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Nutritional profiling reveals the effects of dietary zeolite on Asian seabass *Lates calcarifer*

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ABSTRACT

As costs for nutrition in aquaculture farming escalate, using affordable feeds that provide an optimal combination of nutrients for the fish species of choice is paramount. Natural zeolites have been applied in aquaculture as early as 1977 including feeds in animal nutrition, and moisture and ammonia control of animal manure. In this study, five experimental diets: control, Z1, Z2, Z4 and Z8 containing 0, 1, 2, 4 and 8% of dietary zeolite respectively were investigated for growth performance and immune responses in *Lates calcarifer*. In general, dietary zeolite supplementation boosted overall growth performance, particularly with Z4 of 4% zeolite supplementation showing the highest Body Weight Gain (BWG) over the other diets. Feed Conversion Ratios (FCR) and Protein Efficiency Ratio (PER) of fish were found to be significantly improved ($P < 0.05$) by the supplementation of zeolite. Furthermore, no significant changes in the immune and stress responses of fish at both 4% and 8% zeolite supplementation were exhibited, indicating that the presence of zeolites did not impair the immune state of *L. calcarifer*. This study suggests the suitability of dietary zeolite supplementation and recommends 4% zeolite supplementation to be the most ideal for improved growth performance for *L. calcarifer*.

Keywords: dietary zeolite, *Lates calcarifer*, growth performance, immune response

INTRODUCTION

The development of a suitable fish feed does not only include excellent growth and nutritional properties as well as economical, it must also be safe for the fish to consume without any side effects. An important parameter to be taken into consideration is that the added feed elements do not result in an impaired fish immune state. In all developmental stages of fish, immunity is an essential biological system which provides protection against microorganisms and their products or from the effects of toxic eternal substances via an immune response comprising specialized cells which eliminates the invading pathogens or foreign 'non-self' substances from the host fish (Delves et al., 2016). Immunity is critical for fish survival and growth, however environmental factors such as pathogens and toxic chemicals can threaten the immunity of the fish and result in immunotoxicity and ultimately death (Segner et al., 2011).

Zeolites, also known as clinoptilolite, are minerals of volcanic origin made from crystalline aluminosilicates. Zeolites are useful in agriculture, environmental management as well as industrial chemistry for many purposes: as an absorbent for fertilizers to remain longer in the soil without leaching, as soil pH neutralizer, for cationic ion exchange in groundwater purification as well as additives and deodorizers. Natural zeolites have also been applied in animal science and aquaculture as early as 1977 (Mumpton & Fishman, 1977) with various uses including as feeds in animal nutrition, moisture and ammonia control of animal manure as well as the purification of recirculated hatchery waters in aquaculture tanks. The addition of zeolites has been very efficient against the malodour of animal wastes as well as increased feed efficiency by as much as 25% (Mumpton, 1999). Supplementation of zeolite as fish feed to some fish species such as catfish and salmon has shown positive effects by increasing growth performance, better feed utilization and improving the general health of the fish (Obradović et al., 2006; Khodanazary et al., 2013; Kanyılmaz et al., 2015; Ghiasi & Jasour, 2012).

The Asian seabass (*Lates calcarifer*), commonly known as Barramundi or 'Siakap' (in Malaysia) is a large euryhaline fish species that is widely distributed in the Indo-Pacific regions. They are important aquaculture species which are usually cultured in ponds, cages and recirculating tanks in both fresh and seawater. In 2014, the annual global aquaculture production of this species was recorded at more than 70 thousand tonnes, where Thailand, Indonesia, Malaysia and Taiwan Province of China are the primary producers (FAO, 2016). The Asian seabass is a carnivorous fish species and require about 45 to 55% protein for its optimum growth [juvenile stage of less than 200 g] (Glencross et al., 2016). The high protein requirement of this species directly makes them costly to be produced. Numerous studies have been conducted to optimize the growth performance and feed utilization of this species by either replacing some or the total amount of the protein and lipid source from fish-based to plant sources, incorporating dietary prebiotics or improving feeding technique by pre-soaking feed pellet (Irvin et al., 2016; Wattanakul et al., 2017; Glencross et al., 2016; Ali et al., 2016; Boonyaratpalin et al., 1998).

