USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

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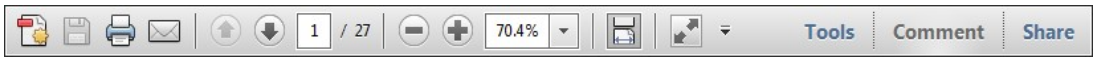
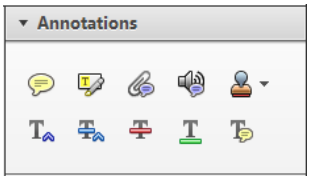
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

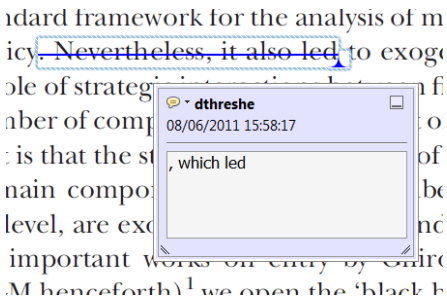


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

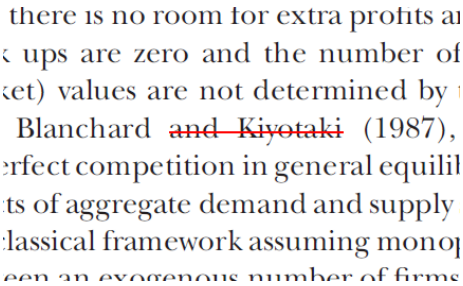


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

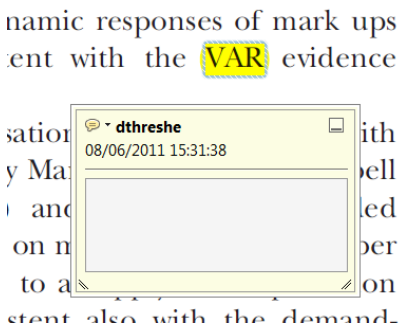


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

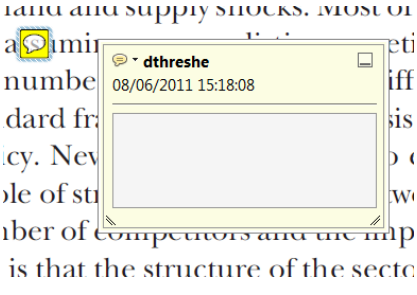


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

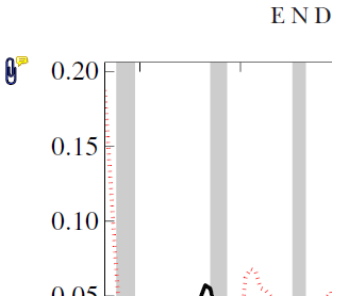


5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

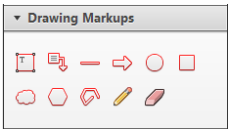
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



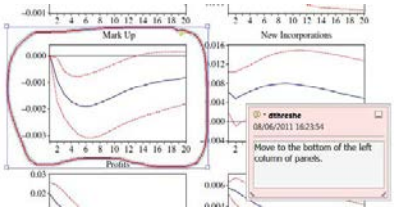
6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



Received: 27 February 2016 | Accepted: 27 September 2016

DOI: 10.1111/anu.12518

ORIGINAL ARTICLE

WILEY Aquaculture Nutrition

Effects of replacing fishmeal with different cottonseed meals on growth, feed utilization, haematological indexes, intestinal and liver morphology of juvenile turbot (*Scophthalmus maximus* L.)

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Funding information
Special Fund for Agro-scientific Research in the Public Interest, Grant/Award Number: 201303053; Shandong Provincial Natural Science Foundation, Grant/Award Number: JQ201206

Abstract

A ten-week feeding trial was conducted to evaluate the effect of replacing fishmeal with two differently processed cottonseed meals (CSM), namely XC and SC, separately in turbot (5.28 ± 0.02 g). Nine isonitrogenous and isoenergetic diets were formulated with 0% (FM), 15% (XC15, SC15), 25% (XC25, SC25), 35% (XC35, SC35) and 45% (XC45, SC45) of fishmeal replaced by CSM. Fishmeal was successfully replaced by XC in turbot diets without growth reduction at 35%, but not by SC even at 15%. The apparent digestibility coefficients of SC-included diets were significantly lower than XC-included diets at same replacement level. The activities of aspartate aminotransferase and superoxide dismutase were significantly affected in XC45 and SC45 group. The XC45 and SC-included diets caused shortened distal intestine villi height and structural damage of liver compared with FM diet. This study indicated that different processing methods could affect the nutritional value of CSM and effect as a protein source for turbot. CSM with high quality could be an important alternative dietary protein source for juvenile turbot.

KEYWORDS
apparent digestibility coefficients, cottonseed meal, growth performance, haematological, intestinal and liver morphology, turbot

1 | INTRODUCTION

Fishmeal is the major protein source in aquaculture diet because of its well-balanced amino acid composition, essential fatty acids and high palatability (Miles & Chapman, 2006). However, because of steadily rising prices, fishmeal has become the main limiting factor of aquaculture expansion. It has become an inevitable trend of replacing fish meal with less expensive and locally available plant protein sources.

