Fish and Shellfish Immunology 95 (2019) 697–704

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Dietary non-protein energy source regulates antioxidant status and immune response of barramundi (*Lates calcarifer*)

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ARTICLE INFO

Keywords:
Lates calcarifer
Non-protein energy source
Growth performance
Histology
Antioxidant status
Immune responses

ABSTRACT

This study evaluates the effects of different dietary sources of non-protein energy on growth performance, histological structure, antioxidant status and immune response of barramundi *Lates calcarifer*. Fish were fed with isoenergetic diets (18 kJ/g) with two types of non-protein energy in the experimental groups and a regular diet was used as the control for 56 days. The specific growth rate and survival of fish were not significantly different between experimental diets. Hepatic histology did not reveal significant differences between dietary treatments at cellular level. The activity of most antioxidant enzymes in the lipid group significantly increased, and the antioxidant capacity in the carbohydrate group was significantly higher than that in other treatments. In the TOR pathway, LST8 homolog (mLST8) expression in the high lipid group was downregulated, and the mechanistic target of rapamycin (mTOR) expression in the high carbohydrate group was downregulated and eIF4E expression was upregulated. The C-reactive protein (CRP) expression in the high lipid and high carbohydrate groups was upregulated. The expression levels of heat shock protein genes in the high lipid group and the high carbohydrate group were significantly downregulated. This study indicates that the lipid diet have less effect in fish immunity but is more suitable as a non-protein ingredient for energy supply for barramundi.

1. Introduction

Aquaculture has important contribution to the stable supply of the world's food portfolio [1]. The recent trend is to increase fish production and profitability through intensive or semi-intensive farming using high stocking density and high energy diet, but this practice could lead to deterioration of the environmental condition, thereby resulting in disease outbreak. Disease outbreak could potentially become a major barrier to the development of aquaculture [2]. Traditionally, control and prevention of diseases are usually achieved by using a wide range of antibiotics, pesticides, disinfectants and other chemicals. Such practices are usually accompanied by a consequence that potentially damages human and animal health in addition to accumulating a large amount of veterinary drugs in the environment [3,4]. Recently, researchers have explored the manipulation of dietary formula to improve the health of aquatic animals and disease resistance ability. In intensive aquaculture, majority of operation cost comes from feed supply [5]. To reduce feed cost, dietary lipid and carbohydrate are commonly used to replace protein to a make high-energy feed. As the non-protein energy source, lipid and carbohydrate have been widely used as ingredients in the diet of economic fish to spare dietary protein and improve feed efficiency [6].

Nutritional composition in the diet is closely related to the immune status of fish [8]. Nutrients not only provide the essential material for fish growth, but also stimulate the immune system [9]. Nutritional imbalances have a profound effect on disease resistance of fish [10]. Inclusion of non-protein energy can spare dietary protein from catabolism to provide energy and enhance its utilization for growth [6]. As protein is the most expensive ingredient in fish diet, it is imperative to incorporate only the amount necessary for normal maintenance and growth [11]. Meanwhile, non-protein energy ingredients also have an advantage to reduce ammonia nitrogen released to the environment [12]. Carbohydrates are recognized as an important ingredient for animals, and have been widely used in practical feed as a cheaper source

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https://doi.org/10.1016/j.fsi.2019.11.018
Received 4 September 2019; Received in revised form 6 November 2019; Accepted 7 November 2019
Available online 08 November 2019

of energy [13]. Lipids, as a high energy macronutrient in fish feed, can provide both energy and essential fatty acids for fish requirement [15]. Fish cannot synthesize essential fatty acids but need to acquire them from feed to support growth and metabolic maintenance [16].

During nutrient metabolism, numerous reactive oxygen species (ROS) could be generated during aerobic activities in most animals [17]. Although optimal ROS could act roles in growth and immune defense, excessive accumulation of these biomolecules could induce oxidative stress and cause serious damage to lipids, proteins and nucleic acids in animal cells, including the cells in the immune system [18]. Current studies have shown that the antioxidant system causes changes in liver function through affecting lipid metabolism, thereby affecting immunity [19,20]. In general, the antioxidant enzyme activity and antioxidant content as well as immune-related gene expression levels were measured to evaluate the antioxidant status and immune response of animals. In largemouth bass *Micropterus salmoides*, higher superoxide dismutase (SOD) and catalase (CAT) content in the liver were recorded in fish fed diets 18.1% lipid, while the malondialdehyde (MDA) content in the liver increased with the increase of dietary lipid levels. Dietary carbohydrate can increase the hepatic SOD activities and decrease CAT, GSH and LPO oxidative stress indexes in gilthead sea bream *Sparus aurata* [21]. In juvenile black carp *Mylopharyngodon piceus*, dietary carbohydrate (288.4 g kg^{-1}) can promote growth, reduce oxidative stress, enhance innate immune responses, and improve health and disease resistance [22].

The barramundi *Lates calcarifer* is an important species in aquaculture in Indo-West Pacific region with a production volume over 67,000 tons [23,24]. Barramundi has become a widely cultivated species due to its biological merits including tolerance to high stocking density, easy propagation and acceptance to artificial pellets at a young age [25,26]. Moreover, it grows fast as it merely takes 6 months to 2 years to reach a commercial size of 350–3000 g [27].