This study investigated zeolites as a potential fish feed alternative for the Asian seabass *L. calcarifer* and their effects on growth performance, body composition and immune responses. The main focus of this study was the effects of dietary zeolites on 1) the growth and nutritional necessities of the fish and 2) to determine if the addition of the feeds caused any undesirable immune response to *L. calcarifer* juveniles

MATERIALS AND METHODS

Experimental Diets

Five experimental diets were formulated to be isoproteic (45% crude protein) and isolipidic (14% crude lipid). Natural zeolite was supplemented into each diet at 0, 1, 2, 4, and 8% (w/w) and these diets were labelled as control, Z1, Z2, Z4, and Z8, respectively as seen in Table 1. For the diet preparation, all dry ingredients were finely ground and thoroughly mixed with fish oil. Water was then added to produce moist dough and screw-passed through a 3-mm die. The strands of feeds were oven dried at 45°C for 6 hours. After drying, the strains were broken up, kept in a zip-lock plastic bag and stored in a freezer at −20°C until use. The proximate composition of the experimental diets was determined according to the standard method for analysis of animal feed (AOAC, 1997).

Table 1 Ingredients of experimental diets (g/100 g dry weight)

Ingredients (per 100 g)	Diets (g)				
	Control	Z1	Z2	Z4	Z8
Fish meal[a]	46.3	46.3	46.3	46.3	46.3
Soybean meal	18.4	18.4	18.4	18.4	18.4
Tapioca starch[b]	12.4	12.4	12.4	12.4	12.4
Alphaa-Cellulose	8.0	7.0	6.0	4.0	0.0
CMC[c]	1.5	1.5	1.5	1.5	1.5
Vitamin premixed[d]	3.0	3.0	3.0	3.0	3.0
Mineral premixed[e]	1.0	1.0	1.0	1.0	1.0
Dicalcium phosphate	1.0	1.0	1.0	1.0	1.0
Zeolite	0.0	1.0	2.0	4.0	8.0
Fish oil	8.4	8.4	8.4	8.4	8.4

[a] Danish fish meal, TripleNine 999 Fish meal, Denmark. [b] Golden Fish brand. Bake with Me Sdn. Bhd., Malaysia.[c] Carboxymethyl cellulose (CMC), Sigma. [d] Vitamin mixture (g/kg mixture): Inositol, 5.0; choline chloride, 75.0; niacin, 5.5; riboflavin, 1.0; pyridoxine HCl, 1.0; thiamine HCl, 0.92; d-calcium panothenate, 3.0; retinyl acetate, 0.60; vitamin D3, 0.083; Menadione, 1.67; DL alpha-tocopherol acetate, 8.0; d-biotin, 0.02; folic acid, 0.09; vitamin B12, 0.00135. All ingredients were diluted with alpha-cellulose to 1 kg. [e] Mineral mixture (g/kg mixture): Calcium phosphate monobasic, 270.98; Calcium lactate, 327.0; Ferrous sulphate, 25.0; Magnesium sulphate, 132.0; Potassium chloride, 50.0; Sodium chloride, 60.0; Potassium iodide, 0.15; Copper sulphate, 0.785; Manganese oxide, 0.8; Cobalt carbonate, 1.0; Zinc oxide, 3.0; Sodium selenite, 0.011; Calcium carbonate, 129.275.

Feeding Trial and Experimental Design

The feeding trial was conducted over six weeks at the Fish Hatchery of the Borneo Marine Research Institute, Universiti Malaysia Sabah (UMS), Malaysia. Juvenile Asian seabass (n = 225) with an average body weight of 0.84 ± 0.04 g (mean ± standard deviation) and a total length of 4.14 ± 0.10 cm were used in this study. The fish were randomly distributed into 15 fibreglass tanks with a dimension of 30 cm × 30 cm × 30 cm (27 L) at a stocking density of 15 individuals per tank. Fish were cultured in a flow-through seawater system (1 L/min) and each tank was supplied with aeration. Each dietary treatment was fed to triplicate tanks of fish, thrice a day at 0800, 1200, and 1600 hours, respectively. The fish were fed until apparent satiation in each feeding session. The amounts of feed consumed and fish mortality, if any, were recorded every day for the calculation of feed utilization efficiency and survival. The bulk weight of the fish from all dietary treatments was measured once every two weeks.

