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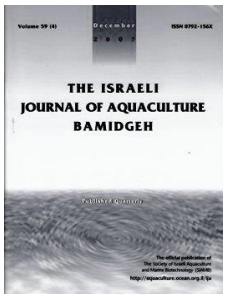

Effects of Dietary Corn Gluten Meal on Growth, Immunity, Enzyme Activity, and Protein Metabolism in Relation to TOR Gene Expression in Juvenile Large Yellow Croaker *Larimichthys crocea*

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Keywords: *Larimichthys crocea;* corn gluten meal; growth; immunity; digestive enzyme; TOR gene expression

Abstract

A series of six isonitrogenous (45% crude protein) and isolipidic (10% crude lipid) diets were formulated by replacing 0% (control), 15%, 30%, 45%, 60% and 75% of fish meal (FM) with corn gluten meal (CGM), including 40% FM. In addition, all diets were supplemented with crystalline amino including lysine and methionine in all diets except the control. Results showed that there were no significant effects on survival, weight gain ratio (WGR), specific growth rate (SGR), and feed conversion ratio (FCR) among all dietary diet groups (P>0.05). There were also no significant differences (P > 0.05) among all diets in the serum immune enzyme, Alkaline Phosphatase (AKP), Lysozyme (LYZ) and Complement 3 (C3), but in Complement 4 (C4), there was a significantly higher content in C75 compared with the other diets (P < 0.05). There were no significant differences in alpha-amylase activities of digestive enzymes in hindgut among the dietary treatments (P > 0.05), while the activities of lipase were significantly lower in C0 than in the other groups (P < 0.05). There were no significant differences in trypsin activity in fish fed the C15, and C75 diets than the control group (P > 0.05). There were no significant differences (P > 0.05) among all the diets in aspartate amino transferase (AST), but in alanine aminotransferase (ALT) the activities of C75 diet were significantly lower compared with the other diets (P < 0.05). mRNA expression levels of target of rapamycin (TOR) in dietary groups were significantly lower than the control group (P < 0.05), however, there were no significant differences in C15 to C75 groups (P > 0.05).

Results of the present study suggested that CGM could substitute up to 75% of FM without influencing the growth of juvenile *Larimichthys Crocea*.

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Introduction

Protein requirement studies on fish are crucial to aquaculture as world aquaculture depends on fish meal (FM) as the main protein source in aqua feeds (Perera and Yúfera, 2016). It is generally known that FM is the most important ingredient for aquaculture feeds due to its high protein quality and more balanced amino acids composition, higher vitamins, minerals, and essential n-3 fatty acids (Bendiksen et al., 2011). However, decades of wild fisheries stagnation and limited availability of marine products has increased pressure for the introduction of alternative dietary sources of protein (FAO 2008-2015).

In recent years, some studies have been tested to derive plant alternative protein sources for FM, such as from grains, pulses, and oilseeds (Gatlin et al., 2007). Some of the main problems in utilization of plant protein sources are unbalanced amino acid composition and the anti-nutritional factors (ANFs) in most plant protein (Li and Robinson, 2006). For example, corn gluten meal (CGM) has high protein content (60%-70% dry matter), is low in fiber, and rich in vitamins B and E. It also contains no ANFs (Luo et al., 2013) and has been widely studied (Yigit et al., 2012). However, CGM is deficient in amino acids, such as lysine, methionine, and arginine (Pereira and Oliva-Teles, 2003). Thus, in this study, where CGM was included, supplemented lysine and methionine were required.

Large yellow croaker (*Larimichthys Crocea*) is a representative species of Perciformes, Sciaenidae, distributed in the Yellow sea, East China sea and South sea, which constitute one of the four most important marine fisheries in China. Farming of large yellow croaker boomed after the success of their reproduction in fish hatcheries in the late 1980s (Lin et al., 1991). The formulation of a nutritionally adequate and cost-effective feed is most important for its successful culture. There are some studies on the nutrition for juvenile large yellow croaker (Ai et al., 2008; Xie et al., 2011, 2012; Yu et al., 2012). The present study was designed to determine the influence of replacement fish meal with corn gluten meal on growth, immunity, enzyme activities, and expression of protein metabolism related gene in juvenile large yellow croaker.

Materials and Methods

Diet preparation and fish culturing.

Six isonitrogenous (45% crude protein) and isoclipidic (10% crude lipid) practial diets were formulated to meet the protein and lipid requirements of juvenile large yellow croaker (Duan et al., 2001). The experimental diets contained FM, corn gluten meal (CGM; Ningbo, China), and wheat gluten meal, soybean (Ningbo, China) as major protein sources, fish oil and soybean oil as well as soybean lecithin as the lipid source, and wheat-starch as the carbohydrate source, crystalline amino acids such as lysine and methionine were supplemented, and FM was replaced at increasing levels: 0 (diet C0), 15% (diet C15), 30% (diet C30), 45% (diet C45), 60% (diet C60) and 75% (diet C75). The ingredients and proximate composition of diets are presented in Table 1. The production progress and fish culturing were referenced to Wang *et al.*, 2017.

