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Dietary Fishmeal Replacement by Methanol-Extracted Cottonseed Meal with Amino Acid Supplementation for Juvenile Cobia *Rachycentron canadum*

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Abstract: The present study aims to evaluate methanol-extracted cottonseed meal (CSM) as a potential replacement for fishmeal (FM) in aquafeeds for juvenile cobia *Rachycentron canadum*. Five isonitrogenous (41% crude protein) and isolipidic (11% crude lipid) diets were formulated with 0 (i.e., the full fishmeal diet, as Control), 25%, 50%, 75% and 100% of the dietary protein from FM replaced by methanol-extracted CSM with L-lysine (L-Lys) and DL-methionine (DL-Met) and supplemented to the established requirement levels for cobia. Diets were fed to triplicate groups of juvenile fish with an average initial weight (±SEM) of 11.35 ± 0.23 g/fish for 9 weeks. Percent weight gain (WG), feed efficiency (FE) and protein efficiency ratio (PER) of fish fed diets with 25% and 50% of FM protein replaced by methanol-extracted CSM were higher or comparable to those of fish fed the Control diet. Those responses were gradually reduced with increasing levels of CSM substitution, resulting in significant ($p < 0.05$) negative linear trends. Condition factor (CF) and hepatosomatic index (HSI) values significantly decreased with increasing dietary CSM inclusion, as did whole-body protein and lipid composition. Activities of superoxide dismutase (SOD) of fish fed CSM diets were not significantly different compared to that of fish fed the Control diet. The glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) levels, as well as serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities from fish fed diets with 50% or greater of CSM were lower than those of fish fed the Control and CSM25 diets. These results indicated that the inclusion of CSM did not induce any apparent stress on juvenile cobia. Additionally, methanol-extracted CSM with Lys and Met supplementation was able to replace up to 20~30% of crude protein provided by FM in the diet of cobia without drastically affecting the growth performance or body composition.

Keywords: fishmeal replacement; cottonseed meal; cobia *Rachycentron canadum*

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1. Introduction

Aquaculture continues to be one of the fastest growing food-producing sectors throughout the world. Many aquatic organisms, particularly the carnivorous fish species, require relatively high protein levels in prepared diets compared to land animals. The protein component of prepared diets is typically the most expensive, and thus feed costs generally represents at least one-half of the variable operating costs in commercial fish production. Fishmeal rendered from forage fish species has long been considered the most preferable protein feedstuff for inclusion in prepared diets of various cultured fish, especially carnivorous species, and is the most commonly used protein source in aquafeed. However, due to the increasing demand for fishmeal to support the continued global expansion

of aquaculture, the price of fishmeal has tended to rise over time, and its supply has become restricted and unstable in certain instances. Therefore, it is urgent to develop more economic supplies of protein feedstuffs to replace and/or lessen the reliance on fishmeal and allow for the continued and more sustainable development of aquaculture. A variety of protein feedstuffs derived from the processing of oilseeds and other plant crops have become increasingly used in aquafeeds [1,2].

Cottonseed meal (CSM) is one such feedstuff generated by the removal of oil from the cottonseed. The global production of cotton for fiber is quite substantial, and the CSM resulting from oil extraction is substantial on a global scale, and is much less expensive per unit of protein than fishmeal and other protein feedstuffs of animal and plant origin, including soybean meal. For example, the price of CSM is only about one-fourth the price of fishmeal [3]. Therefore, aquaculture production costs could be reduced substantially if CSM can be efficiently used in aquatic feeds. However, the presence of anti-nutritional factors such as gossypol and relatively low levels of lysine in CSM are two main constraints restricting its use in high proportions in aquafeeds [4]. Indeed, higher substitution levels of CSM have been shown to depress growth, mainly due to gossypol, the compound that at high levels can be toxic to animals, including various fish species reviewed by [5,6]. However, in recent years, various processing technologies, including methanol extraction, solid state fermentation and the use of gossypol-degrading enzymes, have been applied to cottonseed meal and have markedly reduced or eliminated the presence of antinutrients [7,8].

Previous studies have revealed that dietary fishmeal substitution with various CSM products at certain levels did not significantly impair the growth performance or health of various freshwater and marine fish species such as the black sea bass *Centropristis striata* [9], *Pseudobagrus ussuriensis* [10], European flatfish *Scophthalmus maximus* [11], grass carp *Ctenopharyngodon idellus* [12], red drum *Sciaenops ocellatus* [13], largemouth bass *Micropterus salmoides* [14,15], and Asian red-tailed catfish *Hemibagrus wyckioides* [16]; differences in the tolerance of various fish species to anti-nutritional factors such as gossypol in CSM also have been observed. Therefore, species-specific evaluations are needed to assess the potential utility of CSM products in the diet of a given fish species.

Cobia *Rachycentron canadum* (L.) is a high quality, economically important, marine finfish that is globally distributed in tropical and subtropical waters [17] around the world, including Asia. They are also suitable for intensive aquaculture, particularly in offshore, net/cage systems, because of their hardiness in a crowded environment and rapid growth [18]. Due to their high price and increasing market demand, the aquaculture of cobia is continuing to develop. There is a pressing need to develop more cost-effective diet formulations for this fish. Thus far, the dietary requirements of cobia for protein, amino acids, and lipids have been investigated [18–31]. However, there are limited studies evaluating practical feedstuffs with this species. Despite numerous studies focusing on the application of CSM in the diet of several aquatic animals, until now, there have been no studies carried out to evaluate the feasibility of partially replacing fishmeal with CSM in the diet of juvenile cobia.

Thus, the present study replaced fishmeal with methanol-extracted CSM, in which the gossypol content was markedly reduced, and we aimed to examine the effects of methanol-extracted CSM substitution for fishmeal on the growth performance, feed utilization, body composition, body indices, and immune responses of cobia. The results provide useful information for the development of more cost-effective formulated diets for the optimal aquaculture of cobia.