At the end of the feeding trial, all the fish were placed on a fasting diet for 24 hours and weighed individually. Six primary nutritional and growth parameters of the fish were determined: Body Weight Gain (BWG), Specific Growth Rate (SGR), Survival, Feed Intake (FI), Feed Conversion Ratio (FCR) and Protein efficiency ratio (PER) using the following formula:

1. Body weight gain (BWG) (%) = [(Final weight (g) – Initial weight (g)/Initial weight (g)] ×100
2. Specific growth rate (SGR) (%/d) = [ln (Final weight (g)) – ln (Initial weight (g))/ days] ×100
3. Survival (%) = (Final fish number – Initial fish number)/Initial fish number × 100
4. Feed Intake (FI) (g) = Total feed intake for 14 weeks
5. Feed conversion ratio (FCR) = Dry feed consumed (kg)/wet weight gain (kg)
6. Protein efficiency ratio (PER) = Gain in body mass (g)/protein intake (g)

Other parameters determined in this study include those related to the general health state of the fish which are Condition Factor (CF), Hepatosomatic Index (HSI) and Viscerosomatic Index (VSI) with the following formula:

1. Condition Factor (CF): 100 – (Body Weight [g] / Total length [cm]^3)
2. Hepatosomatic Index (HSI): 100 – (Liver weight [g] / Body weight [g])
3. Viscerosomatic Index (VSI): 100 – (Viscera weight [g] / Body weight [g])

Immune Gene Profiling by qPCR

Isolation of Total RNA and cDNA synthesis

At the end of the six-week feeding trial, three fish from diets Z4 and Z8 from each tank were selected and killed by an overdose of 0.25 ml stabilizer solution (Nika, Transmore), 30 mg of fish tissues were then excised, weighed and placed into mortar containing liquid nitrogen and immediately crushed and ground thoroughly with a pestle. The ground samples were then transferred into 1.5 ml microcentrifuge tubes and RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Three technical replicates were performed from control, 4% and 8% of zeolite treatments. The RNA integrity and concentration were determined using the Nanodrop spectrophotometer. cDNA was synthesized using 10 ng of total RNA for each treatment using the ReverTra Ace qPCR RT kit (Toyobo) according to the manufacturer's protocol. The synthesized cDNA samples were stored at –80°C freezer for further analysis.

qPCR Analysis of Immune Genes

Twenty per cent (v/v) of the cDNA solution from the ReverTra Ace qPCR RT kit was assayed in a real-time PCR reaction using primers as outlined in Table 2. The qPCR analysis was performed using Thunderbird® SYBR® qPCR Mix according to the manufacturer's protocol and analyzed in a real-time cycler (Bio-Rad). Briefly, 20 µl of reaction was initially denatured according to the manufacturer's protocol (Thunderbird® SYBR® qPCR Mix and Bio-Rad CFX96 RT-PCR Detection System) at 95°C for 3 min and then amplified for 40 cycles (95°C, 15 s, 60°C, 30 s and 72°C, 30 s). Data were normalized to the reference gene, 18S rRNA (Mohd Shaharuddin et al., 2013). A no-template control sample (NTC) was tested to ensure the qPCR master mix has no contamination.

Oligonucleotide Primers

The oligonucleotide primers used in this study are Elongation factor-1α (Ef1α), Complement 3 (C3), Heat shock protein kDa70 (HSP70), Heat shock protein kDa90 (HSP90), Perforin, Serum amyloid A, (SAA) and 18S rRNA housekeeping gene. The genes are listed in Table 2.