Although the effects of nutrients on immune function and health status of fish have been investigated, most studies are mainly focusing on the change of amino acids, fatty acid and micronutrients [28,29]. In barramundi, most studies are mainly focusing on the impact of non-protein content on its growth efficiency, but the effect on non-protein energy on its immunity is little known [30–32]. In the present study, fish oil and α-starch were used as the potential energy sources to evaluate the antioxidant status and immune responses of barramundi to dietary lipids and carbohydrate. Results from the present study will improve our understanding on the use of lipid and carbohydrate in fish feed to spare protein and reduce feed cost, but without compromising fish health and immunity.

2. Materials and methods

2.1. Experiment design and system

Barramundi juveniles (33.69 ± 3.63 g, 12.08 ± 2.13 cm) were produced by Tropical Aquaculture Research and Development Center, Sanya, China, and reared in the indoor recirculating aquaculture system. A total of 117 fish were randomly assigned to 9-tanks (800 L) for a 12-day acclimation and fed with experimental diets. In each experiment tank, 13 fish were assigned, and the diet treatments were randomly assigned to the experimental tanks. Upon completion of acclimation, feeding trial was conducted and the experiment lasted 8 weeks. Fish were fed ad libitum twice a day at 08:00 and 16:00 h. The experiment was conducted in outdoor seawater tanks. In each tank, water was changed 1 h after feeding with the water exchange rate over 50% of the tank volume each time. Filtered natural seawater was used in this study. During the experimental period, the water quality parameters were measured daily and maintained at ammonia nitrogen < 0.1 mg L^{-1}, nitrite nitrogen < 0.02 mg L^{-1}, pH 7.8, and dissolved oxygen > 7.0 mg L^{-1}. At the end of the experiment, all fish were anaesthetized in 7 mg L^{-1} eugenol (Shangchi Dental Material Co., Ltd., Changshu, China) before handling and sampling to comply with the animal ethic protocol approved by the Animal Welfare Committee (E437-16). All fish in each tank were weighed to determine growth performance and three fish were sampled from each tank (i.e. nine fish per treatment) for somatic parameters, histological, biochemical and gene expression analyses. The samples of biochemical and gene expression were snap-frozen in liquid nitrogen, then preserved in −80 °C until use.

Table 1
Feed composition (g kg^{-1}), proximate composition of the diets (air-dry basis g kg^{-1}) and gross energy content (MJ kg^{-1}) in three experimental diets.

Ingredients	C	HL	HC
Fish meal	560	560	560
Peeled soybean meal	236	176	186
α-starch	100	100	150
Fish oil	60	120	60
Choline chloride	2	2	2
Vitamin premix[a]	10	10	10
Mineral premix[b]	10	10	10
Yttrium oxide	2	2	2
Calcium dihydrogen phosphate	10	10	10
Ethoxy quin	10	10	10
Total	1000	1000	1000
Proximate composition			
Dry matter	929.9	914.1	932
Crude protein	474.9	445.7	451.4
Lipid	99.9	153.1	94
Ash	173.6	167	167.2
Nitrogen-free extract	179.99	145.04	218.10
Gross energy(kJ/g)	18.26	19.08	18.13

The dietary energy was calculated as protein: 23.64 MJ kg^{-1},lipid: 39. 54 MJ kg^{-1},carbohydrate: 17.15 MJ kg^{-1}

C, Control; HL, High lipid; HC, High carbohydrate.

[a] Vitamin premix (mg kg^{-1} diet or specified): vitamin A 900 0000 (IU kg^{-1} diet), vitamin D 250 0000 (IU kg^{-1} diet), vitamin K3 600 (IU kg^{-1} diet), vitamin E 500 (IU kg^{-1} diet), vitamin B1 3200, vitamin B2 1 0900, vitamin B5 2 0000, vitamin B6 5000, vitamin B12 1160, vitamin C 5 0000, niacin 400, folic acid 50, calcium pantothenate 200,phaseomannite 1500, biotin 2.

[b] Mineral premix(mg kg^{-1} diet or specified): MgSO4·7H2O 300; KCl 70; KI 1.5; ZnSO4·7H2O 14; MnSO4·4H2O 3; CuCl2 5; CoCl2·6H2O 0.5; FeSO4·7H2O 15; KH2 PO4·H2O 4.5 (g kg^{-1} diet); CaCl2 2.8 (g kg^{-1} diet).

Three iso-energy diets (Table 1) were prepared in Tropical Aquaculture Research and Development Center. According to the general nutritional requirements of barramundi, the control group (C) used the diet formula according to crude protein 475 g kg^{-1}, crude fat 100 kg^{-1}, and nitrogen-free extract 180 kg^{-1}. In the experimental group, fish oil and α-starch were used as the source of lipid and carbohydrate, respectively [33]. The crude fat in the lipid group (HL) increased to 160 kg^{-1}, and the nitrogen-free extract in carbohydrates group (HC) increased to 220 kg^{-1}. All ingredients were crushed and sifted (0.2 mm mesh size), mixing through a commercial food mixer (Guangdong Lifeng Co., Ltd., China) and then blended with oil. Pellets (4 mm in diameter) were then produced through a pelletizer (Shandong Hengfeng Co., Ltd., China), and air-dried at room temperature (25 °C). All diets were sealed in plastic bags and stored at −20 °C until use.