2. Materials and Methods

2.1. Diet Formulations

A Control diet was formulated to contain, on a dry-matter (DM) basis, 41% crude protein contributed by fishmeal (23% by weight), soy protein concentrate (25% by weight) and 6.95% bone meal (Table 1). The concentration of free gossypol in CSM varies among cotton species and varieties and is affected by CSM processing methods. The present study

used low-gossypol cottonseed meal (CSM), which was provided by TYCOON, Xinjiang Taikun Group, Changji city, China. The CSM is prepared following these steps: firstly, the shell of cottonseed was peeled off and about 97% of the shell was removed, and then it was grounded into cottonseed meal. At this stage, the gossypol content was ~8000 mg kg^{-1} CSM. The second step is softening, where the CSM was softened at 55–60 °C for 10–15 min, after which the gossypol content was reduced to 7000–7500 mg kg^{-1}. The third step is drying, i.e., the CSM was dried at 65–70 °C for 15–20 min, the moisture content of CSM was lowered to 3–4%, and the gossypol content was lowered to 6500–7000 mg kg^{-1}. The fourth step was solvent extractions, in which the CSM was firstly extracted with n-Hexane for 80 min and secondly extracted with methanol at 50–55 °C for 20 min to free and solubilize the gossypol; the gossypol content in CSM was lowered to 400–500 mg kg^{-1}. Finally, the meal was recovered by filtering and washed with 95% ethanol; then, it was re-filtered. Residual ethanol was eliminated by first air drying and then toasting it in an oven at 50 °C. The hexane and methanol treatments also extracted some residual oil, reducing the lipid content to ~0.6%. These treatments reducing the gossypol content to <400 mg kg^{-1}. The gossypol content of CSM used in the present study was 371 mg kg^{-1}. The dry matter, crude protein, crude lipid, and crude fiber contents of CSM are 94.9%, 63.4%, 0.6%, and 4.9%, respectively.

Table 1. Formulation and proximate composition of the Control and experimental diets (% dry weight).

Ingredient	Diet Designation (Cottonseed Meal/% Fishmeal Replacement)				
	Control (0)	**25**	**50**	**75**	**100**
Fishmeal [1]	35.00	26.25	17.50	8.75	0.00
Cottonseed meal [2]	0.00	9.92	19.83	29.75	39.67
Soy Protein Conc.	25.00	25.00	25.00	25.00	25.00
Menhaden Oil	5.00	5.50	6.00	6.50	6.80
Premix [3,4]	29.3	29.3	29.3	29.3	29.3
Bone meal [5]	6.95	5.05	3.16	1.3	0
L-Lysine	0.00	0.15	0.30	0.45	0.60
DL-Methionine	0.00	0.15	0.30	0.45	0.60
Analyzed proximate composition (% dry weight) [6]					
Moisture	10.5	10.4	10.6	10.2	10.0
Crude protein	41.1	41.1	41.3	42.6	42.0
Crude lipid	10.5	10.4	11.7	11.2	10.6
Ash	17.0	14.7	12.3	9.3	9.4
Metabolized energy (kJ/g) [7]	19.3	19.6	20.3	20.8	20.6
Free gossypol (mg·kg^{-1})	0	31.04	62.00	92.91	123.32

[1] Fish meal, obtained from Tecnológica de Alimentos S.A., Peru, belongs to TASA, and is super steam dried. [2] Cottonseed meal (CSM) was supplied by TYCOON, Xinjiang Taikun Group, Changji city, China. The CSM was processed by methanol extraction to reduce the content of gossypol. The dry matter, crude protein, crude lipid, crude fiber and gossypol contents of CSM are 94.9%, 63.4%, 0.6%, 4.9% and 317 mg kg^{-1}, respectively. [3] Premix contained: 20% dextrinized starch; 2.5% lecithin; 0.3% feed attractants; 2.0% choline chloride; 2.0% $Ca(H_2PO_4)$; 0.5% Vitamin C; 2.0% vitamin mineral premix. [4] Vitamin and mineral premix according to Wang et al. [32]. [5] Made from bone of swine, supplied by Junyou Feed Corporation, Guangzhou, China. [6] Values are means of triplicate analysis. [7] Obtained by calculation following Shiau and Chen [33]. The estimated energy values for each energy nutrient were the following: protein, 23.64 kJ/g; lipid, 39.33 kJ/g; carbohydrate 17.20 kJ/g.

Four experimental diets were formulated with different inclusion levels of CSM to replace 25, 50, 75 and 100% of the fishmeal protein in the Control diet (referred to as CSM25, CSM50, CSM75 and CSM100, respectively) along with incremental reductions in bone meal (Table 1). L-lysine and DL-methionine were added to the diets in increasing concentrations as FM protein was replaced to ensure the established requirements of cobia were met [17]. The amino acid compositions of fishmeal, CSM meal, experimental diets, and fish whole-body were quantified. For each sample, ~400 mg sample was homogenized in a 10% sulfosalicylic acid solution, hydrolyzed in 6 N HCl, and centrifuged at 6000× *g* at 4 °C for 10 min. The collected supernatants were percolated through 0.22 µm filters for free

amino acids measurements. The amino acid content was analyzed by high-performance liquid chromatography (HPLC) (Table 2).

Table 2. Amino acid composition of fishmeal, cottonseed meal and experimental diets (g/100 g, dry weight) [1].

Amino Acid	Fish Meal	Cottonseed Meal	Control	CSM25	CSM50	CSM75	CSM100
His	2.10	1.71	1.11	1.13	1.15	1.20	1.17
Arg	3.49	7.19	2.25	2.65	3.02	3.33	3.59
Thr	2.41	1.81	1.81	1.78	1.77	1.77	1.69
Lys	4.23	2.28	2.54	2.46	2.42	2.43	2.30
Met	1.57	0.81	0.76	0.72	0.62	0.53	0.78
Val	2.87	2.45	1.81	1.78	1.77	1.77	1.69
Ile	2.41	1.75	1.58	1.51	1.46	1.42	1.32
Leu	4.18	3.21	2.78	2.70	2.61	2.59	2.41
Phe	2.31	3.16	1.76	1.85	1.94	2.05	2.05
Tyr	2.09	1.18	1.13	1.11	1.1	1	1.01
Asp	6.31	5.32	3.64	3.65	3.67	3.75	3.62
Ser	2.79	2.57	1.69	1.73	1.75	1.81	1.77
Glu	8.28	10.86	6.81	7.28	7.75	8.3	8.39
Pro	2.98	2.32	1.89	1.88	1.85	1.87	1.77
Gly	4.34	2.39	2.08	1.96	1.85	1.78	1.61
Ala	3.96	2.22	2.09	1.95	1.83	1.74	1.56

[1] Values are means of two replicate determinations (n = 2).