Cottonseed meal (CSM) is often cited as one of the protein sources for its relatively high protein content, low cost per unit of protein and high availability throughout the world. The application of CSM as a substitute for fish meal has been assessed in many fish species, such as channel catfish, *Ictalurus punctatus* (Barros, Lim, & Klesius, 2002; Robinson, 1991), tilapia, *Oreochromis* sp. (Mbahinzireki, Dabrowski, Lee, El-Saidy, & Wisner, 2001), rainbow trout, *Oncorhynchus mykiss* (Rinchard, Lee, Czesny, Ciereszko, & Dabrowski, 2003; Rinchard, Lee, Dabrowski et al., 2003) and so on. These results showed that CSM could be used in aquatic animal diets but was limited by the potential toxic effects of free gossypol and its low levels of lysine and methionine content. However, the free gossypol content can be reduced by certain cooking procedures (Thurber, Vix, Pons, Crovetto, & Knoepfler, 1954). Gossypol toxicity can be effectively reduced by fermentation (Zhang, Xu, Zhao, Sun, & Yang, 2007) or by adding ferrous sulphate (El-Saidy & Gaber, 2004) to diets. The protein quality of cottonseed meal can be improved by supplementation of lysine and methionine (Li & Robinson, 2006).

Turbot (*Scophthalmus maximus* L.) is the widely cultivated commercial flatfish around the world, and its annual production has been up to 76,000 tons in 2013 according to FAO. The aquaculture of turbot

has been developed into one of the dominant mariculture industries in northern China from its introduction in 1992 till now, and the annual production has been over 60,000 tons since 2009 (Lei, Liu, & Guan, 2012). Meanwhile, a series of studies have reported that the turbot has a high protein requirement cat about 500–600 g/kg of the diet (Burel et al., 2000; Lee, Cho, Park, Kim, & Lee, 2003; Regost, Arzel, & Kaushik, 1999). This needs high content of fishmeal in its diet.

To date, there have been no preliminary studies on the replacement of fishmeal by CSM in turbot. Many reports pointed out that the nutritional value of CSM varies depending upon the method of oil extraction, proportion of husks and lint and degree of cortication (Nagalakshmi, Rao, Panda, & Sastry, 2007). In this study, upland cotton (*Gossypium hirsutum* L.), a worldwide spread cotton species (Wendel, Brubaker, & Percival, 1992), was processed differently in Xinjiang and Shandong, respectively, thereby named XC and SC, were evaluated as potential substitute for fishmeal protein in turbot diet.

2 | MATERIALS AND METHODS

All experimental protocols for animal care and handling used in this study were approved by the Animal Care Committee of Ocean University of China.

2.1 | Diet formulations

Using red fish meal, CSM and wheat gluten meal as main protein sources, fish oil and soy lecithin as main lipid source, wheat meal as carbohydrate source, nine isonitrogenous (approximately 500 g/kg) and isoenergetic (approximately 21.0 kJ/g diet of gross energy) diets were formulated. Two different CSM, XC and SC, were used. The experimental diets were formulated to produce diets in which 0% (FM), 15% (XC15, SC15), 25% (XC25, SC25), 35% (XC35, SC35) and 45% (XC45, SC45) of proteins from fishmeal were replaced with that from CSM, respectively. The diets were supplemented with lysine, methionine, isoleucine, leucine and threonine (crystal amino acid) so that they were similar to the control (FM). Y_2O_3 (1 g/kg) was supplemented as the indicator for the dry matter and crude protein digestibility determination following previous studies (Glencross et al., 2007). Procedures for diet preparation and storage were as previously described (Ai et al., 2011). All formulations are shown in Table 1.

The processes of producing CSM involved three major phases, dehulling, cooking and pressing. The XC retained lower hull content than SC. The dehulled cottonseed were cooked under the usual conditions employed at each mill, where cooking temperatures ranged from 100°C to 105°C and time at about 40 min in the process of XC, while cooking temperatures from 115°C to 120°C, and time at about 75 min in the process of SC. Proximate and amino acid composition of XC and SC were shown in Table 2.

2.2 | Experimental procedure

Juvenile turbots were purchased from a fish rearing farm (Yantai, China). Experiments were carried out in Haiyang Yellow Sea Aquatic Product ~~CO~~ Ltd (Yantai, China). All fish were acclimated to laboratory conditions for 2 weeks feeding the commercial diets before experiments. After being fasted for 24 hr, fish with an average initial weight of 5.28 ± 0.02 g were selected and randomly assigned to 27 experimental fibreglass tanks (500 L capacity, with a bottom diameter of 0.8 m and depth of 1 m) with 35 fish per tank. Seawater passed through sand filters flowing into each tank at approximately 3.0 L/min. Each treatment was randomly assigned to three replicate groups. Fish were manually fed to apparent satiety two times daily at 7:00 and 19:00 for 10 weeks. The faeces waste was cleaned after feeding. During the experimental period, the water temperature ranged from 20°C to 23°C, salinity ranged from 29 to 32 g/L, and dissolved oxygen was above 6 mg/L.