2.2. Proximate composition analysis

Moisture was determined by oven dry to a constant weight at 103 °C in an air-blower-driven drying closet (Nocchi Instrument Co., Ltd., China). Total nitrogen (% dry weight) was determined using a rapid N exceed (Elementar Co., Ltd., Germany). Crude protein content was calculated as % nitrogen × 6.25. Ash (% dry weight) was quantified by burning 2 g samples at 650 °C for 3 h in a muffle furnace (Laboratory Instrument Co., Ltd., China). Crude lipid (% dry weight) was determined by ether extraction using a Soxtec System (Zhejiang Tuopu Instrument Co., Ltd., China).

2.3. Histological analysis

The liver was collected and fixed in 4% paraformaldehyde. The fixed tissues were embedded in paraffin blocks and sliced in a series of transverse sections (4 μm thick) using a Leica RM 2016 rotary microtome (Shanghai Leica Instrument Co., Ltd., China). A hematoxylin–eosin (HE) stain was used for general histological analysis. Each slide with tissue sections was mounted permanently using neutral balsam. The sections were scanned using a Pannoramic 250/MIDI scanner (3D HISTECH Co., Ltd., Hungary), and Caseviewer2.0 (3D HISTECH Co., Ltd., Hungary) was used for image analysis and measurement. For each liver sample, three cross-sections were quantified for hepatocyte dimension. Each cross section was ere quantified by taking 10 measurements.

2.4. Hepatic oxidative status and serum non-specific immune assays

For each assay, pooled samples from each tank were partially thawed, weighed and homogenized using a tissue homogenizer on ice in five volumes of 0.2 M NaCl (w/v). The suspensions were centrifuged at 3500 rpm for 10 min at 4 °C. The protein content in the homogenate was determined using Coomassie blue stain [34]. The activities of total superoxide dismutase (T-SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6), peroxidase (POD, E.C. 1.11.1.7) and glutathione peroxidase (GSH-Px, E.C. 1.11.1.9) in the liver were quantified based on the methods in previous studies [35–37]. The reduced glutathione (GSH) in the liver was determined enzymatically through recycle reactions of GSH with DNTB (5,5′-dithios -2-205 nitrobenzoic acid) in the presence of an excess of glutathione reductase. The malondialdehyde (MDA) content in liver was determined using the thiobarbituric acid test [38]. The total anti-oxidant capacity (T-AOC) in the serum was measured by the ABTS method [39]. Serum lysozyme (LZM, E.C. 3.2.1.17) activity was determined using turbidimetric assay to Ref. [40]. All assays were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and performed in triplicate.

2.5. Gene expression analysis

The harvested frozen tissues were homogenized in 1 mL Trizol (Invitrogen) on ice using a hand-holding homogenizer (Greenprima Instrumenta Co., Ltd., UK), and RNA was separated in the chloroform layer and precipitated by isopropanol. The RNA pellet was washed in 1-mL 75% ethanol, and air-dried before resuspension in RNase-free water. The concentration of the RNA was quantified by spectrophotometry (Bioteke Corporation Co., Ltd., China). Finally, the integrity of RNA was assessed using agarose gel electrophoresis. The RNA was immediately used for cDNA synthesis. Subsequently, reverse transcription was performed on 1 μg of total RNA using TransScript-Uni One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech Co., Ltd., China). The synthesized cDNA samples were stored at −20 °C until further use.

The immune-related genes chosen for analysis by qPCR were selected from the *L. calcarifer* NCBI database (https://www.ncbi.nlm.nih.gov/). The Primer Premier 5 program was used for designing the primers of mTOR, mLST8, eIF4E, CRP and β-actin. The primers of HSP70 and HSP90 were previously designed and validated by Ref. [41] (Table 2). The qPCR on the immune-related genes was performed with the Real-time qPCR analysis (Hangzhou Longgene Scientific Instrument Co., Ltd., China) using SYBR Green (Tiangen Biotech Co., Ltd., China). The 20 μl of reaction including 10 μl 2 × RealUniversal PreMix, 0.6 μl of each primer (10 μM) and 2 μl of diluted cDNA was initially denatured at 95 °C for 15 min and then amplified for 40 cycles (95 °C, 10 s, 58 °C, 20 s and 72 °C, 30 s). For each sample, the PCR reactions were performed in triplicate. At the end of each cycle, the melting curve analysis of the primers was performed to ensure only specific products were obtained with no formation of primer dimers. No template control was included with each assay to verify that PCR master mixes were free of contamination. The expression levels of immune-related genes were normalized based on the level of housekeeping gene (β-actin) [42]. After verification of PCR efficiency to be around 100%, the gene expression data were analyzed based on the $^{\Delta\Delta}C_t$ method [43].