The diets were prepared and handled as described by Wang et al. [7]. Briefly, dry ingredients were first ground into a fine powder through a 320 µm mesh. All the ingredients were then thoroughly mixed with soybean oil, and water was added to produce a stiff dough. The dough was then extruded using an experimental feed mill [F-26 (II), South China University of Technology, Guangzhou, China] with a long single-screw and a die size of 2 mm in diameter. Subsequently, noodle-like strands were broken up and sieved into pellets with a diameter of ~2 mm. The pellets were air dried to approximately 90% dry matter and broken into appropriate sizes ranging from 1 to 3 mm in diameter to match the mouth gape of the fish. The pellets were stored at −20 °C until fed.

2.2. Facilities, Feeding Trials and Fish

The feeding trial was conducted at the Marine Laboratory of the South China Sea Fisheries Research Institute, Hainan Province, China using a completely random design. The experimental fish were cultured in 12, 300-L cylindrical fiberglass tanks operated as a recirculating system located in an indoor, climate-controlled laboratory with the water supply provided by a closed recirculating system with a common reservoir of natural seawater. Adequate water quality was maintained by mechanical and biological filtration, and aeration was provided continuously by one air stone per tank to maintain dissolved oxygen levels close to air saturation. Water quality parameters, including temperature, salinity, dissolved oxygen and pH were monitored daily using a multi-parameter probe (YSI Inc., Yellow Springs, OH, USA). During the experimental period, temperatures ranged from 28 °C to 31 °C, salinity was 27.2–30.2 ppt, pH was 7.5–7.9, ammonia nitrogen was <0.05 mg/L and DO was not less than 6.0 mg/L. A 12 h light–12 h dark photoperiod was maintained with fluorescent lights controlled by automatic timers.

Juvenile cobia were obtained from a local commercial hatchery. Upon arrival, they were placed in a 500 L plastic tank for 1 week to acclimate to laboratory conditions. During acclimation, they were fed a commercial diet (with 42% protein and 11% lipid, Guangdong Evergreen Group, Zhanjiang, China). At the beginning of the experiment, each tank was stocked with 20 fish with an average weight of 11.4 ± 0.2 g. Three replicate groups of fish were randomly assigned to each diet. During the feeding trial, fish were hand-fed to apparent satiation twice daily (8:00 h and 17:00 h). Fish were considered satiated when they ceased consuming the pellets, such that some fell to the bottom of the tank, and after which

the excess feed was removed and dried. The amount of diet consumed by fish in each tank was recorded after each feeding. The culture tanks were cleaned weekly, and the feeding trial continued for 9 weeks, after which all fish from each tank were weighed and counted.

2.3. Sample Collection and Chemical Analyses

After the feeding trial, fish were starved for 24 h and anesthetized with eugenol (1:10,000; Shanghai Reagent, Shanghai, China) before weighing and counting. Three fish were randomly sampled from each aquarium, i.e., nine fish for each dietary treatment. The proximate composition analyses of feed ingredients, experimental diets and fish were performed by the standard methods [34]. Samples of the diets and fish were dried to a constant weight at 105 °C to determine moisture content. Protein levels were determined by measuring nitrogen (N × 6.25) using the Kjeldahl method. Lipid levels were quantified by an ether extraction using a Soxhlet apparatus. Ash levels were measured by combustion at 550 °C. Livers, viscera and fat on the intestine were collected from an additional three fish per aquarium for subsequent enzymatic assays and body condition index calculations.

Blood samples for alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activity measuring were obtained from the caudal vein of each of these fish by a 1 mL syringe fitted with a 25 gauge needle and allowed to clot at 4 °C for 4 h before centrifugation at 5000× *g* for 10 min at 4 °C. Serum (supernatant) from the three fish per tank was sampled in equal amounts and mixed before being frozen at 80 °C. The activities of AST and ALT in serum were assayed by commercial kits (Jiancheng Bioengineering Institute, Nanjing City, China) following the manufacturers' instructions. One unit of ALT is defined as the amount of enzyme that generates 1 μmol of pyruvate per min at 37 °C. One unit of AST is defined as the amount of enzyme that will generate 1 μmol of glutamate per min at 37 °C.

Intestinal samples for lipase, amylase and trypsin enzyme activity measuring were cut from the middle part of the intestine. The amylase activity was determined by the starch hydrolysis method, according to the Somogy–Nelson colorimetric method, described by Hidalgo et al. [35]. Activities of the intestinal lipase and trypsin enzymes were determined using the commercial kits from the same company, as mentioned above.

Liver samples were dissected from three fish per aquarium after bleeding, immediately frozen in liquid nitrogen, and stored at −80 °C before analysis. The samples were obtained by the homogenization of frozen tissue in ice-cold 0.86% (*w*/*v*) NaCl solution. Hepatic antioxidant enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), as well as malondialdehyde (MDA), one of the metabolites derived from lipid peroxidation, were measured using a thiobarbituric acid reactive substance (TBARS) assay kit [32,36] from the same company as mentioned above, following the manufacturer's protocol.

The protein content in the samples was quantified by the Bradford [37] method using a total protein quantification kit (Jiancheng, Nanjing, China) with bovine serum albumin as the standard. All analyses were conducted in triplicate.

