer* were arcsine transformed for statistical comparisons to satisfy normal distribution and homoscedasticity requirements. Survival data were subjected to one way ANOVA followed by Tukey's multiple comparison range using the statistical software SPSS version 21.0 to determine significant differences among treatments. All significance levels of the statistical analysis were set at $p<0.05$.

RESULTS AND DISCUSSION

Growth Performance

In the current study, the influence of functional hydrolysates in Asian seabass during the nursery phase including total weight gain (TWG), % relative weight gain (% RWG), % specific growth rate (% SGR), Final weight (g) and Final length are summarized in **Table 1**.

Table 1. Growth performances of *L. calcarifer* fed dietary treatments for 6 weeks during the nursery phase.

Treatments	TWG (g)	RWG (%)	SGR (%)	Final weight (g)	Final length (cm)
AQUATIV 3 % FPH	18.25±0.47[a]	530.35±44.39[a]	6.57±0.25[a]	21.71±0.39[a]	11.76±0.21[a]
AQUATIV 2 % FPH	16.64±0.45[b]	483.65±39.81[b]	6.29±0.25[a]	20.10±0.39[a]	11.00±0.17[a]
Control	9.87±0.89[c]	287.13±35.99[c]	4.82±0.34[b]	13.33±0.84[b]	10.33±0.32[b]

TWG: Total Weight Gain (g), RWG: Relative Weight Gain (%), SGR: Specific Growth Rate
Significant differences among the treatments and control are indicated by different letter (n=2, *P<0.05*). Following from this initial nursery phase, the dietary test went on for another 8 weeks in grow-out units.

Table 2. Growth performances of *L. calcarifer* fed dietary treatments for 9 weeks during the grow-out phase.

Treatments	TWG (g)	RWG (%)	SGR (%)	Final weight (g)	Final length (cm)
AQUATIV 3 % FPH	40.74±1.23[a]	187.80±7.40[a]	1.92±0.05[a]	62.94±1.47[a]	16.56±0.23[a]
AQUATIV 2 % FPH	36.76±2.60[b]	182.99±13.84[a]	1.89±0.09[a]	56.94±2.26[b]	15.55±0.28[b]
Control	26.31±1.99[c]	198.77±26.21[a]	1.98±0.15[b]	39.60±1.49[c]	13.77±0.31[c]

TWG: Total Weight Gain (g), RWG: Relative Weight Gain (%), SGR: Specific Growth Rate
Significant differences among the treatments and control are indicated by different letter (n=2, *P<0.05*). During the grow-out feeding trial, SGR were lower for supplemented diet fish groups due to their higher average weight and length. TWG however remained higher for fish groups receiving FPH supplemented diets.

Immune Performance

Blood white cell counts are detailed in Table 3 (nursery) and Table 4 (grow out).

Table 3. Cellular immune response of *L. calcarifer* fed dietary treatments for 6 weeks during the nursery phase.

Parameter	Control	FPH Treatments	
		2 %	**3 %**
Neutrophile (%)	5.07±0.19[b]	6.31±0.15[a]	6.73±0.12[a]
Monocyte (%)	1.93±0.2[b]	2.65±0.1[a]	2.8±0.13[a]
Lymphocyte (%)	50.87±1.49[b]	63.74±1.19[a]	66.71±0.71[a]
Macrophage (10^5 cell/mL)	1.83±0.25[b]	2.62±0.12[a]	2.83±0.15[a]
Leukocyte (10^3 cell/mL)	48.33±1.36[b]	55.67±1.14[a]	56.34±1.17[a]
Phagocytes activity (%)	15.77±1.18[b]	37.95±1.01[a]	40.23±0.52[a]

Significant differences among the treatments and control are indicated by different letter (n=6, *P<0.05*).

Table 4. Cellular immune response of *L. calcarifer* fed dietary treatments for 9 weeks during the grow-out phase.

Parameter	Control	FPH Treatments	
		2 %	3 %
Neutrophile (%)	6.03±0.12	6.60±0.10	6.63±0.12
Monocyte (%)	2.40±0.10	2.87±0.12	2.93±0.06
Lymphocyte (%)	55.21±0.76	65.25±1.09	66.30±1.07
Macrophage (10^5 cell/ml)	2.30±0.20	2.60±0.10	2.67±0.12
Leukocyte (10^3 cell/ml)	49.68±0.56	57.01±2.36	54.67±0.43
Phagocytes activity (%)	28.29±0.67	39.64±1.03	40.48±0.95

Significant differences among the treatments and control are indicated by different letter (n=6, *P<0.05*).