Table 2 Primers used in qPCR

No.	Genes	Sequences (5' – 3')	References
1	18S rRNA, 18S	F: TGGTTAATTCCGATAACGAACGA R: CGCCACTTGTCCCTCTAAGAA	Mohd Shaharuddin et al., 2013
2	Elongation factor-1α, Ef1α	F: AAATTGGCGGTATTGGAAC R: GGGAGCAAAGGTGACGAC	Mohd Shaharuddin et al., 2013
3	Complement 3, C3	F: GCAATCCTCCACAACTACAG R: ACTCTGACCTCCTGACGATAC	Mohd Shaharuddin et al., 2013
4	Heat shock protein kDa70, HSP70	F: AAGGCAGAGGATGATGTC R: TGCAGTCTGGTTCTTGTC	Mohd Shaharuddin et al., 2013
5	Heat shock protein kDa90, HSP90	F: ACCTCCCTCACAGAATACC. R: CTCTTGCCATCAAACTCC	Mohd Shaharuddin et al., 2013
6	Perforin	F: CTCCAGGTGCCAATCTG R: TGTCCTTGCGTTTCCAC	Mohd Shaharuddin et al., 2013
7	Serum amyloid A, SAA	F: TTGTTTGTGGCAGGAGTTGTTC R: TCTGGGCAGCATCATAGTTCC	Mohd Shaharuddin et al., 2013

Statistical Analysis

All data obtained were subjected to one-way analysis of variance (ANOVA) using the computer program IBM SPSS Statistics version 22 for Windows. Homogeneity of variance was tested using Levene's test, and multiple comparisons among treatments were performed using Tukey's multiple range test. Significant differences were assumed when $P < 0.05$. Statistical analyses for RT-PCR were carried out using a one-way analysis of variance using NCSS Statistical software.

RESULTS

Nutritional Profiling Analyses

As seen in Table 3, the growth performance of Asian seabass fed with different levels of dietary zeolite presented in Table 3 shows the proximate compositions of graded zeolite diets of Z1, Z2, Z4 and Z8 diets for moisture, protein, lipid and ash for were not significantly different from the control non-zeolite diet.

Table 3 Proximate compositions of experimental diets

Diet	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Control	7.84±0.39	44.51±0.20	12.58±1.29	11.20±0.17
Z1	7.34±0.17	45.25±0.21	12.33±0.61	11.28±0.06
Z2	7.38±0.19	43.94±0.79	11.58±0.26	11.35±0.10
Z4	7.92±0.21	43.38±0.45	11.51±0.08	11.58±0.21
Z8	7.29±0.28	43.91±0.68	12.75±0.93	12.07±0.16

As seen in Table 4, at the end of the feeding trial Z2, Z4 and Z8 showed higher BWG than that of the control group (738.60±30.71%). Z4 showed the highest BWG in all groups tested. The BWG of Z1 was significantly lower than Z4 (950.10±144.05%) and Z1 (654.40±45.16%). However, when compared to the control, this reduction was not statistically significant. For SGR and percentage survival, a similar trend was observed. Apart from Z1 (3.61±0.11%/d), the SGR of Z2, Z4 and Z8 were higher than the control group (3.80±0.06%/d) however the differences for all groups were not statistically significant. The percentage of fish survival was determined to be between 95.56 – 100 %, fish fed Z1 was also found to show the lowest survival percentage compared to the control group, while Z4 and Z8 showed 100% survival percentages. The survival percentage of Z2 was similar to the control group at 97.78±3.85%. The survival percentages between Z1, Z2, Z4, Z8 and control were not found to be statistically different.

Table 4 Growth performances of juvenile Asian seabass

Diet	Initial Body Weight IBW(g)	Final Body Weight FBW(g)	Body Weight Gain [BWG] (%)	Specific Growth Rate [SGR] (%/d)	Survival (%)
Control	0.88±0.02	7.38±0.18^{ab}	738.60±30.71^{ab}	3.80±0.06	97.78±3.85
Z1	0.86±0.03	6.46±0.31^{a}	654.40±45.16^{a}	3.61±0.11	95.56±7.70
Z2	0.82±0.04	7.82±0.48^{ab}	859.27±81.67^{ab}	4.03±0.15	97.78±3.85
Z4	0.80±0.02	8.44±1.23^{b}	950.10±144.05^{b}	4.19±0.15	100.00±0.0
Z8	0.82±0.06	8.01±0.77^{ab}	872.14±47.93^{b}	4.06±0.09	100.00±0.0

Significant differences are indicated by different superscripted letters between each column (P<0.05).

As seen in Table 5, after six weeks, no significant difference was detected in the total FI of fish, with the fish-fed control diet gaining the highest total FI (10.75 g/fish) (Table 5). The best FCR (the lowest FCR ratio) was observed in diet Z8 (1.20) and this value was significantly better compared to Control (1.66), Z1 (1.76), and Z2 (1.35) diets, respectively. Although the FCR of diet Z8 was highest, it not significantly different from Z4. A similar trend was also observed for PER where the best PER value (highest PER ratio) was observed for Z8 (1.90±0.00) and was statistically higher than the control (1.33±0.00), Z1 (1.20±0.07) and Z2 (1.65±0.06).