Analyses and measurement.

After the 56-day feeding trial, the total number and mean body weight of fish in each tank was determined after the fish were fasted for 24h before harvest. Another four fish from each cage were anesthetized with eugenol (1:10000), blood was collected from the caudal vine with a 1-mL heparinized syringe and centrifuged at 4000g for 10 min at 4°C and immediately stored at -80°C until analysis. Samples of hind-intestine were excised to measure digestive enzyme activities. Samples of liver were collected to measure gene expression.

The serum immune enzymatic activities, hindgut digestive, and liver protein metabolism enzymatic activities, and real-time quantitative polymerase chain reaction (TOR) were referenced to (Wang *et al.*, 2017).

Table 1. Formulation and proximate composition (% dry weight) of six experimental diets fed to juvenile large yellow croaker.

Ingredients	*Diets*					
	C0	*C15*	*C30*	*C45*	*C60*	*C75*
Fish meal（Danish）	40.00	34.00	28.00	22.00	16.00	10.00
Corn gluten meal	0.00	6.84	13.69	20.53	27.37	34.21
Wheat gluten meal	10.55	10.55	10.55	10.55	10.55	10.55
Soybean meal	13.00	13.00	13.00	13.00	13.00	13.00
Wheat-starch	15.00	15.00	15.00	15.00	15.00	15.00
Fish oil	1.96	2.20	2.44	2.86	2.91	3.34
Soybean oil	1.96	2.20	2.44	2.86	2.92	3.34
Soy bean lecithin	1.50	1.50	1.50	1.50	1.50	1.50
Vitamin premix[a]	3.00	3.00	3.00	3.00	3.00	3.00
Mineral premix[b]	2.00	2.00	2.00	2.00	2.00	2.00
Lys	0.00	0.25	0.49	0.74	0.99	1.24
Met	0.00	0.07	0.14	0.20	0.27	0.34
Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30
Monocalcium phosphate	1.50	1.50	0.07	1.50	1.50	1.50
Attractant[c]	0.30	0.30	0.30	0.30	0.30	0.30
Cellulose	8.93	7.29	7.09	3.66	2.39	0.38
Total	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition (% Dry matter)						
Crude Protein	45.26	45.28	44.63	46.21	45.99	46.26
Crude Lipid	10.54	10.73	10.65	11.34	10.61	11.23
Ash	10.38	9.54	8.39	7.70	7.13	6.39

[a] Supplied the following (mg/kg diet): thiamin 25 mg; riboflavin, 45 mg; pyridoxine HCL, 20 mg; vitamin B 12, 0.1 mg; vitamin K 3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg, biotin, 1.20 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; alpha-tocopherol, 120 mg; ascorbic acid, 2000 mg; choline chloride, 2500 mg, ethoxyquin 150 mg, wheat middling 14.012 g.

[b] Supplied the following (mg/kg diet): NaF, 2 mg; KI, 0.8mg; $CoCl_2.6H_2O$ (1%), 50mg; $CuSO_4.5\ H_2O$, 10mg; $FeSO_4.H_2O$, 80mg; $ZnSO_4.H_2O$, 50mg; $MnSO_4.H_2O$, 60mg; $MgSO_4.7H_2O$, 1200mg; $Ca(H_2PO_3)_2.\ H_2O$, 3000mg; Nacl,100mg; Zoelite, 15.447g.

[c] Supplied the following (% diet): glycine and betaine (1:2).

Statistical analysis.

The following variables were calculated:

Survival (%) =100 × (final number of fish)/(initial number of fish)

Weight Gain Ratio (WGR, %) = 100 × (W_t - W_0)/W_0

Specific growth rate (SGR, %/day) =100 × ($\ln W_t$-$\ln W_0$)/t

Feed conversion ratio (FCR, %) = dry feed intake/(W_t - W_0)

W_t and W_0 represents the final and initial weight of juvenile large yellow croaker, while t represents rearing days.

All data were subjected to analysis of variance using SPSS 17.0 for Windows. Differences among the means were tested by Tukey's multiple range tests. The level of significance chosen was $P<0.05$.

Results

Growth performance. The effects of different diets on juvenile large yellow croaker are presented in Table 2. Compared with fish fed the C0 diet, none of the experimental diets had a significant effect on Survival and WGR, SGR, FCR ($P>0.05$).