Table 2
Primers of mechanistic target of rapamycin (mTOR), mTOR associated protein, LST8 homolog (mLST8), eukaryotic translation initiation factor 4E (eIF4E), C-reactive protein(CRP), heat shock cognate 70 kDa protein (HSP70), heat shock cognate 90 kDa protein (HSP90) and actin beta (β-actin) genes in barramundi used in qPCR.

Gene abbreviation	Primer sequence (5′-3′)	Amplicon size (bp)	Accession number
mTOR	F: GTTTCTTCCGCTCCATTTC R: CAGGGCTTCATTCACTTCA	110	XM_018675222
mLST8	F: TGATTCAACACTATTAGCCACA R: TTTCCACGCACCACAGG	212	XM_018687802
eIF4E	F: TGACGACTACAGCGATGAT R: GTGTCTGCGTGGGATTG	183	XM_018697729
CRP	F: ACCGAACTGAAGACCACGAT R: TGGGGCACCTCAAACAAA	106	HQ652974
HSP70	F: AAGGCAGAGGATGATGTC R: TGCAGTCTGGTTCTTGTC	186	XM_018691298
HSP90	F: ACCTCCCTCACAGAATACC R: CTCTTGCCATCAAACTCC	197	XM_018661637
β-actin	F: AACCAAACGCCCAACAACT R: ATAACTGAAGCCATGCCAATG	112	XM_018667666

2.6. Calculations and statistical analysis

Specific growth rate (SGR) was calculated as: SGR = 100 × (ln(W_f) − ln(W_i))/ΔT, where W_f was the final body weight; W_i was the initial body weight; andΔT was the experimental duration. Feed conversion ratio (FCR) was calculated as FCR = dry feed fed/body wet weight gain. Feed intake (FI) was calculated as FI = (feed consumed per tank/fish)/days, Protein efficiency ratio (PER) = total weight gain/protein intake. Condition factor (CF) = 100 × [(body weight)/(body length)^3]. Viscera ratio (VR) = 100 × [(viscera weight)/(whole-body weight)]. Hepatosomatic index (HIS) = 100 × [(liver weight)/(whole-body weight)]. Intraperitoneal fat ratio (IPF) = 100 × [(intraperitoneal fat weight)/(whole-body weight)].

The data were expressed as the mean ± standard deviation (SD). Statistical analyses were carried out by PASW Statistics (version 18). Comparisons between different groups were conducted by one-way ANOVA and LSD test, and significant difference was set at $P < 0.05$. All percentage data were transformed using square root to satisfy the assumptions of ANOVA.

3. Results

3.1. Growth performance

Weight gain and feed intake showed significant difference between treatments ($P < 0.05$, Table 3). The difference between the HL and control groups was significant ($P < 0.05$), but there was no significant difference between other groups ($P > 0.05$). The highest HIS was observed in HL, which was significantly higher than that in the HC group ($P < 0.05$). The highest IPF was observed in the HL group ($P < 0.05$). SGR, survival, FCR, PER, CF and VR were no significant differences between any feeding groups ($P > 0.05$, Table 3).

3.2. Hepatic histology

Quantitative morphometric analysis of the liver did not reveal any significant difference between dietary treatments in histological parameters. The hepatocyte length was not significantly different between

Table 3
Effects of three experimental diets on growth performance of *Lates calcarifer*.

Productivity index	Experimental diets		
	C	HL	HC
Initial weight (g fish^{-1})	32.85 ± 0.67	32.76 ± 2.15	35.31 ± 6.19
WG (g fish^{-1})	11.51 ± 2.41^{b}	15.41 ± 1.98^{a}	14.15 ± 0.71^{ab}
SGR (% d^{-1})	0.53 ± 0.10	0.69 ± 0.07	0.61 ± 0.11
Survival (%)	100 ± 0	97.44 ± 4.44	92.31 ± 7.69
FI (g fish^{-1} d^{-1})	0.63 ± 0.07^{b}	0.83 ± 0.13^{a}	0.64 ± 0.08^{ab}
FCR	3.12 ± 0.46	3.09 ± 0.76	2.54 ± 0.21
PER(%)	0.68 ± 0.10	0.76 ± 0.20	0.88 ± 0.07
CF	3.42 ± 0.05	3.62 ± 0.04	3.39 ± 0.47
VR	8.28 ± 0.47	9.46 ± 0.66	8.04 ± 2.22
HIS	1.47 ± 0.27^{ab}	1.96 ± 0.27^{a}	1.36 ± 0.25^{b}
IPF	1.91 ± 0.24^{b}	3.84 ± 0.59^{a}	1.48 ± 0.32^{b}

Data are given as the mean ± SD. In the same row, values with same small letter superscripts or no letter superscripts mean no significant differences ($P > 0.05$); different small letter superscripts mean significant differences ($P < 0.05$).
C, Control; HL, High lipid; HC, High carbohydrate.
WG, Weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion rate; PER, protein efficiency ratio; CF, Condition factor; VR, Viscera ratio; HIS, Hepatosomatic index; IPF, Intraperitoneal fat ratio.