2.3 | Sample collection

Faecal samples were collected from the fifth week from each tank, with an automatic faecal collector by siphoning after feeding 3–4 hr. Collected faecal samples were stored at −20°C until analysis.

When the feeding trial was completed, all of the fish were starved for 24 hr. Then, total number and total body weight of fish in each tank were measured. Six fish were randomly sampled from each tank and stored at −20°C for whole body composition analysis.

Two fish from each tank (six fish per treatment) were randomly sampled and anaesthetized with benzocaine (30 mg/L) to measure individual body weight, body length, visceral weight and liver weight so as to calculate condition factor (CF), viscerosomatic index (VSI) and hepatosomatic index (HSI).

Blood samples were taken from the caudal vein of another anaesthetized six fish from each tank using heparinized syringes. Then, the blood were centrifuged at 4,000 *g* for 10 min at 4°C to obtain serum samples and stored at −80°C until analysis. Meanwhile, the liver and back muscle were sampled and frozen in liquid nitrogen, then at −80°C for tissue gossypol analysis.

2.4 | Analytical methods

2.4.1 | Biochemical analysis

Proximate composition of ingredients, diets, fish samples and faeces was preformed according to the methods described before (Liu et al., 2014). Dry matter was determined by drying the samples at 105°C for 24 hr. Crude protein content was analysed using the Kjeldahl method (Kjeltec TM 8400, FOSS, Sweden) to measure the nitrogen and calculated as $N \times 6.25$. Crude lipid content was examined by diethyl ether extraction using Soxhlet method (Buchi 36680, Switzerland). Ash content was measured after combustion at 550°C for 16 hr in a muffle furnace. Crude fibre content was detected according to AOAC (1995). Protein solubility of CSM was tested according to the method described before (Araba & Dale, 1990) with some modifications. It was expressed as a percentage of the total protein soluble in a 0.2% solution of KOH. Amino acids in ingredients and diets were analysed by an amino acid analyser (L-8900, Hitachi).

TABLE 1 Formulation and proximate compositions of the experimental diets (g/kg dry matter)

	Amount (g/kg dry diet) in each treatment								
Ingredients	**FM**	**XC15**	**XC25**	**XC35**	**XC45**	**SC15**	**SC25**	**SC35**	**SC45**
Fish meal [a]	620.0	527.0	465.0	403.0	341.0	527.0	465.0	403.0	341.0
Cottonseed meal(XC) [a]	0.0	106.7	177.8	249.0	320.1	0.0	0.0	0.0	0.0
Cottonseed meal(SC) [a]	0.0	0.0	0.0	0.0	0.0	118.4	197.4	276.4	355.3
Wheat meal [a]	280.0	245.3	224.6	202.8	180.7	231.0	199.5	167.0	134.3
Wheat gluten meal [a]	0.0	0.5	0.8	1.1	1.4	0.5	0.8	1.1	1.4
Fish oil	40.0	48.0	53.0	59.0	64.0	48.0	53.0	59.0	64.0
Soy lecithin	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
$Ca(H_2PO4)_2$	0.0	5.5	9.0	12.5	16.0	6.0	10.0	14.0	18.0
Amino acid premix [b]	0.0	7.0	9.8	12.6	16.8	9.1	14.3	19.5	26.0
Choline chloride	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Taurine	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin premix [c]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mineral premix [d]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Calcium propionic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ethoxyquinoline	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Attractants [e]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Sodium alginate	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Yttrium oxide	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Proximate composition									
Crude protein (g/kg)	504.9	504.0	502.7	500.1	504.0	504.4	507.3	498.6	506.6
Crude lipid (g/kg)	121.8	115.2	112.7	115.3	109.5	116.1	110.4	123.0	124.9
Ash (g/kg)	116.4	101.2	110.7	111.1	107.7	115.9	116.1	114.5	113.3
Gross energy (kJ/g) [f]	21.16	21.27	21.04	21.07	21.03	21.04	20.92	21.18	21.29
Free gossypol(mg/kg)	ND	120.99	193.11	295.89	350.18	130.07	227.33	300.22	378.44
Amino acids (g/kg)									
Arg	25.2	28.1	30.0	32.0	33.9	24.9	24.7	24.4	24.2
His	11.0	10.9	10.9	10.8	10.8	10.5	10.2	9.8	9.5
Ile	16.9	17.2	17.1	17.0	17.1	16.2	15.8	15.4	14.9
Leu	31.2	31.6	31.4	31.1	31.2	31.4	31.1	30.9	31.1
Lys	29.4	30.5	30.4	30.4	30.9	29.5	29.3	29.2	29.4
Met	11.5	11.6	11.5	11.3	11.3	11.6	11.5	11.4	11.5
Phe	17.0	17.4	17.8	18.1	18.4	16.8	16.8	16.7	16.6
Thr	16.6	17.0	17.0	17.0	17.2	16.8	16.8	16.8	17.0
Val	18.7	18.2	17.9	17.5	17.2	18.5	18.3	18.1	17.9
Trp	5.3	5.2	5.1	5.1	5.0	5.1	5.0	5.0	4.9