Gossypol analyses: The free gossypol content in CSM and the diets was determined using high performance liquid chromatography-tandem mass spectrometry (Waters Aquity TQD, Taunton, USA) according to the method by Wang et al. [38], with some modifications. The diets or CSM samples were freeze-dried and ground in a motor and pestle. Approximately 0.2 g dry CSM or diet samples were weighed and added with 2 mL extraction solution (acetonitrile:water = 7:3), then shook for 2 min, extracted by ultrasonic for 5 min, and centrifuged at 4000 r/min for 5 min; then, the supernatant was collected. The residue was extracted again according to the above steps, and the supernatant was combined. The supernatant was filtered by a 0.22 μm filter and measured on the machine. The gossypol standard (purity > 98%) was provided by ANPEL Laboratory Technologies (Shanghai city, China) Inc.

2.4. Response Measurements and Calculations

The following responses were calculated:

$$\text{Survival (\%)} = 100 \times N_t/N_0$$

$$\text{Weight gain (WG\%)} = 100 \times (W_t - W_0)/W_0$$

$$\text{Feed intake (FI, g fish}^{-1}\text{ day}^{-1}\text{)} = D_f/N_t/t$$

$$\text{Feed efficiency (FE)} = (W_t - W_0)/D_f$$

$$\text{Protein efficiency ratio (PER)} = (W_t - W_0)/\text{protein intake}$$

$$\text{Condition factor (CF)} = (W_t \times 100)/(\text{fish body length}^3)$$

$$\text{Hepatosomatic index (HSI)} = (\text{liver weight} \times 100)/W_t$$

where BW is the wet body weight, W_t is the mean final body weight (g), W_0 is the mean initial body weight (g) and t is the experimental duration in days. N_0 and N_t represent the initial and final numbers of fish in each tank, respectively. D_f is the dry diet intake (g).

2.5. Statistical Analysis

All evaluated variables were subjected to an analysis of variance (ANOVA) to determine whether the inclusion levels of CSM significantly ($p < 0.05$) affected the observed responses. Normality and homoscedasticity assumptions of all evaluated variables were confirmed with Levene Test before any statistical analysis. When the ANOVA identified differences among groups ($p < 0.05$), a follow-up trend analysis using orthogonal polynomial contrasts was performed to determine whether the effect was linear and/or quadratic [39]. Differences between the means were tested by Duncan's multiple range test. A second-order polynomial regression based on weight gain (WG), feed efficiency (FE) and protein efficiency ratio (PER) was performed to estimated the optimal level of fishmeal replacement by CSM. All tests were performed in SPSS 18.0 for Windows (IBM Corp., Armonk, NY, USA). Values are presented as means ± standard error of mean (SEM) of triplicate groups.

3. Results

3.1. Growth Performance

The survival of cobia fed the experimental diets, in which methanol-extracted CSM protein contributed 75% or less, was relatively high; whereas, at the highest CSM inclusion level, the survival was significantly ($p < 0.05$) reduced (Table 3). The weight gain (WG), feed efficiency (FE) and protein efficiency ratio (PER) were highest for fish fed the CSM25 diet, followed by those fed the Control and CSM50 diets. However, there was a linear decrease with increasing CSM levels. Significant negative linear or quadratic trends were found with increasing increments of CSM, with fish fed the CSM100 diet having the most pronounced reductions. The feed intake (FI) was the lowest in fish fed the CSM75 and CSM100 diets, with the FI of the CSM75 group significantly ($p < 0.05$) lower than that of fish fed the diets with 50% or less protein contributed by CSM. Significant positive linear and quadratic trends in WG, FE and PER were found with increasing increments of CSM.

Regression equations based on second-order polynomial regression analyses of WG, FE and PER for estimating the optimal level of fishmeal replacement by CSM were: $Y = -0.15\,X^2 + 5.63\,X + 1072.6$ ($R^2 = 0.84$), $Y = -0.00008X^2 + 0.005X + 0.49$ ($R^2 = 0.85$) and $Y = -0.0002\,X^2 + 0.01\,X + 1.20$ ($R^2 = 0.86$), respectively (Figure 1). Optimal CSM replacement levels derived with the polynomial regression method for WG, FE and PER were 19.2%, 31.3% and 29.8%, respectively (Figure 1).

Table 3. Survival, percent weight gain (WG), feed intake (FI), feed efficiency (FE) and protein efficiency ratio (PER) of cobia fed the Control and experimental diets containing graded levels of methanol-extracted cottonseed meal (CSM) [1].

Diet Designation	Survival (%)	Final Weight (g)	WG (%)	FI (g fish^{-1} d^{-1})	FE	PER
Control	90.0 ± 0.0 [a]	120.1 ± 5.7 [ab]	957.6 ± 46.1 [ab]	3.79 ± 0.16 [bc]	0.45 ± 0.02 [b]	1.10 ± 0.05 [b]
CSM25	96.7 ± 1.7 [a]	166.7 ± 5.7 [a]	1371.8 ± 61.3 [a]	3.69 ± 0.12 [bc]	0.67 ± 0.01 [a]	1.62 ± 0.01 [a]
CSM50	88.3 ± 10.1 [a]	115.4 ± 16.4 [bc]	926.2 ± 44.1 [bc]	3.95 ± 0.43 [c]	0.46 ± 0.09 [b]	1.11 ± 0.05 [b]
CSM75	90.0 ± 5.0 [a]	68.6 ± 4.2 [cd]	498.5 ± 41.8 [cd]	2.21 ± 0.13 [a]	0.40 ± 0.00 [b]	0.95 ± 0.01 [b]
CSM100	37.5 ± 7.5 [b]	40.8 ± 3.0 [d]	260.4 ± 23.8 [d]	2.90 ± 0.42 [ab]	0.18 ± 0.05 [c]	0.42 ± 0.09 [c]
ANOVA (Pr > F) [2]	0.000	0.000	0.000	0.003	0.000	0.000
Linear Trend (Pr > F)	0.000	0.000	0.000	0.005	0.000	0.000
Quadratic Trend (Pr > F)	0.004	0.003	0.003	0.705	0.000	0.001

[1] Values represent mean ± SEM of three replicate aquariums (n = 3). Numbers within a column with the same superscript letter are not significantly different (p > 0.05). [2] Significance probability associated with the F-statistic.