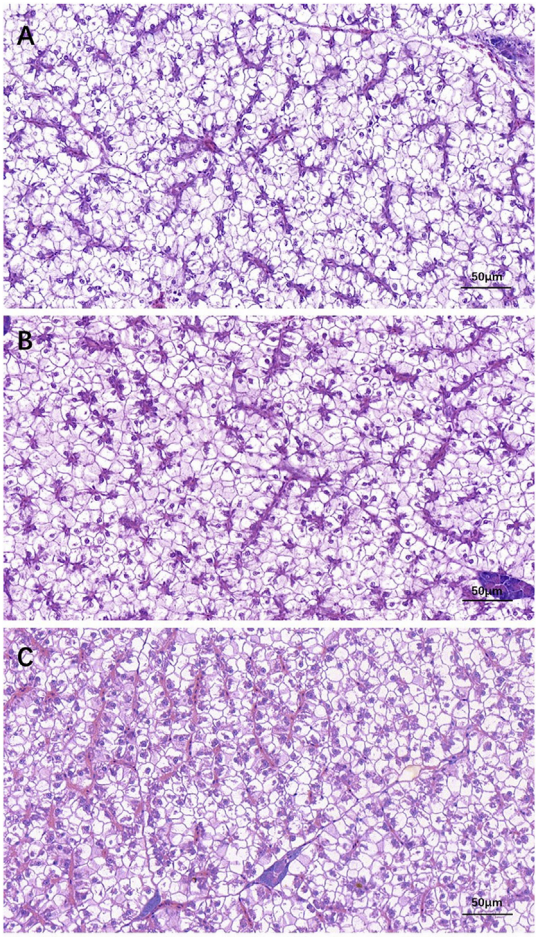

Fig. 1. Hepatocytes morphology of barramundi subjected to the different feeds (C-Control, HL-High lipid, HC-High carbohydrate). Hepatocytes histological sections (400× magnification) of C (A), HL (B) and HC (C).

any feeding groups ($P > 0.05$, Fig. 1). The hepatocyte length in C, HL and HC was 15.42 ± 0.95 μm, 15.74 ± 1.50 μm and 16.59 ± 1.68 μm, respectively.

3.3. Hepatic oxidative status and serum non-specific immune assays

T-SOD and CAT activities were significantly affected by experimental diets ($P < 0.05$, Fig. 2A and B), and the highest activity was found in the HL group. Liver POD showed no significant difference among treatments ($P > 0.05$, Fig. 2C). However, the GSH content and GSH-Px activities were significantly affected by the dietary treatment ($P < 0.05$). In HL, GSH was significantly lower than the other two treatments ($P < 0.05$), while the GSH-Px activity in HL was the highest in the three groups (Fig. 2D and E). The T-AOC increased in both HL and HC as compared with the control, and its value in HC was significantly higher than in the control ($P < 0.05$, Fig. 2 F). There was no significant difference in the content of MDA ($P > 0.05$, Fig. 2 G). The LZM in the HL group was significantly lower than the other two groups ($P < 0.05$, Fig. 2H).

3.4. Gene expression of liver

In the mechanistic target of rapamycin (mTOR) signaling pathway, the mTOR expression in the HC group and the mLST8 expression in the HL group were significantly down-regulated ($P < 0.05$), while the eIF4E expression in the HC group was significantly up-regulated ($P < 0.05$, Fig. 3 A). There were significant differences in expression levels in the three treatments of CRP ($P < 0.05$). The expression level of CRP in the HC group was highest, the lowest expression level of CRP was with the control treatment (Fig. 3 B). In heat shock protein genes, the HSP70 and HSP90 expression levels were similar in each treatment, and the expressions were significantly down-regulated in both HL and HC ($P < 0.05$, Fig. 3C).

4. Discussion

Protein is the most expensive ingredient in fish feed [12]. When the lipid content in the diet is insufficient, the protein will be used as a source of energy for metabolic maintenance and routine activities [44]. A high-efficient and economic diet should be provided with energy from non-protein energy sources as much as possible to reduce feed cost and pollution to the environment due to the use of high protein diet [12]. It is now widely accepted that nutritional approach is possible to improve fish health and alleviate disease infection. The concept that better nutrition leads to better health is very familiar to human nutrition and is also applicable to aquatic animals in aquaculture [10]. Efforts have been made on farmed fish to understand the link between nutrition, immune response and resistance to diseases. Studies have found that an optimal level of dietary protein can improve the function of intestinal physical barriers and reduce enteritis morbidity in grass carp *Ctenopharyngodon idella* [45]. The diets with a CHO: lipid ratio of 2.45–5.58 (24.5–33.5% carbohydrate and 6–10% lipid) are optimal to maintain health status of juvenile yellow catfish *Pelteobagrus fulvidraco* [46].