[a]Red fish meal (dry mater, g/kg): protein 738.0, crude lipid 100.5; wheat gluten meal (dry mater, g/kg): crude protein 842.0, crude lipid 13.4; wheat meal (dry mater, g/kg): crude protein 184.0, crude lipid 17.9. These ingredients were obtained from Great seven Bio-Tech (Qingdao, China). Cottonseed meal (XC) (dry mater, g/kg): crude protein 643.2, crude lipid 14.5. The ingredient was offered from ChangJi, XinJiang, China. Cottonseed meal (SC) (dry mater, g/kg): crude protein 579.5, crude lipid 14.5. The ingredient was purchased from Qingdao, Shandong, China.

[b]Amino acid premix of XC-based diets: lysine: methionine: isoleucine: leucine = 5:2:3:4; Amino acid premix of SC-based diets: lysine: methionine: threonine: leucine = 4:2:2:5.

[c]Vitamin premix (mg/kg diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 10; vitamin K, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 60; retinol acetate, 32; cholecalciferol, 5; alpha-tocopherol, 240; ascorbic acid, 2,000; microcrystalline cellulose, 1,473.

[d]Mineral premix: (mg/kg diet): $CoCl_2$ (1%), 50; $CuSO_4 \cdot 5H_2O$, 10; $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $MnSO_4 \cdot H_2O$, 45; $MgSO_4 \cdot 7H_2O$, 1,200; H_2NaOSe (1%), 20; H_2CaIO_4 (1%), 60; Zeolite powder, 8,485.

[e]Attractants (g/kg dry diet): betaine, 2; DMPT, 1; glycine, 1; alanine, 0.5; inosine-5′-diphosphate trisodium salt, 0.5.

[f]Gross energy of experimental diets was calculated according to gross energy values 23.64 kJ/g crude protein, 39.54 kJ/g crude fat, 17.57 kJ/g carbohydrate, respectively.

ND, not detected.

2.4.2 | Digestibility determination

Yttrium oxide contents in the diets and faeces were determined by acid digestion with perchloric acid and subsequently measured by inductively coupled plasma-atomic emission spectrophotometer (ICP-OES, VISTA-MPX).

2.4.3 | Haematological analysis

Activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analysed by Automatic biochemical analyser (CHEMIX-800, Sysmex Corporation, Kobe, Japan) using the Diagnostic Reagent Kits (Sysmex Corporation, Wuxi, China). The superoxide dismutase (SOD) was determined using WST-1 methods (the kit was obtained from Nanjing Jiancheng Engineering Institute Co., Ltd., China).

2.4.4 | Gossypol analysis

Free gossypol was analysed for ingredients, experiment diets, liver and muscle using the aniline method (Shawrang, Mansouri, Sadeghi, & Ziaie, 2011). In the presence of 3-amino-1-propanol, gossypol was extracted with a mixture of 2-propanol and hexane. It was then subsequently converted into gossypol-aniline with aniline. Finally, the absorbance of the compound was measured at the wavelength of its maximum absorbance (440 nm) by spectrophotometer (Ultrospec 2100 *pro*).

2.4.5 | Phytic acid analysis

Phytic acid content in CSM was determined according to modified colorimetric (Wade Regent) method (Gao et al., 2007) with minor modifications. Samples were thoroughly mixed with 2.4% HCl in 15-ml tubes for 16 hr. Then, the crude acid extracts were transferred to 15-ml tubes containing 1 g NaCl. The contents were shaken for 20 min and settled at −20°C for 20 min. The mixtures were centrifuged at 1,000 *g* at 10°C for 20 min. The clear supernatants were then determined by reaction with the Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ + 0.3% sulfosalicylic acid). Final colour development was measured at 500 nm with a spectrophotometer (Ultrospec 2100 *pro*).

2.4.6 | Intestinal and liver histology

Intestinal and liver were sampled and placed in Bouin's fixative solution (picric acid saturated solution: formalin: glacial acetic acid = 15:5:1) for histological evaluation and then transferred to 70% ethanol after 24 hr. About 5-mm-length segments of the fixed distal intestinal and liver tissue in 70% ethyl alcohol were cut off and then dehydrated in a series of alcohol solutions and embedded in paraffin, then sliced the tissue into 7-μm sections and mounted them onto albumin-coated slides. After stained the slices with haematoxylin and eosin (H&E), the morphological structures of these tissues were observed using an imaging microscope (Olympus, DP72; Nikon, ECLIPSE, E600, Japan).