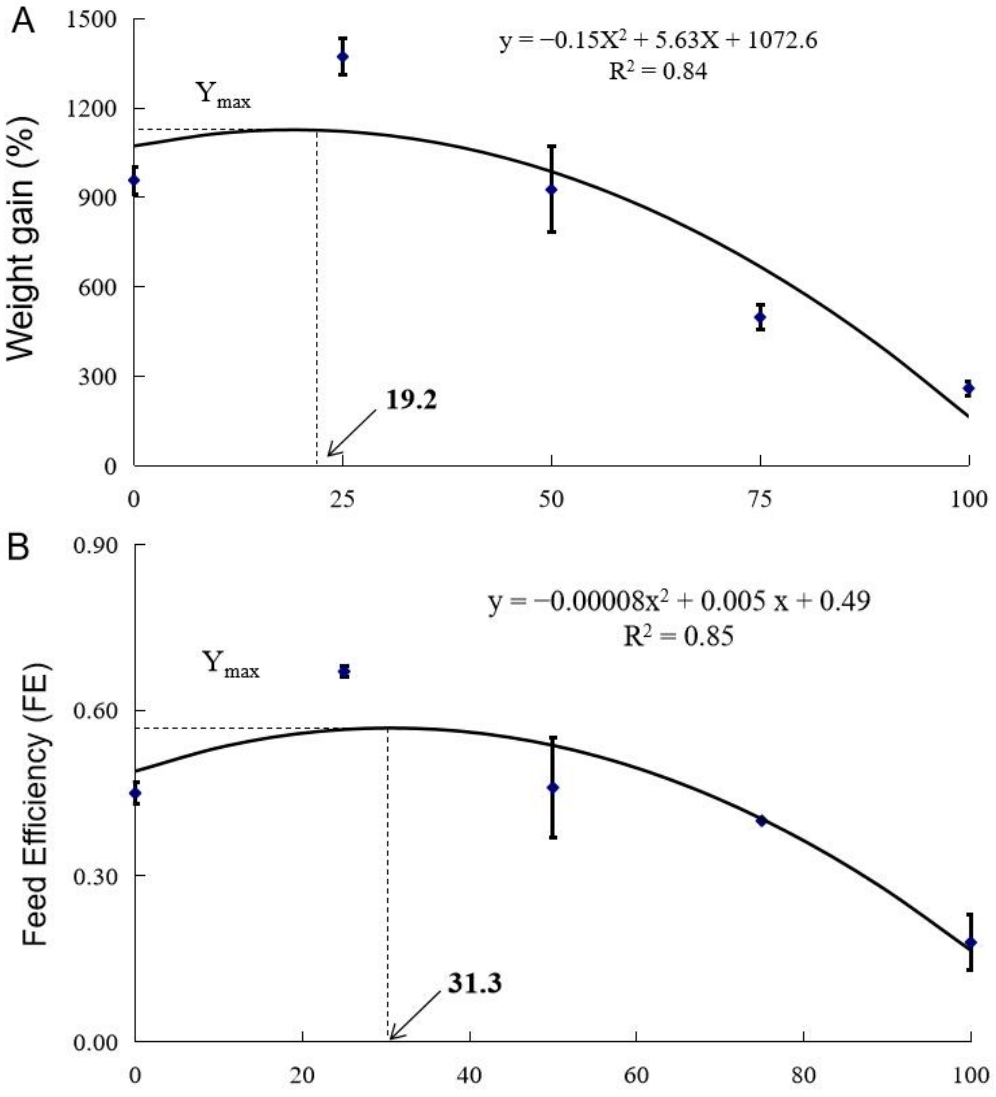

Figure 1. *Cont.*

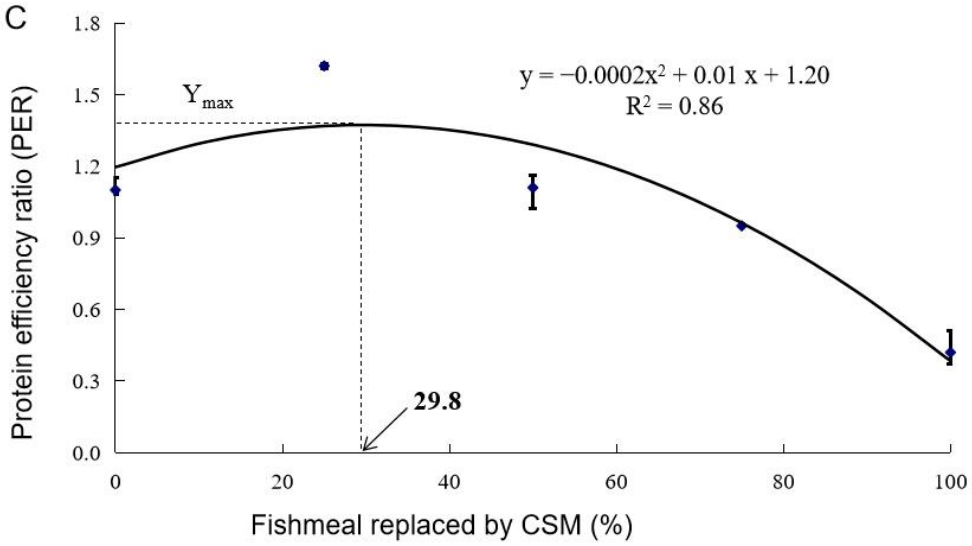

Figure 1. Effect of replacement of fishmeal by cottonseed meal (CSM) on (**A**) weight gain (WG), (**B**) feed efficiency (FE) and (**C**) protein efficiency ratio (PER) of juvenile cobia *Rachycentron canadum*. Each point represents the mean ± S.E. of three groups of fish (n = 3), with 20 fish per group. The optimal CSM replacement level derived with the linear regression method for WG, FE and PER were 19.2%, 31.3% and 29.8%, respectively.

3.2. Body Condition Indices and Whole-Body Composition

Significant negative linear trends were observed for condition factor (CF) and hepatosomatic index (HSI) values of cobia in response to increasing CSM levels (Table 4). Significant linear or quadratic trends were found between CSM levels and whole-body composition (Table 4). The moisture and ash in whole-body tissues increased with the increasing dietary CSM levels, with the moisture value of fish fed CSM100 being significantly higher than the other groups. Both whole-body protein and lipid decreased with the increasing dietary CSM levels.