Table 2. Effects of CGM on the growth performance of juvenile large yellow croaker.*

Dietary	*Survival(%)*	*WGR(%)*	*SGR(%/day)*	*FCR*
C0	97.22±2.55	318.00±0.01	2.55±0.01	0.98±0.03
C15	96.67±3.33	320.00±0.15	2.56±0.07	0.99±0.02
C30	93.89±4.81	299.00±0.17	2.47±0.08	1.09±0.14
C45	96.11±3.47	317.00±0.21	2.55±0.09	1.00±0.06
C60	98.33±1.67	306.00±0.16	2.50±0.07	0.99±0.02
C75	99.44±0.96	298.00±0.05	2.47±0.02	1.03±0.02

Where WGR = Weight gain ratio, SGR = Specific growth rate, and FCR = Feed conversion ratio. *Data are means ± S.D. (n = 3). Values in the same column with different superscripts represent significant difference ($P< 0.05$), Values in the same row with same or no superscripts are not significant difference ($P > 0.05$). The same are as follows.

Serum immune response.

Serum immune enzyme AKP increased and then decreased with increasing protein levels, but there were no significant differences among diets ($P > 0.05$). LYZ decreased and then increased with increasing protein levels, but there were no significant differences among the diets ($P < 0.05$). There were no significant differences among diets of C3 ($P > 0.05$), but in C4, there was a significantly higher ($P < 0.05$) content in C75 compared with the other diets (Table 3).

Table 3. Effects of CGM on immune response of juvenile large yellow croaker.

	Dietary					
	C0	*C15*	*C30*	*C45*	*C60*	*C 75*
AKP (Um/L)	20.89±0.58	18.09±0.25	16.29±4.66	18.01±1.41	36.16±0.00	34.95±2.76
LYZ (Um/L)	179.21±8.40	105.75±3.10	100.59±1.56	94.69±3.54	101.33±0.45	132.30±15.49
C3(Um/L)	0.22±0.00	0.36±0.12	0.31±0.07	0.45±0.21	0.14±0.04	0.40±0.02
C4(Um/L)	0.25±0.01^{ab}	0.28±0.01^{ab}	0.14±0.02^{a}	0.17±0.00^{ab}	0.52±0.18^{b}	1.01±0.17^{c}

Where AKP = Alkaline Phosphatase, and LYZ = Lysozyme.

Hindgut digestive enzyme and liver protein metabolism enzyme activities.

There were no significant differences in alpha-amylase activities of digestive enzyme in hindgut among the dietary treatments ($P > 0.05$), while the lipase activity was significantly lower in C0 than in the other groups ($P < 0.05$). Trypsin activity in fish fed C15 and C75 showed no significant differences compared to the control group ($P > 0.05$) while in C30 it was significantly lower than in the control group ($P<0.05$), but in C45, C60 it was significantly higher than in the control group ($P < 0.05$). Liver protein metabolism enzymes were not significantly different among all the diets. There were no significant differences among all the diets in aspartate amino transferase (AST) ($P > 0.05$), but in alanine aminotransferase (ALT) the activities of C75 diet were significantly lower diets ($P < 0.05$) compared with the other diets (Table 4).

Table 4. Effects of CGM on alpha-amylase, lipase, and trypsin of juvenile large yellow croaker.

Enzyme	*Diets*					
	C0	*C15*	*C30*	*C45*	*C60*	*C75*
Alpha-amylase (Um/g protein)	0.04±0.00	0.04±0.01	0.05±0.00	0.04±0.00	0.04±0.00	0.04±0.0
Lipase (U/g protein)	93.78±38.60^{a}	676.43±32.60^{b}	353.06±20.68^{c}	464.41±145.49^{bc}	528.55±4.65^{bc}	635.21±10.98^{b}
Trypsin (U/g protein)	2.70±0.07^{a}	2.94±0.01^{a}	1.45±0.02^{b}	5.47±0.19^{c}	4.13±0.04^{d}	3.05±0.02^{a}
AST (U/g protein)	154.47±67.47	50.48±14.64	75.66±38.71	167.12±2.99	178.28±19.43	136.4±84.33
ALT (U/g protein)	263.82±3.66^{a}	226.95±17.00^{a}	273.06±9.40^{a}	286.60±7.83^{a}	236.93±56.67^{a}	108.85±14.88^{b}

Where AST = Aspartate amino-transferase, and ALT = Alanine aminotransferase.

Expression of TOR gene in the liver.

Relative mRNA expression of TOR gene in the liver of juvenile large yellow croaker is presented in Figure 1. mRNA expression level of TOR in dietary groups was significantly lower than the control group ($P < 0.05$) by about 0.020-fold, 0.045-fold, 0.026-fold, 0.012-fold, 0.020-fold, respectively. However, there were no significant differences in the groups from C15 to C75 ($P > 0.05$).