All digital images were analysed using Image J version 1.36 (National Institutes of Health). At least six images from each group were analysed. Intestinal images were analysed to determine the ratio (R) between the villi height (VH) or microvilli height (MH) and the lumen diameter (LD) of the gut [R_1 = VH/LD, R_2 = MH/LD, arbitrary units (AU)]. A high R value indicates high villi height or microvilli height. The method to measure villi height and microvillus height was described previously (Peng et al., 2013). Liver images were analysed for hepatocyte area (HA).

2.5 | Calculations and statistical methods

The following variables were calculated:

$$\text{Survival rate (SR, \%)} = (\text{final fish number}/\text{initial fish number}) \times 100\%$$

$$\text{Weight gain rate (WGR, \%)} = (\text{final body weight} - \text{initial body weight})/\text{initial body weight} \times 100\%$$

$$\text{Specific growth rate (SGR, \%)} = \text{Ln}(\text{final body weight}/\text{initial body weight})/\text{days} \times 100\%$$

$$\text{Daily feed intake (DFI, \%)} = \text{dry feed intake}/[(\text{final body weight} + \text{initial body weight})/2]/\text{days} \times 100\%$$

$$\text{Feed efficiency ratio (FER)} = \text{wet weight gain (g)}/\text{dry feed intake (g)}$$

$$\text{Protein efficiency ratio (PER)} = \text{wet weight gain (g)}/\text{protein ingested (g)}$$

$$\text{Protein retention} = 100 \times (\text{final body weight} \times \text{final carcass protein content} - \text{initial body weight} \times \text{initial carcass protein content})/\text{protein intake.}$$

$$\text{Condition factor (CF, \%)} = \text{final body weight (g)}/\text{body length (cm)}^3 \times 100\%$$

$$\text{Hepatosomatic index (HSI, \%)} = \text{liver weight (g)}/\text{whole body weight (g)} \times 100\%$$

$$\text{Viscerosomatic index (VSI, \%)} = \text{viscera weight (g)}/\text{whole body weight (g)} \times 100\%$$

$$\text{Apparent digestibility coefficients (ADC, \%)} = (1 - Y_2O_3 \text{ in the diet}/Y_2O_3 \text{ in faeces} \times \text{nutrient in faeces}/\text{nutrient in diets}) \times 100\%$$

All statistical evaluations were analysed using one-way analysis of variance (ANOVA) by the software SPSS 19.0. Prior to the statistical tests, data were examined for homogeneity of variances. Differences

TABLE 2 Proximate composition and essential amino acid level of XC and SC (g/kg dry matter)

Ingredients	Crude protein	Crude lipid	Ash	Crude fibre	Protein solubility (%)[a]	Free gossypol	Phytic acid	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Try
XC	643.2	14.5	71	41.1	65.5	1.12	37.1	60.7	14.4	15.9	29.1	20.4	7.3	27.8	15.9	22.4	6.5
SC	579.5	14.5	75	72.4	53.1	1.1	34.8	29.3	9.7	16.1	11.4	14.9	3.3	20.8	10.9	21.7	5.7

[a]% crude protein.

between the means were tested by Tukey's multiple range tests. Differences were regarded as significant when $p < .05$. Data were expressed as means ± standard error.

3 | RESULTS

3.1 | Composition of XC and SC

As shown in Table 2, XC has higher crude protein, protein solubility and lower crude fibre content than SC. Most of the essential amino acids content in XC was higher than in SC, especially arginine (60.7 g/kg of dry matter versus 29.3 g/kg of dry matter) and leucine (29.1 g/kg of dry matter versus 11.4 g/kg of dry matter). Levels of antinutritional factors such as free gossypol (1.12 g/kg of dry matter in XC and 1.10 g/kg of dry matter in SC) and phytic acid (37.1 g/kg of dry matter in XC and 34.8 g/kg of dry matter in SC) were almost the same.

3.2 | Growth performance and feed utilization

As shown in Table 3, survival rate and daily feed intake (DFI) showed no difference among groups ($p > .05$). Fish fed diets containing 106.7 g/kg dry matter to 249.0 g/kg dry matter of XC (XC15-XC35, free gossypol of 120.99–295.89 mg/kg) showed no significant difference in final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), feed efficiency ratio (FER), protein efficiency ratio (PER) and protein retention (PR) compared with fish fed FM diet ($p > .05$). However, when the dietary XC level reached to 341.0 g/kg dry matter (XC45, free gossypol of 350.18 mg/kg), FBW of this group had 19.2% reduction than control group (FM). FER (−12.4%), PER (−12.5%) and PR (−14.8%) in this group were also significantly reduced compared with those of control group ($p < .01$). All SC-included diets, in which SC levels ranged from 118.4 g/kg dry matter to 355.3 g/kg dry matter (SC15-SC45) and the free gossypol content ranged from 130.07 to 378.44 mg/kg, significantly reduced FBW, WGR, SGR and PR of the turbot ($p < .01$), with lower FER and PER.

3.3 | Body index

No significant differences were found in the moisture (about 760 g/kg dry matter) of turbots among different groups ($p > .05$). Body crude protein content was reduced significantly when 45% of fishmeal protein was replaced by XC ($p < .05$). All the XC-included diets significantly reduced the body crude lipid content ($p < .05$) (Table 4), but body ash was not significantly affected ($p > .05$). All the SC-included diets significantly reduced the body crude protein and crude lipid content and improved the ash content ($p < .05$).