Table 4. Body condition indices and whole-body composition of cobia fed the Control and experimental diets containing graded levels of methanol-extracted cottonseed meal (CSM) [1].

Diet Designation	Condition Factor (CF)	Hepatosomatic Index (HSI) (%)	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Control	0.94 ± 0.03 ^{ab}	2.43 ± 0.54 ab	71.2 ± 0.4 ^{a}	16.78 ± 0.19 ^{ab}	7.80 ± 0.36 ^{a}	3.30 ± 0.03 ^{b}
CSM25	1.03 ± 0.02 ^{a}	2.47 ± 0.10 a	70.1 ± 0.4 ^{a}	17.32 ± 0.10 ^{a}	8.63 ± 0.28 ^{a}	3.28 ± 0.08 ^{b}
CSM50	0.95 ± 0.05 ^{ab}	2.04 ± 0.20 abc	70.8 ± 0.9 ^{a}	16.93 ± 0.25 ^{a}	7.66 ± 1.07 ^{ab}	3.81 ± 0.24 ^{ab}
CSM75	0.90 ± 0.07 ^{ab}	1.96 ± 0.10 bc	72.1 ± 1.8 ^{a}	16.78 ± 0.22 ^{ab}	6.11 ± 1.25 ^{ab}	3.83 ± 0.19 ^{ab}
CSM100	0.82 ± 0.02 ^{b}	1.80 ± 0.10 c	74.8 ± 0.3 ^{b}	15.81 ± 0.23 ^{b}	4.57 ± 0.01 ^{b}	4.01 ± 0.04 ^{a}
ANOVA (Pr > F) [2]	0.000	0.009	0.000	0.009	0.018	0.012
Linear Trend (Pr > F)	0.001	0.001	0.000	0.006	0.004	0.001
Quadratic Trend (Pr > F)	0.003	0.554	0.006	0.005	0.05	0.166

[1] Values represent mean ± SEM of three replicate aquariums (n = 3). Numbers within a column with the same superscript letter are not significantly different (p > 0.05). [2] Significance probability associated with the F-statistic.

3.3. Amino Acid Profiles of Whole Bodies

Significant linear or quadratic trends were found between CSM levels and fish whole-body essential amino acids concentrations, except for Lysine. These essential amino acid values were lowest in fish fed the 25% and 50% CSM diets, and then increased with increasing dietary CSM levels (Table 5).

Table 5. Whole-body amino acid profile (% of sample, mean ± SEM, n = 3) of juvenile cobia fed the Control and experimental diets containing graded levels of methanol-extracted cottonseed meal (CSM) [1].

Amino Acid	Control	CSM25	CSM50	CSM75	CSM100	ANOVA (Pr > F) [2]	Linear Trend (Pr > F)
His	1.35 ± 0.05	1.33 ± 0.01	1.30 ± 0.00	1.38 ± 0.06	1.40 ± 0.04	0.130	0.067
Arg	3.59 ± 0.06	3.48 ± 0.02	3.47 ± 0.01	3.66 ± 0.18	3.84 ± 0.05	0.063	0.024
Thr	2.35 ± 0.09	2.28 ± 0.01	2.31 ± 0.06	2.45 ± 0.12	2.47 ± 0.06	0.087	0.027
Lys	4.00 ± 0.10	3.92 ± 0.03	3.86 ± 0.03	4.08 ± 0.19	4.15 ± 0.16	0.146	0.084
Met	1.46 ± 0.05 ^{bc}	1.43 ± 0.01 ^{c}	1.43 ± 0.03 ^{c}	1.54 ± 0.06 ^{b}	1.55 ± 0.03 ^{a}	0.041	0.011
Val	2.50 ± 0.07 ^{ab}	2.43 ± 0.07 ^{b}	2.40 ± 0.10 ^{b}	2.59 ± 0.02 ^{a}	2.63 ± 0.06 ^{a}	0.041	0.023
Iso	2.09 ± 0.05 ^{ab}	2.02 ± 0.06 ^{b}	2.01 ± 0.08 ^{b}	2.19 ± 0.02 ^{ab}	2.21 ± 0.05 ^{a}	0.019	0.010
Leu	3.73 ± 0.10 ^{ab}	3.63 ± 0.02 ^{b}	3.65 ± 0.11 ^{b}	3.91 ± 0.12 ^{a}	3.89 ± 0.11 ^{a}	0.044	0.018
Phe	2.04 ± 0.06 ^{ab}	1.98 ± 0.2 ^{b}	1.99 ± 0.06 ^{b}	2.13 ± 0.78 ^{a}	2.14 ± 0.04 ^{a}	0.038	0.016

[1] Values represent mean ± SEM of three replicate aquariums (n = 3). Numbers within a column with the same superscript letter are not significantly different (p > 0.05). [2] Significance probability associated with the F-statistic.

3.4. Digestive Enzyme Activities

As shown in Table 6, the activities of trypsin amylase and lipase for fish fed the CSM50, CSM75 and CSM100 diets were generally lower compared to those fed the Control and CSM25 diet, but differences did not reach a significant level (Table 6).

Table 6. Activities of intestinal digestive enzymes, hepatic antioxidant and immune-related enzymes, and serum MDA and GSH-Px concentrations of cobia fed the Control and experimental diets containing graded levels of methanol-extracted cottonseed meal (CSM) [1].