Table 5 Feed utilization of Asian seabass

Diet	Total Feed Intake [FI] (g/fish)	Feed Conversion Ratio [FCR]	Protein Efficiency Ratio [PER]
Control	10.75±0.31	1.66±0.06^{c}	1.33±0.00^{a}
Z1	9.90±0.87	1.76±0.06^{c}	1.20±0.07^{a}
Z2	9.49±0.88	1.35±0.03^{b}	1.65±0.06^{b}
Z4	9.70±1.26	1.27±0.04^{ab}	1.81±0.03^{bc}
Z8	8.61±0.89	1.20±0.00^{a}	1.90±0.00^{c}

Significant differences are indicated by different superscripted letters between each column (P<0.05).

As seen in Table 6, the body indices of fish supplemented with Z1, Z2, Z4 and Z8 diets were not significantly different from the control non-zeolite diet for Hepatosomatic Index (HSI), Visceral Somatic Index (VSI) and Condition Factor (CF).

Table 6 Body indices of Asian seabass

Diet	Hepatosomatic Index (HSI)	Visceral Somatic Index (VSI)	Condition Factor (CF)
Control	1.65±0.15	9.85±0.40	1.19±0.03
Z1	1.55±0.09	9.43±1.42	1.22±0.04
Z2	1.61±0.07	9.91±1.02	1.20±0.02
Z4	1.60±0.04	9.67±0.60	1.16±0.03
Z8	1.60±0.13	9.87±1.21	1.22±0.01

Immunogenetic Response Profiling Analysis

As seen in Figure 1, for Z4 (4% zeolite supplementation), the expressions of all immune genes analysed were observed to be downregulated compared to the control. At Z8 (8% zeolite treatment), *HSP70, SAA* and *perforin* genes were observed to be upregulated as seen in Figure 1 with fold change values of 1.25, 1.16 and 1.58 respectively; while relative expression of genes *HSP90, Ef1a* and *C3* at 8% zeolite treatment were downregulated. Despite variations in gene expression observed between 4% and 8% zeolite treatment, no significant differences in the relative expression of target genes were observed when a One-Way Analysis of Variance, Turkey-Kramer's multiple comparison tests and the P-value were carried out between the zeolite treatments and control (see Appendix 1).

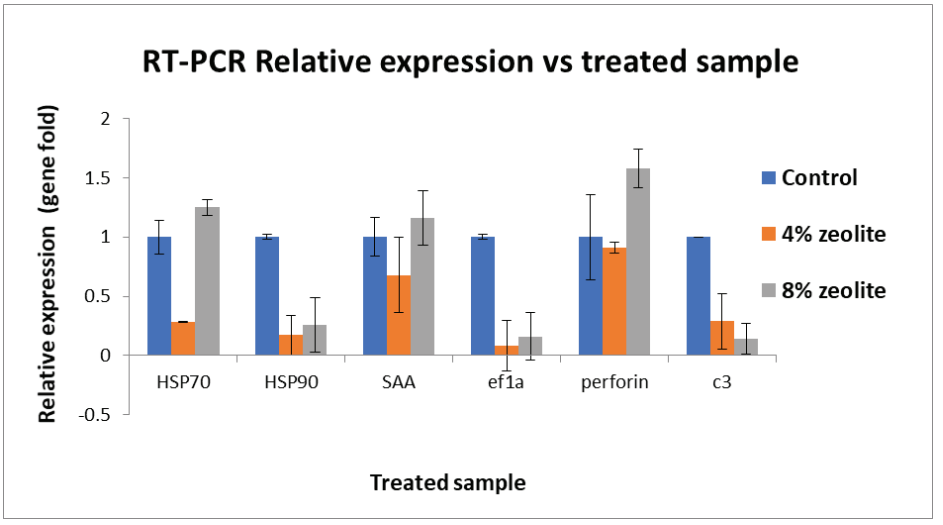

Figure 1 Relative expression (gene fold) of different genes with increasing zeolite treatments. Data above showed no significant differences observed for gene expressions of HSP70, HSP90, SAA, ef1a, perforin and C3 between Control, 4% zeolite and 8% zeolite samples.