Survival Rates

As illustrated by Figure 1, at the end of nursery feeding trial, the 2 and 3% hydrolysate supplementations resulted in 96.75±0.28 % and 97.28±0.18 % survival rate respectively, while the control diet yielded 93.65±0.13 %. More contrasted results were observed at the end of the grow-out feeding trial, most likely resulting from a disease outbreak, which impacted more the control diet group (20.1±21.1%) compared to the supplemented diet groups (78.4±7.7% and 86.0±4.32 % for 2 and 3% FPH supplementation respectively).

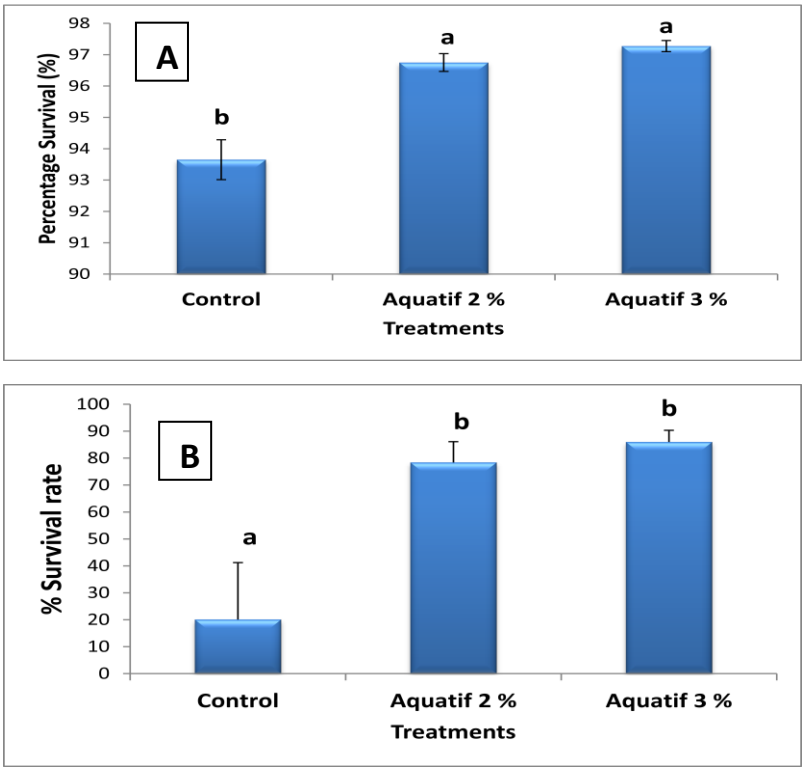

Figure 1. Histogram of the mean percentage survival (%) of *L. calcarifer* during experiment period in nursery phase (a) and grow out phase (b). Significant differences among the treatments and control are indicated by different letter (*p< 0.05*).

Resistance to the bacterial challenge

At the end of the nursery feeding trial, fish from each experimental groups (±20 g, n=20) were challenged by immersion with *V. parahaemolyticus* at a density of 10^5 cells/ml and survival was observed for 5 days. Figure 2 indicated that the supplementation of functional hydrolysates was able to induce significantly ($p<0.05$) higher survival rate in comparison to control diet. In addition, no significant difference was observed between 2 % and 3 % application of functional hydrolysates ($p<0.05$) even though the 3% FPH supplemented group yielded the highest survival rate with 78.33±2.89 %.