XC-included and SC-included diets significantly reduced fish condition factor (CF) compared to FM diet except for the SC15 group ($p < .05$) (Table 5). High levels of XC (XC35, XC45) and all SC-included diets significantly reduced fish hepatosomatic index (HSI) ($p < .05$), while viscerosomatic index (VSI) showed no differences among treatments.

TABLE 3 Growth parameters and feed utilization of juvenile turbot fed the experimental diets*

Treatments	Initial body weight (g)/IBW	Final body weight (g)/FBW	Weight gain rate (%)/WGR	Specific growth rate (%/day)/SGR	Survival rate (%)/SR	Feed efficiency ratio (g/g)/FER	Protein efficiency ratio (g/g)/PER	Daily feed intake (% body weight/day)/DFI	Protein retention (%)
FM	5.28 ± 0.01	63.1 ± 1.46^{ab}	1,094.27 ± 28.34^{ab}	3.54 ± 0.03^{ab}	100 ± 0.00	1.45 ± 0.02^{a}	2.88 ± 0.04^{a}	1.66 ± 0.03	48.38 ± 0.81^{a}
XC15	5.27 ± 0.01	67.03 ± 1.21^{a}	1,171.28 ± 22.11^{a}	3.63 ± 0.02^{a}	100 ± 0.00	1.45 ± 0.02^{a}	2.88 ± 0.03^{a}	1.68 ± 0.02	47.63 ± 0.75^{a}
XC25	5.28 ± 0.01	58.94 ± 0.87^{b}	1,015.64 ± 18.51^{b}	3.44 ± 0.02^{ab}	100 ± 0.00	1.41 ± 0.04^{ab}	2.80 ± 0.09^{ab}	1.70 ± 0.05	46.01 ± 1.32^{ab}
XC35	5.29 ± 0.02	58.5 ± 0.12^{b}	1,005.59 ± 2.92^{b}	3.44 ± 0.00^{b}	100 ± 0.00	1.38 ± 0.03^{abc}	2.77 ± 0.06^{abc}	1.72 ± 0.04	45.32 ± 0.56^{abc}
XC45	5.30 ± 0.01	50.98 ± 2.09^{c}	862.38 ± 40.19^{c}	3.23 ± 0.06^{c}	98.10 ± 1.90	1.27 ± 0.04^{cd}	2.52 ± 0.08^{cd}	1.82 ± 0.04	41.2 ± 0.55^{d}
SC15	5.28 ± 0.01	47.44 ± 1.27^{c}	798.14 ± 24.54^{c}	3.13 ± 0.04^{c}	100 ± 0.00	1.33 ± 0.02^{abcd}	2.64 ± 0.04^{abcd}	1.71 ± 0.02	42.49 ± 0.57^{bcd}
SC25	5.28 ± 0.01	45.28 ± 1.01^{c}	756.59 ± 17.20^{c}	3.07 ± 0.03^{c}	100 ± 0.00	1.33 ± 0.02^{abcd}	2.62 ± 0.03^{bcd}	1.70 ± 0.03	41.64 ± 0.57^{cd}
SC35	5.28 ± 0.00	50.28 ± 1.53^{c}	851.55 ± 28.88^{c}	3.22 ± 0.04^{c}	100 ± 0.00	1.29 ± 0.02^{bcd}	2.59 ± 0.04^{bcd}	1.79 ± 0.03	42.06 ± 1.06^{cd}
SC45	5.28 ± 0.01	45.50 ± 1.86^{c}	761.80 ± 36.19^{c}	3.08 ± 0.06^{c}	100 ± 0.00	1.25 ± 0.01^{d}	2.47 ± 0.02^{d}	1.81 ± 0.02	39.1 ± 0.50^{d}
ANOVA									
F	0.42	33.57	32.93	29.75	1.00	8.84	8.807	3.23	16.24
p	.89	.00	.00	.00	.47	.00	.00	.06	.00

*Values show mean ± standard error, $n = 3$; values in the same column with different superscripted small letters mean significant difference ($p < .05$).

3.4 | Apparent digestibility coefficients

As shown in Table 6, the dry matter apparent digestibility coefficients (ADC) of XC diets (63%–71%) and SC diets (31%–66%) were much lower than that of FM (90.17 ± 0.73%). In addition, the ADC of crude protein decreased significantly with increasing fishmeal-replacing levels in diets ($p < .01$).

3.5 | Haematological indexes

The activities of the serum enzymes are presented in Table 7. XC diets improved ALT significantly when the fishmeal protein was replaced at or above 25% level ($p < .05$), while SC-based diets did not affect ALT activity. Both XC45 and SC45 diets significantly increased the activity of AST and SOD ($p < .05$).