DISCUSSION

This is the first study which reports zeolites as a potential fish feed alternative for the Asian seabass *L. calcarifer* and their effects on growth performance, body composition and immune responses. This study addressed two main focuses: the effects of dietary zeolites on (1) the nutritional and growth performance of the fish and (2) the immune response to indicate perturbance to the immune state of the juveniles.

In the first instance, the similar proximate compositions of fish fed with zeolite and the control non-zeolite group indicated that zeolite supplementation in the fish diets did not negatively impact fish nutritional composition. As seen in Table 4, except for Z1, growth performance was seen to be improved in zeolite-fed fish, as observed by the BWG and SGR of Z2, Z4 and Z8 which were higher than the non-zeolite control diet. Indeed, Khodanazary et al. (2013) demonstrated that dietary supplements of zeolite and pearlite at 5% are suitable as an aquafeed ingredient for common carp (*Cyprinus carpio*). *Tilapia zilii* fed with zeolite showed excellent growth performance and positive feed conversion, at up to 2% addition of zeolite (Yildirim & Senel, 2009). Even freshwater aquarium fish, Angel (*Pterophyllum scalare*) showed improvement in growth with the addition of 10 g/L of zeolite as supplements (Ghiasi & Jasour, 2012). For growth performance parameters BWG, SGR and percentage survival, fish-fed Z1 showed lower values compared to control, while increases in Z2 compared to control were statistically insignificant (please refer to Table 4). It is not known why Z1 showed lower growth performance than the control, however, a combination of host and environmental factors such as the initial health state of the fish may be plausible reasons for the reduced growth rates seen. Z2 showed higher BWG than Z1 and control with the same survival percentages as the control group. However, Z4 comprising 4% zeolite showed the highest proportions of BWG, SGR and percentage survival compared to all other groups. Although statistically similar, fish fed Z8.0 showed decreased BWG, SGR and percentage survival values compared to Z4. This indicated that 8% zeolite was not suitable for the Asian seabass fish feed and that 4% was the maximum threshold for zeolite feed supplementation.

Body indices of fish generally indicate the overall health of a fish. It is usually measured via three determinative parameters; Condition Factor (CF), Hepatosomatic Index (HSI) and Viscerosomatic Index (VSI). The observation was that the three parameters were statistically similar to the control group for Z1, Z2, Z4 and Z8 as seen in Table 5. This concurred with observations that zeolite supplementation did not result in an overall negative impact on the health state of the fish.

An elevated immune response occurs when an organism encounters pathogenic bacteria or foreign materials that threaten its health state and increases in proportion to the degree of invasion of the host. HSP 70, HSP 90, perforin, Complement (C3), Serum Amyloid Protein (SAA) and Elongation Factor 1a (ef1a) comprise immune and

stress proteins involved in the orchestration of an immune response and are selected in this study to indicate stress and immune response of host fish cells to zeolites. These proteins work synergistically to respond accordingly to eliminate the invading foreign materials from the host. The general observation seen in Figure 1 that Z4 and Z8 were not significantly different in expression for these proteins when compared to the control group; indicated that the presence of zeolites was insufficient to invoke a significant immune response during the 6-week feeding trial. Although an increase in gene expressions of HSP70, SAA and perforin was observed for Z8 these increases were statistically insignificant. Therefore it is not possible to conclude the presence of an immune or stress response to relate to the Z8 reduction in values of BWG, SGR and percentage survival as the data suggests. To obtain a clearer picture of the fish immune response, it is therefore recommended that in future studies, the period of trial feed of the fish juveniles is extended to a minimum of 8 weeks.

Taken together, this study indicated that zeolite supplementation positively contributed to the growth performance of the fish with little adverse effects on its immunological profile; and that Z4 comprising 4% zeolite was observed to be the most optimum level for a dietary feed of *L. calcarifer*.

CONCLUSION

The present study revealed a positive effect of dietary zeolite supplementation in the diets for Asian seabass at percentages of 1 – 8%. The optimum inclusion level of dietary zeolite is proposed at 4% for optimum BWG, SGR, and feed efficiency of Asian seabass.

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