4.1. Effects of non-protein energy source on growth parameters

In the present study, there was no significant differences in most growth parameters between experimental groups. The growth performance of the barramundi was not significantly affected by the types of energy substance, and the proportion of non-protein energy substance in the diet could be increased appropriately. Similar results have been reported in barramundi [30]. In the present study, WG and Fi increased to a higher level in HL. The weight gain in HL was related to high feed intake, but there was no significant difference in FCR. Although, feed efficiency was not improved, growth rate was accelerated, which is still important for feeding and growth management. All three treatments resulted in higher survival, suggesting that an appropriate increase in the proportion of lipid or carbohydrate would not significantly affect the survival of barramundi. Similar results have also been reported in

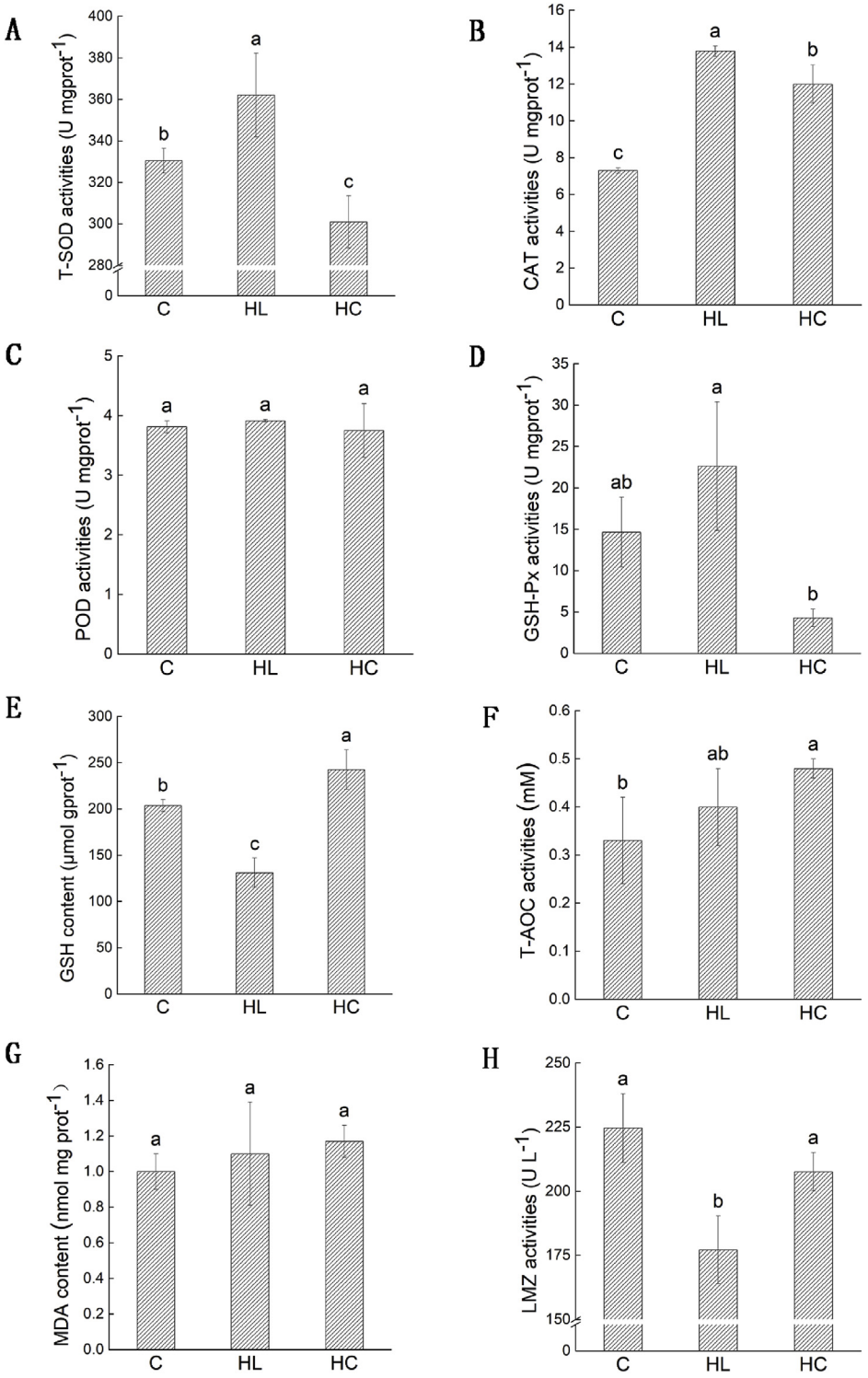

Fig. 2. Activities of total superoxide dismutase (T-SOD, A), catalase (CAT, B), peroxidase (POD, C), glutathione peroxidase (GSH-Px, D), lysozyme(LZM, H), glutathione (GSH, E), malondialdehyde (MDA, G) content and total anti-oxidant capacity (T-AOC, F) in barramundi fed experimental diets (C-Control, HL-High lipid, HC-High carbohydrate).
Different superscript letters indicate significant differences between treatments ($P < 0.05$). Error bars represent standard error.

barramundi and other fish species [47–49].