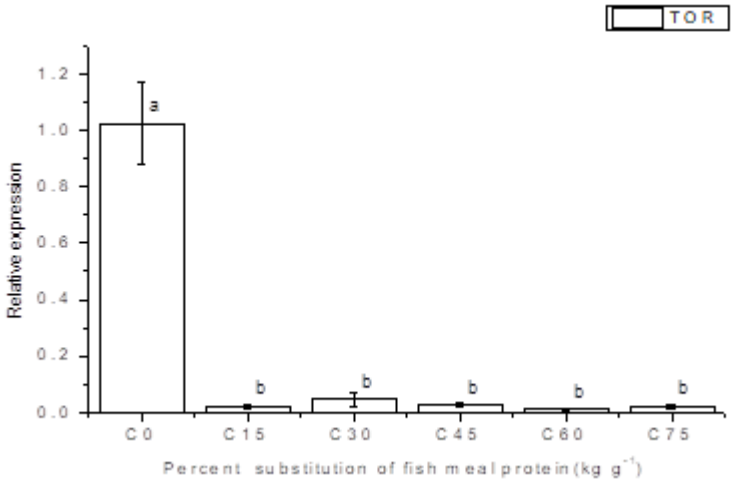

Fig.1. Proximal TOR mRNA expression for diets.

Discussion

CGM is known not to contain anti-nutritional factors (ANFs), and it also lowers costs (Regoast et al.,1999). However, the effect of CGM varies with species, such as *Sparus aurata L*. which can utilize 60% CGM without adverse effects on growth (Pereira et al.,2003), while results with turbot (*Psetta maxima*) suggest that protein from CGM can replace one third of FM in diets (Regost et al., 1999). In the current study, there were no significant differences in survival, SGR, WGR, and FCR of juvenile large yellow croaker. This result is not in line with results found in Japanese flounder (*Paralichthys olivaceus*). Results in fish fed the diet with 60% FM protein were significantly lower than the control diet (Kikuchi et al., 1999). These inconsistent results could be attributed to a dietary amino acid deficiency in the diet. In this study, apart from the control group, the diets of the other groups were supplemented with appropriate amounts of crystal lysine and methionine to meet growth requirements.

The innate immune system of fish is the first line of defense (Khosravi et al., 2011). In the present study, serum LYZ content decreased and then increased, whereas, there were no significant differences in all diets. This is similar to results with juvenile gilthead sea bream (Kokou et al., 2012). Complement C3 and C4 play an important role in activating the immune system by tagging the foreign molecules using their internal thioester bond that reacts with nearby hydroxyl or amino groups to form a covalent bond (Dodds and Law, 1998). In our paper, there were no significant effects in C3 among all the diets, but there were significant differences in C75 compared with the control group in C4, whereas C3 content was the highest in the C45 diet, suggesting that appropriate levels of CGM could improve immune function.

Digestive enzymes play a key role in digesting nutrients; they can affect growth and health of fish (Shan et al., 2008). The wheat-starch content in the control diet was identical to that of all the other diets and there were no significant differences in alpha-amylase activity. In our case, when the substitution level was equal to 45%, the trypsin activity was significantly higher compared to the control group. When the substitution levels were equal to or above 15%, lipase activity was significantly higher compared with the control group. These findings differ from previous studies on juvenile meager where there was decreased protease and lipase activity in fish fed CSGM150 or CSGM225, while there was no significant effect in amylase activity (Couto et al., 2016).

In the present study, there were no significant differences in AST activity in all dietary groups. But ALT activity was reduced when the dietary replacement reached 75%. This suggests that lower dietary protein damaged the liver to some extent. This result was similar to the findings of Cheng et al., (2010). Lysine, arginine, and methionine are the limiting amino acids in CGM (Pereira and Oliva-Teles, 2003). In the present study unbalanced amino acid absorption may partially explain the relatively lower growth rate of fish fed high plant protein-based diets.

mRNA expression of TOR in fish from the C15 to C75 groups was not significantly affected by these diets. Compared to the control, it was significantly lower and different diets had no effect on the transcription level of TOR gene in fish (Li et al., 2013; Luo et al., 2012). This is probably due to regulation of the TOR gene expression which is controlled by the translation and/or post translation level rather than the transcription level. It was reported that kinase activity of the TOR protein was regulated by a variety of factors, such as phosphorylation (Lansard et al., 2009). However, there is no direct evidence that dietary CGM levels can affect TOR protein levels in the liver of juvenile large yellow croaker. This needs to be verified in further studies.

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