Diets	Control	CSM25	CSM50	CSM75	CSM100	ANOVA (Pr > F) [2]	Linear Trend (P)	Quadratic Trend (P)
Trypsin (U mg prot^{-1})	810.18 ± 83.01	904.40 ± 164.48	676.23 ± 20.73	620.05 ± 190.05	587.25 ± 84.16	0.268	0.051	0.603
Amylase (U mg prot^{-1})	0.13 ± 0.01	0.14 ± 0.01	0.10 ± 0.01	0.11 ± 0.00	0.12 ± 0.00	0.071	0.055	0.845
Lipase (U g prot^{-1})	26.05 ± 0.86	29.35 ± 1.40	24.93 ± 0.51	24.22 ± 0.72	26.97 ± 0.86	0.058	0.628	0.585
SOD (U mg prot^{-1})	82.26 ± 5.12 ^{ab}	77.03 ± 4.52 ^{b}	70.09 ± 3.46 ^{b}	105.11 ± 5.22 ^{a}	85.08 ± 5.79 ^{ab}	0.050	0.217	0.569
GSH-Px (μmol L^{-1})	363.18± 98.76 ^{ab}	459.10 ± 102.93 ^{a}	151.27 ± 8.61 ^{bc}	201.98 ± 5.55 ^{abc}	98.81 ± 25.54 ^{c}	0.039	0.008	0.494
MDA (nmol mg prot^{-1})	14.73 ± 3.13 ^{a}	15.13 ± 1.19 ^{a}	2.22 ± 0.43 ^{b}	1.38 ± 0.33 ^{b}	5.38 ± 1.23 ^{b}	0.000	0.000	0.222
ALT (U L^{-1})	4.00 ± 0.00	4.20 ± 0.74	2.50 ± 0.29	2.50 ± 0.29	3.33 ± 0.33	0.069	0.057	0.168
AST/(U L^{-1})	52.67 ± 13.69	67.75 ± 12.29	48.50 ± 9.23	53.00 ± 7.91	51.50 ± 10.50	0.715	0.594	0.834

[1] Values represent mean ± SEM of three replicate aquariums (n = 3). Numbers within a column with the same superscript letter are not significantly different (p > 0.05). [2] Significance probability associated with the F-statistic. SOD, total superoxide dismutase; GSH-PX, glutathione peroxidase; MDA, malondialdehyde.

3.5. Antioxidant Enzyme Activities and Serum Parameters

Fish fed diets with 75% and 100% of the dietary protein from CSM had the highest SOD activity, but only a marginally significant difference was detected (Table 6). The GSH-Px and MDA levels, as well as the serum ALT and AST activities, were lower in fish fed diets with 50% or greater CSM than that of fish fed the Control and CSM25 diets. Significant negative linear trends were observed for GSH-Px and MDA of cobia in response to the increasing CSM levels (Table 6).

4. Discussion

According to previous studies, the inclusion of regular CSM in fish diets typically has been limited to about 10–15% by weight (reviewed by Li and Robinson [5]). Whereas, in the present study, the survival, growth rate, FE and PER of cobia fed diets in which methanol-extracted CSM replaced 25% and 50% of dietary protein provided by fishmeal was higher or similar to fish fed the Control diet, in which all protein was provided by fishmeal and soy protein concentrate. These results suggest that the substitution of methanol-extracted CSM, which had a relatively low gossypol content for FM protein, at levels of up to 50% (with supplemental lysine and methionine), provided adequate nutrition. This methanol-

extracted CSM can be used as an alternative to fishmeal and possibly other costly protein feedstuffs for juvenile cobia.

However, it is noteworthy that weight gain was significantly reduced in cobia fed diets in which 75% or 100% of their protein was from CSM. The reduced performance of cobia fed higher levels of CSM could be due to several reasons, including lowered feed intake (FI) and/or feed efficiency (FE), the toxic effects of gossypol in these diets and lower nutritional value, particularly the essential amino acid profile of CSM.

For cobia fed the CSM75 diet, given that their FE was not significantly different compared to fish fed the Control diet, a reduction in feed intake could be a possible reason for their lower growth. For the CSM100 group, the lower performance could be due to either the reduced FI or FE or both, as the two factors were significantly lower than that observed for fish fed the Control diet. Our previous study established that the palatability of experimental diets containing elevated levels of CSM products and consequently FI of red drum was reduced [7]. The lower levels of desirable flavors and/or higher levels of anti-nutritional factors such as gossypol could be possible reasons for poor palatability. Our previous study suggested that the lower levels of desirable flavors can be compensated for by supplementing palatability attractants such as IMP and citric acid to improve the performance of red drum [7]. In the present study, although palatability attractants were provided in CSM diets, the FI of CSM75 and CSM100 groups was still significantly lower than those fed the Control diet. Therefore, the levels of free gossypol probably exceeded the threshold of causing poor palatability in these diets. A similar result was found by Anderson et al. [9] in black sea bass, in which the reduction of FI was consistent with the level of gossypol in the diets. Furthermore, in the present study, the FE of cobia in the CSM100 group was significantly lower than the other groups. However, Anderson et al. [9] found no significant differences in FE and PER of black sea bass fed either high- or low-gossypol CSM substitution diets. These inconsistent findings could be due to a species-specific difference in their tolerance to anti-nutritional factors.

The poor performance of fish fed high CSM diets in some previous studies may also be attributed to a deficiency of essential amino acids, because Lys and Met can be relatively low in CSM [40,41], and gossypol can reduce the bioavailability of Lys [42]. However, in the present study, Lys and Met were supplemented in CSM-containing diets to exceed the established requirements of juvenile cobia for Lys at 2.33% of the diet [23] and for Met at 1.24% of the diet [32]. In the present study, the analyzed concentrations of Lys and Met in the diets did not vary greatly, with values of 2.30–2.54% and 0.53–0.78%, respectively (Table 2). Lys was higher than the previously quantified requirement values. Met was lower than the quantified requirement values; however, given that the Met level of CSM100 was even higher than that of Control, the lower performance of fish in these groups was unlikely to be due to the amino acid limitation. Moreover, the whole-body Lys and Met concentrations of fish in CSM75 and CSM100 groups were even higher than the Control (Table 5), indicating that the uptake of Lys and Met from the diets was not affected by CSM, and these supplemented amino acids were effectively assimilated and deposited in cobia.