4.2. Effects of non-protein energy source on hepatic oxidative status and serum non-specific immune response

Reactive oxygen species (ROS) include O^{2-}, H_2O_2 and –OH molecules that are produced during metabolism and can cause damage to protein, lipid and nucleic acid in animal cells [50]. The ROS scavenging ability is correlated with the enzymatic and non-enzymatic antioxidant defense systems [37]. In the enzymatic antioxidant defense system, SOD is the enzyme that catalyzes dismutation of the superoxide anion to O_2 and H_2O_2, and CAT and POD react with H_2O_2 to form water and oxygen molecules [51–53]. The GSH-Px detoxifies H_2O_2 or organic hydroperoxides that are produced in lipid peroxides [54], and GSH is

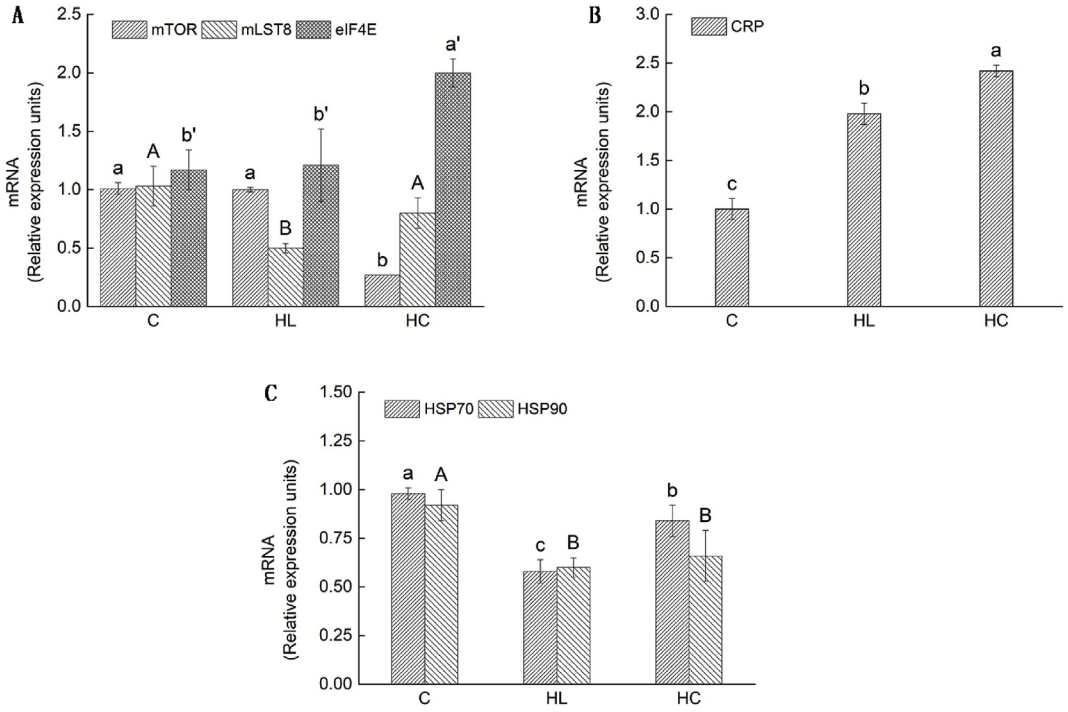

Fig. 3. Relative expression of mechanistic target of rapamycin (mTOR), MTOR associated protein, LST8 homolog (mLST8), eukaryotic translation initiation factor 4E (eIF4E), heat shock cognate 70 kDa protein (HSP70), heat shock cognate 90 kDa protein (HSP90) and C-reactive protein (CRP) genes in barramundi fed different experimental diets (C-Control, HL-High lipid, HC-High carbohydrate).
Different superscript letters indicate significant differences among treatments ($P < 0.05$). Error bars represent standard error.

the antioxidant substrate that catalyzes the enzymatic decomposition of lipid peroxides [55]. The non-enzymatic antioxidant defense system is composed of endogenous antioxidants (e.g., NADPH, NADH, GSH and ubiquinol), dietary antioxidants (e.g., vitamin C, vitamin E and carotenoids), and metal binding proteins (e.g., ceruloplasmin and myoglobin) [50]. The T-AOC is an indicator of antioxidant capacity that is applicable to both lipophilic and hydrophilic antioxidants such as flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants [39]. MDA is the final product of lipid peroxidation and directly reflects the ROS scavenging ability in fish [56]. In the present study, a range of the high lipid diet enhanced the activity of antioxidant enzymes. The content and relative antioxidant capacity of antioxidants and antioxidant enzymes maintained a relative balance, while the content difference of MDA between three treatments was not significant. Guo et al. (2019) found that dietary lipid in the *Micropterus salmoides* provides more energy, but lipid peroxidation aggravates. In their, the activities of SOD and CAT was significantly higher in high lipid group (18.1%) than that in other low lipid groups (3.3%, 8.2% and 13.2%) [20], which is similar to the results of the present study. In order to protect organelle integrity from the damage of lipid peroxides, the antioxidant system will respond by maintaining the balance of antioxidants and ROS. The synthesis of antioxidant enzymes is likely to maintain a high activity to avoid severe oxidative stress [57,58].