3.6 | Liver free gossypol level

With increased levels of dietary cottonseed, the free gossypol levels in liver also increased. Fish fed SC-included diets (13–91 mg/kg dry matter) had relatively higher liver free gossypol level than fish fed XC-included diets (11–63 mg/kg dry matter, Table 8).

3.7 | Intestinal and liver histology

Intestinal and liver samples of fish from different treatments were compared to those of the control group. As shown in Table 9 and Figure 1, the heights of villi and microvilli were shorter in all SC-included treatments ($p < .05$). In XC-included groups, shorter length of microvilli occurred in XC35 and XC45 group, while shorter length of villi occurred in XC45 group ($p < .05$).

Parenchyma structure of liver was damaged of fish fed XC45 and all SC-included diets. Also, there were a growing number of hepatic cells in smaller size (Figure 2) with increased levels of plant protein (Table 9). XC45 and SC-included diets significantly reduced the area of hepatic cells ($p < .05$).

4 | DISCUSSION

This experiment evaluated the potential of CSM as fishmeal substitutes in diets for turbot. The results of the present study indicated that CSM processed in Xinjiang could be incorporated in diets for juvenile turbot up to a level of 249.0 g/kg, which would replace 35% of fish meal, without significant negative effects on growth and feed utilization. However, CSM processed in Shandong province, significantly reduced the growth of turbot at 15% of the fishmeal protein replacement level (118.4 g/kg formulas content) in the turbot diets. In this study, fish presented a specific growth rate (SGR) higher than 3%, a relatively high SGR than many other studies (Day & Gonzalez, 2000; Oliva-Teles, Cerqueira, & Gonçalves, 1999; Yigit et al., 2006). This maybe attribute to appropriate temperature, water flow, stocking density, diets and some other reasons. In a study conducted by Irwin,

TABLE 4 Whole body composition of juvenile turbot fed the experimental diets (% wet weight)*

Treatments	Moisture	Crude protein	Crude lipid	Ash
FM	75.85 ± 0.15	16.46 ± 0.05^{a}	4.11 ± 0.11^{a}	3.68 ± 0.07^{a}
XC15	75.96 ± 0.28	16.24 ± 0.07^{ab}	3.70 ± 0.06^{b}	3.71 ± 0.03^{a}
XC25	77.22 ± 0.47	16.13 ± 0.04^{ab}	3.43 ± 0.02^{bcd}	3.65 ± 0.06^{a}
XC35	77.13 ± 0.48	16.05 ± 0.16^{abc}	3.47 ± 0.04^{bc}	3.81 ± 0.01^{ab}
XC45	76.77 ± 0.38	15.7 ± 0.14^{bc}	3.09 ± 0.10^{de}	3.88 ± 0.07^{ab}
SC15	76.75 ± 0.05	15.71 ± 0.04^{bc}	3.08 ± 0.03^{de}	4.00 ± 0.07^{b}
SC25	77.39 ± 0.73	15.53 ± 0.18^{c}	3.18 ± 0.09^{cde}	4.00 ± 0.03^{b}
SC35	76.91 ± 0.15	15.87 ± 0.16^{bc}	3.03 ± 0.07^{e}	4.00 ± 0.04^{b}
SC45	77.44 ± 0.20	15.49 ± 0.10^{c}	3.08 ± 0.08^{de}	4.06 ± 0.06^{b}
ANOVA				
F	2.29	8.01	26.08	9.51
p	.07	.00	.00	.00

*Values show mean ± standard error, $n = 3$; values in the same column with different superscripted small letters mean significant difference ($p < .05$).

TABLE 5 Hepatosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF) of juvenile turbot fed the experimental diets (%)*

Treatments	HSI	VSI	CF
FM	1.18 ± 0.01^{a}	5.20 ± 0.04^{ab}	3.40 ± 0.03^{a}
XC15	1.20 ± 0.04^{a}	5.04 ± 0.02^{a}	3.21 ± 0.03^{bc}
XC25	1.07 ± 0.01^{ab}	5.08 ± 0.05^{ab}	3.02 ± 0.02^{de}
XC35	1.03 ± 0.03^{b}	5.20 ± 0.06^{ab}	2.96 ± 0.02^{e}
XC45	0.99 ± 0.04^{b}	5.26 ± 0.05^{b}	3.07 ± 0.02^{cde}
SC15	0.75 ± 0.03^{d}	5.03 ± 0.02^{a}	3.29 ± 0.02^{ab}
SC25	0.95 ± 0.02^{bc}	5.04 ± 0.02^{a}	3.07 ± 0.02^{cde}
SC35	0.85 ± 0.03 ^{cd}	5.12 ± 0.01^{ab}	3.22 ± 0.06^{bc}
SC45	0.78 ± 0.03^{d}	5.23 ± 0.01^{b}	3.15 ± 0.04^{bcd}
ANOVA			
F	31.28	6.05	20.23
p	.00	.00	.00

*Values show mean ± standard error, $n = 3$; values in the same column with different superscripted small letters mean significant difference ($p < .05$).