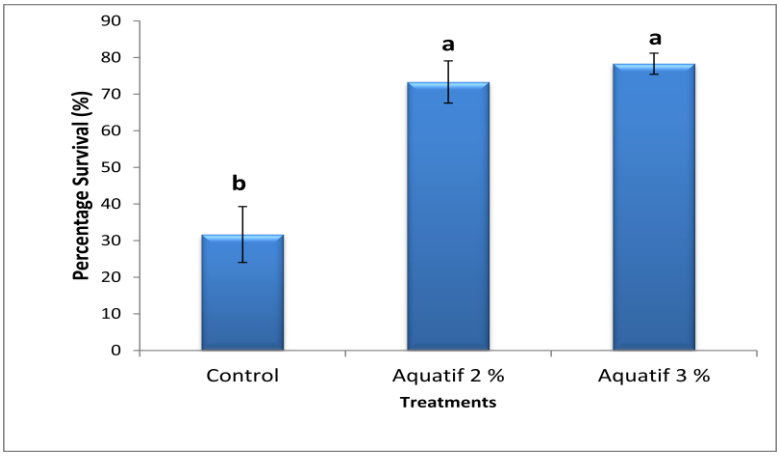

Figure 2. Histogram of the mean survival (%) of *L. calcarifer* (n=20, weight: ±20g) challenged with *V. parahaemolyticus* at 10^5 cells/ml. Survival was scored after 5 days challenge with *V. parahaemolyticus*. Significant differences among the treatments and control are indicated by different letter (n=2, $p< 0.05$).

Water Quality Analysis

Table 5 Water quality analysis during AQUATIV nursery feeding trial. Sampling campaign was performed on weekly basis at 9 AM at the middle point of experimental cages.

Parameter	Unit	Test Results			Method Specification
		Control	2 %	3 %	
Total Bacteria	CFU/mL	1.1 – 2.7x10^2	1.4 – 2.7x10^2	1.1 – 2.8x10^2	IKM/5.4.10/BBL-B
Total Vibrio	CFU/mL	0.2 – 1.1x10^2	1.4 – 2.7x10^2	1.1 – 2.8x10^2	Convensional
pH		7.76 – 8.25	7.72 – 8.18	7.73 – 8.19	SNI 06-6989.11-2004
Nitrate (NO_3)	mg/L	<0.01	<0.01	<0.01	Colorimetry
Nitrite (NO_2)	mg/L	<0.1	<0.1	<0.1	Colorimetry
Ammonia (NH_3)	mg/L	<0.02 –0.094	<0.02 –0.094	<0.02 –0.094	IKM/5.4.6/BBL-B
Posphate (PO_4)	mg/L	0.012–0.024	0.018-0.027	0.015-0.022	IKM/5.4.8/BBL-B
Salinity	‰	30 – 31	30 – 31	30 - 31	IKM/5.4.4/BBL-B
Turbidity	NTU	0.65 – 1.42	0.81 – 1.44	0.65 – 1.42	IKM/5.4.9/BBL-B
Temperature	^{0}C	30.1 – 30.4	30.1 – 30.4	30.1 – 30.4	Thermometer

Table 6. Water quality analysis during AQUATIV grow-out feeding trial. Sampling campaign was performed on weekly basis at 9 AM at the middle point of experimental cages.

Parameter	Unit	Test Results							Metoda Analisa
		15 Juli	22 Jul	29 Jul	5 Aug	12 Aug	19 Aug	26 Aug	
Total Bacteria	CFU/mL	173	$1.2x10^3$	$5.2x10^3$	$1.4x10^3$	$2x10^2$	$2.7x10^2$	$3.7x10^3$	IKM/5.4.10/BBL-B
Total Vibrio	CFU/mL	23	131	$2.7x10^2$	$3.4x10^2$	$1.9x10^2$	$3.1x10^2$	$1.9x10^2$	Convensional
pH		8.18	7.78	8.26	7.76	8.18	8.37	8.25	SNI 06-6989.11-2004
Nitrate (NO_3)	mg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	Colorimetry
Nitrite (NO_2)	mg/L	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	Colorimetry
Ammonia (NH_3)	mg/L	<0.009	<0.009	<0.009	0.357	<0.009	<0.009	<0.009	IKM/5.4.6/BBL-B
Posphate (PO_4)	mg/L	0.027	0.025	0.047	<0.033	<0.033	<0.033	<0.033	IKM/5.4.8/BBL-B
Salinity	‰	30	30	31	30	30	31	31	IKM/5.4.4/BBL-B
Turbidity	NTU	7.15	2.63	5.33	27	2.44	6.52	7.26	IKM/5.4.9/BBL-B
Temperatu-re	^0C	29.1	30.5	28.7	29.6	30.5	29.7	30.7	Thermometer

While observed water quality was controlled and satisfying during nursery feeding trial (Table 5), it was not possible to guarantee a good water quality during the grow-out feeding trial (Table 6). Water quality observed during this latter period is however representative of conditions which may apply to any Asian seabass cage rearing within this area. Most impacted water quality indicators were the water turbidity, i.e. the load of organic matters and their associated pollutants and bacterial communities, including a high load of *Vibrio* spp. As illustrated above, degraded water quality may be the origin of the disease outbreak, which occurred during grow-out feeding trial.