Another possible reason for the lower performance in the feed utilization and growth of cobia fed CSM 75 and CSM 100 diets could be the negative effects of increased gossypol in the diet. The anti-nutritional effects of gossypol in cottonseed are well established. Fish have a suitable tolerance range for dietary-free gossypol, and performance may be damaged when the dietary-free gossypol varies over this range. However, the gossypol concentration in the CSM used in the present study was relatively low, much lower than regular CSM, and thus far less than the toleration limitation. Previous studies found that dietary incorporation of regular CSM in the replacement of soybean meal at a level of 100% (647 mg gossypol/kg diet) did not impair the survival of the common carp *Cyprinus carpio* [43], and the replacement of FM with regular CSM up to 100% (9160 mg gossypol/kg diet) did not impair the survival of juvenile tilapia *Oreochromis* sp. [44]. The dietary gossypol concentration up to 900 mg/kg diet produced no reduction in the growth of channel catfish [45]. Alam et al. [46] found that a gossypol level of a 3466 mg/kg diet did

not significantly affect the survival of the juvenile southern flounder *Paralichthys lethostigma*. Anderson et al. [9] was able to replace 100% of the FM protein with low-gossypol CSM protein supplemented with lysine, without negatively affecting the growth performance of the juvenile black sea bass *Centropristis striata*. These findings suggest that a variety of freshwater and marine finfish species can tolerate relatively high levels of gossypol. In the present study, the gossypol content in experimental diets ranged from a 29 to 120 mg /kg diet, which is much lower than the values in the studies mentioned above.

The activity of AST and ALT could be closely related to the transferring of amino acids. Elevated levels of AST and ALT have been used as indices for the diagnosis of liver function and damage [47]. Zhang et al. [48] found significant elevations in serum AST and ALT activities in crayfish fed with high cottonseed meal diets. Whereas, in the present study, the plasma AST and ALT activities of cobia fed CSM75 and CSM100 diets were similar to that of fish fed the Control. This result was consistent with Bu et al. [10], who found that the aminotransferase activities of AST and ALT in juvenile Ussuri catfish *Pseudobagrus ussuriensis* decreased with the increasing dietary cottonseed meal inclusion levels, and the lowest ALT and AST activities were observed in fish fed a diet with 60% of protein from fishmeal replacement by CSM. Li et al. [16] also found that the plasma AST activity of catfish gradually declined with the raising dietary CSM content. Therefore, in the present study, it was unlikely that the gossypol content would be a factor limiting the performance of cobia.

In the present study, the condition indices, whole-body protein and lipid composition of juvenile cobia were decreased with the increasing CSM which appeared to be associated with reduced weight gain. These responses are in line with our previous findings with red drum fed incremental levels of cottonseed flour [7]. Reductions in these indices could be due to several reasons, although the specific reason was not identified. The first possibility is that the potential toxicological effects of dietary gossypol have been shown to impair the protein and lipid deposition of fish. For example, black sea bass juveniles fed a 100% regular-CSM and protein-based diet showed a lower whole-body lipid level than fish fed a control FM-protein-based diet [9], suggesting the lower utilization of lipids from CSM in this species. Also, lipid peroxidation can hinder the normal metabolism and deposition of lipids. MDA is a final product of lipid peroxidation in the cells, and has a strong biotoxicity, causing body damage [49]. MDA is widely used as indicators for lipid peroxidation and as a marker of oxidative stress [50]. Zhang et al. [48] found that the dietary high-CSM significantly increased hepatopancreas MDA content in crayfish, which suggested that oxidative stress was induced when subjected to high cotton meal diets. Bu et al. [10] found the MDA concentrations of catfish *Pseudobagrus ussuriensish* fed diets with 30% or less of the dietary protein from FM replaced by CSM were lower than the Control, and the MDA concentrations were significantly higher than the Control only when the CSM levels exceeded 50%. In the present study, the MDA concentrations in fish fed diets containing 50% or higher of their protein from CSM were lower than that of the Control, indicating that the inclusion of CSM was unlikely to disturb the lipid metabolism of fish. The detrimental results might be explained by a species-specific difference in tolerance to free gossypol content.

A second possibility is that the potential toxicological effects of dietary gossypol may cause a stress status of fish which leads to an increase in energy consumption; this consequently reduces the deposition of energy substances such as protein and lipid. Antioxidant enzymes constitute the first line of the enzymatic defense mechanism against free radicals to maintain the complex immune system of fish. In the course of the normal metabolism of organisms, the production and elimination of reactive oxygen species such as the superoxide anion radical, hydroxyl radical and hydrogen peroxide maintain the dynamic balance [51]. An increase in activities of antioxidant enzymes suggested that these enzymes were activated or mobilized, and fish were undergoing a stressful status. In a previous study, we fed juvenile large yellow croaker *Larmichthys crocea* with oxidized lipids, and found that the activities of hepatic catalase (CAT), SOD and MDA content were increased,

and the degree of the increase in these factors is positively correlated with the oxidation degree [36]. This finding suggested that anti-nutritional factors in the diet can cause an increment of anti-oxidant enzyme activity, which reflects a stressful condition of fish. In the present study, the SOD activities of fish in CSM75 and CSM100 groups were slightly higher than or comparable to that of the Control. The GSH-PX of fish in these groups was lower than that of the Control. Similarly, Bu et al. [10] found that as dietary CSM levels increased, liver SOD, CAT, GSH-Px and TAC activities of Ussuri catfish decreased. Zhang et al. [48] also found the activities of SOD, CAT and GSH-Px of juvenile crayfish *P. clarkii* were generally decreased with the increasing CSM substitution levels. Consistently, Xu et al. [15] found that the SOD, T-AOC and GSH-Px activities of *M. salmoides* were decreased with the increasing SCM inclusion levels. Li et al. (2023) reported that with the increasing dietary CSM levels, hepatic superoxide dismutase activities of juvenile catfish *H. wyckioides* were decreased Therefore, these results combined with the lower MDA content, as mentioned above, all suggested that the inclusion of CSM did not exert apparent oxidative stress on juvenile cobia in the present study.

5. Conclusions

In conclusion, the results of the present study showed that up to 20~30% of crude protein provided by FM in diets could be readily replaced by methanol-extracted CSM with Lys and Met supplementation, according to the second-order polynomial regression model analysis of WG, FE and PER against dietary CSM replacement level. CSM is a potentially cost-effective alternative plant protein for inclusion in the diet of cobia.

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