The non-specific immune system of fish is considered to be the first line of defense against invading pathogens [59]. In the present study, no liver histological damage was seen in any of the three treatments. The major components of the innate immune system (non-specific) are macrophages, monocytes, granulocytes and humoral elements, like lysozyme [60]. The interaction between nutrition supply and defense capacity has long been known in fish. Nutritional support plays an important role in maintaining health status of organisms by providing the building blocks of non-specific cellular and humoral immunity to defend a wide variety of pathogen organisms [61]. In this study, the serum lysozyme activity was significantly different between treatments, which clearly demonstrates that dietary non-protein energy can server as an immunostimulant for barramundi. Studies have shown that lysozyme contains an 18-amino acid domain that is an advanced glycation end product to generate ROS [62]. Although a high intake of lipid will increase of ROS,. lysozyme activity could counteract the production of ROS in the HL group.

4.3. Effects of non-protein energy sources on gene expression

The mechanistic target of rapamycin (mTOR) signaling pathway can sense and integrate a variety of environmental cues to regulate growth and homeostasis in an organism [63] and affect protein synthesis, lipid synthesis and energy metabolism. The mTOR is an atypical serine/threonine protein kinase and interacts with several proteins to form two distinct complexes, namely, mTOR complex 1 (mTORC1) and 2 (mTORC2). In the present study, mTOR was significantly down-regulated in the HC group. That may affect the complexes of mTORC1 and mTORC2, thereby affecting the pathway. The mLST8 is involved in both mTORC1 and mTORC2, and its absence does not affect the activity of mTORC1, but is essential for the mTORC2 activity [64,65]. The mTORC2 is associated with the metabolism and cell survival [63]. Therefore, in the HL group, the decrease of mTORC2 activity would affect the relevant life activities of the organism. Protein synthesis is by far the best-characterized process controlled by mTORC1. The mTORC1 directly phosphorylates the translational regulators, eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1, which, in turn, promote protein synthesis [66]. In this study, in comparison with other two treatments, the eIF4E was significantly upregulated by in the HC group, indicating that carbohydrate can promote protein synthesis.

The CRP is an important inflammatory marker and it has a pleiotropic effect. Both “pro-inflammatory” and “anti-inflammatory” activities have been described [67]. The CRP is capable of activating complement, opsonizing bacteria, fungi and parasites and agglutinating particles [68]. In the present study, CRP expression in the HC group was highest, and the expression in the HL group was higher than that in the

control. But the increase in CRP expression was minor. Typically, when the fish is infected by pathogen bacteria, CRP expression in the liver upregulated by hundreds to a thousand time [69]. Dietary fatty acids may affect inflammatory processes through the change of body weight and adipose tissue mass. The high-carbohydrate diet can lead to excessive protein glycation and activation of oxidative stress and inflammation [70]. A minor increases in CRP levels is associated with a number of physiological conditions with out inflammation symptoms [67].

Heat shock proteins (HSPs) are a suite of highly conserved proteins with varying molecular weight (16–100 kDa) [71]. They are a well-known group of stress proteins which can be triggered by environmental stress, such as microbial infection, hypoxia, starvation and water deprivation. HSP70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins [72]. Its content also changes with diet compositions [73]. HSP90 is active in supporting various components of the cytoskeleton and steroid hormone receptors [74,75], therefore it can form the first line of defense against heat stress [76]. Their production is up-regulated under various conditions of stress and they are released by stressed, infected, necrotic and neoplastic cells, but not by apoptotic cells [77]. Studies on the relationship between HSPs expression and dietary composition in fish are relatively few, but in rats, high-fat and high-sugar diets could induce obesity and reduce the expression of HSPs. In addition, the increase of HSP expression can prevent or soften the damage caused by obesity due to the intake of a hyperlipidic diet, leading to improvement in insulin sensitivity, glucose tolerance and inflammation reduction [78]. In the present study, the decline of HSP70 and HSP90 expressions in the HL and HC groups was synchronized, which supports a hypothesis that the dietary protein can affect the expression of HSPs. The digestion of different types of dietary protein can generate peptides with different effects on metabolism, including the expression of HSPs [79]. In the present study, the dietary protein was from fish meal and soybean meal with a different proportion. However, the effects of protein source and ratio on protein metabolism need further research.

5. Conclusion

This study shows that when barramundi were fed with the same amount of energy, the energy source had little effect on the growth and hepatic histological parameters. In antioxidant enzyme activities and gene expressions relevant to stress response, the HL diet enabled fish to gain a strong antioxidant ability and stress resistance. However, in the expression of immune-related genes, lipids and carbohydrates evoked inflammatory response in barramundi, and the inflammatory response by the carbohydrate diet was stronger. Therefore, between the two types of non-protein energy source, the high lipid diet has less effect on immunity but is more suitable as a non-protein energy source in formulating barramundi diets.

Acknowledgements

This work was supported by Guangxi Innovation Driven Development Special Fund Project [Guike AA18242031], Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS [2017ZD01, 2018ZD01], and Key Research and Development Project of Hainan Province [ZDYF2017036, ZDYF2018096].

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