DISCUSSION

Protein hydrolysis is one promising approach to improve the physiochemical, functional, sensory and nutritional properties of marine by product native proteins (Šližytė et al., 2005). Many studies have revealed that protein hydrolysis can also improve intestinal absorption (Kristinsson and Rasco, 2000a) and microbiota (Kotzamanis et al., 2007). Furthermore, hydrolysates are more readily digested and absorbed in fish digestive tract (Carvalho et al., 1997). In order to replace fish meal, FPH are also widely used as growth enhancers, attractants or palatability enhancer (Hardy, 1991; Aguila et al., 2007). Several authors have reported that good functional properties and nutritive value of protein hydrolysates improve the growth and feed utilization in salmonids (Berge and Storebakken, 1996; Refstie et al., 2004) and in carp larvae (Carvalho et al., 1997). In line with our results, at nursery phase, significant increase of total weight gain (TWG), % relative weight gain (% RWG), % specific growth rate (% SGR), final weight (g) and final length (cm) were recorded in Asian Seabass treated with 2 % and 3 % of fish hydrolysates in comparison to control ($p<0.05$). The positive effects of functional hydrolysates on Asian seabass performances at nursery phase might be explained by the high palatability of FPH stimulating the feed intake (Cahu et al., 1999; Oliva- Teles et al., 1999; Aguila et al., 2007). It resulted in a higher growth rate with higher biomass production. Based on the significant differences observed for zootechnical performances of Asian Seabass at nursery phase (Table 1, 2 and Figure 1), these functional properties of hydrolysates obviously impacted the nutritional metabolism of fish. During

hydrolysate manufacturing process, the capability of enzymatic hydrolysis to decrease the protein size of the raw material and to improve the dietary protein quality bring another advantage of using diet containing functional hydrolysates (Petersen, 1981). Protein hydrolysates will be better absorbed than classical raw materials such as meal (Cissé et al., 1995; Ouellet et al., 1997), resulting in a higher feed efficiency and less waste released into the environment. Survival rate, relative weight gain and specific growth rate in fish fed functional hydrolysates were considerably higher during the nursery phase compared to the grow-out phase. Under the controlled nursery conditions, hydrolysates expressed their full potential. In the grow out phase, fish were exposed to persistent environmental challenges (turbidity, temperature and salinity fluctuations). Those adverse environmental conditions actually resulted in a disease outbreak and slowed growth down as described in other studies (Walters and Plumb, 1980; Robertson et al., 1987). The condition developed by fish in the test strongly relates to a *Tenacibaculum* infection further complicated by opportunistic vibriosis.

There have been reports of biologically active peptides with immuno-stimulating and antibacterial properties being produced during the hydrolysate manufacturing process (Coste et al., 1992; Bøgwald et al., 1996; Gildberg et al., 1996; Daoud et al., 2005; Kotzamanis et al., 2007). The results of the present study showed that feeding Asian Seabass with functional hydrolysates enhanced phagocytosis activity by blood phagocytic cells during the whole experimental period and stimulated the circulating neutrophile, monocyte, lymphocyte and leukocyte number. Observations on macrophages showed that the application of 3 % of functional hydrolysates was the most effective dose among the treatments. It could increase the number of macrophages after the natural bacterial challenge. Macrophages play a major role in both innate and adaptive immune system. Study from Norum et al. (2005) and Joerink et al. (2006) stated that in the innate immune system, macrophages act as phagocytes cells, produce pro inflammation, ROS (by NADPH oxidase enzyme) and RNS (by *nitric oxide* (NO-) *synthase*). Meanwhile, in the adaptive immune system, macrophages act as professional APCs (*Antigen presenting cells*). Moreover, the ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection in aquatic organisms (Selvaraj et al., 2005). Corroboration for our results comes from the work of Tang et al. (2008) who have reported that growth performance and immune parameters of the large yellow croaker can be improved by supplementing functional hydrolysates to their basal diet.

CONCLUSION

In conclusion, this work revealed that the addition of functional hydrolysates in Asian Seabass feed was able to enhance significantly the fish immune system as well as the fish zootechnical performances such as Survival rate, total weight gain (TWG), relative weight gain (RWG) and specific growth rate (SGR) during the experimental period. Therefore, dietary supplementation of functional hydrolysates in Asian Seabass, *Lates calcarifer* feeds should be encouraged as a holistic approach to improving its sustainable culture.

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