TABLE 6 Apparent digestibility coefficients (%, ADC) for dry matter and crude protein of the experimental diets*

Treatments	Dry matter	Crude protein
FM	90.17 ± 0.73^{a}	97.77 ± 0.15^{a}
XC15	70.99 ± 0.33^{b}	93.17 ± 0.13^{b}
XC25	67.70 ± 1.54^{bc}	90.90 ± 0.02 ^{cd}
XC35	63.10 ± 1.09^{d}	90.96 ± 0.26^{c}
XC45	65.17 ± 0.72 ^{cd}	89.08 ± 0.52^{d}
SC15	65.87 ± 0.43 ^{cd}	92.80 ± 0.12^{b}
SC25	32.51 ± 0.67^{e}	85.17 ± 0.42^{e}
SC35	31.51 ± 0.66^{e}	84.81 ± 0.43^{e}
SC45	31.21 ± 0.66^{e}	82.11 ± 0.36^{f}
ANOVA		
F	784.46	178.46
p	.00	.00

*Values show mean ± standard error, $n = 3$; values in the same column with different superscripted small letters mean significant difference ($p < .05$).

O'halloran, and FitzGerald (1999) (Ireland), the SGR of the turbot was higher than 3% with water temperature and stocking density being similar with ours. Also, in another study, water temperature ranged between 17 and 21°C, and the SGR was also higher than 3% (Wang, He, Mai, Xu, & Zhou, 2015).

The reason accounting for the different effects of CSMs might be the different nutritional value (Table 2), which could differ among different processing methods (Forster & Calhoun, 1995). The hull content in the final meals determined the protein and fibre content (Thurber et al., 1954). Usually, high hull content in the CSM gives high fibre content and low protein content and vice versa. Researchers have pointed out that high fibre content of the ingredient resulted in low apparent dry matter digestibility in turbot (Wei et al., 2015) and hybrid striped bass (Sullivan & Reigh, 1995). Increased fibre intake decreased protein digestibility in human (Baer, Rumpler, Miles, & Fahey, 1997). In addition, deficiency of an essential amino acid led to poor utilization of the dietary protein (Halver & Hardy, 2002). Thus, high crude fibre content and amino acid deficiency (Table 2) in SC led to low digestibility of the diets (Table 6), which would finally affect the growth performance. Furthermore, the high content of arginine in XC could be another factor, and it has been reported that arginine supplemented diets had a stimulatory effect on fish growth (Plisetskaya, Buchelli-Narvaez, Hardy, & Dickhoff, 1991). In addition, cooking process usually affected the protein solubility (Milligan & Bird, 1951; Thurber et al., 1954). Generally, meals of high protein solubility resulted in good animal growth and meals of low protein solubility gave poor growth in chicks (Araba & Dale, 1990; Phelps, 1966). Thus, the lower protein solubility of SC might be another reason for the poor growth of turbot in SC-included groups. Thus, under the present experimental condition, turbots fed XC-included diets had better growth performance than turbots fed SC-included diets at the same replacement level.

TABLE 7 Serum indexes of juvenile turbot fed the experimental diets*

Treatments	ALT(U/L)	AST(U/L)	SOD(U/ml)
FM	1.67 ± 0.67^{a}	10.00 ± 0.58^{a}	25.21 ± 1.74^{a}
XC15	3.50 ± 0.50^{ab}	11.50 ± 0.50^{a}	26.48 ± 0.70^{a}
XC25	4.67 ± 0.33^{b}	13.50 ± 2.50^{a}	25.62 ± 0.64^{a}
XC35	4.67 ± 0.67^{b}	14.50 ± 2.50^{a}	26.81 ± 0.53^{a}
XC45	5.00 ± 0.58^{b}	27.33 ± 2.33^{b}	55.75 ± 1.64^{b}
SC15	3.00 ± 0.00^{ab}	12.33 ± 1.45^{a}	24.93 ± 0.84^{a}
SC25	3.00 ± 0.58^{ab}	12.50 ± 2.50^{a}	26.79 ± 0.68^{a}
SC35	4.00 ± 0.58^{ab}	13.67 ± 1.20^{a}	24.06 ± 2.33^{a}
SC45	3.00 ± 0.58^{ab}	32.50 ± 0.50^{b}	54.50 ± 1.42^{b}
ANOVA			
F	4.05	20.19	90.03
p	.01	.00	.00

*Values show mean ± standard error, n = 3; values in the same column with different superscripted small letters mean significant difference (p < .05).

Significant variation in body composition was observed with the increasing levels of substitution in our study. It is in agreement with the results in African bonytongue (Monentcham et al., 2010), common carp (Wang et al., 2014) and hybrid tilapia (Yue & Zhou, 2008). The reasons for the observed lower body protein in groups fed CSM-containing diets in the present study could be the low level of amino acids contents and the gossypol in CSM. Although experimental diets were supplemented with crystalline amino acids to the levels of control diet, differences in the time course of absorption of different amino acids could reduce the efficiency of protein synthesis (Ambardekar, Reigh, & Williams, 2009). Moreover, researchers had claimed that gossypol negatively regulated phospho-mTOR and phospho-p70S6K1 in colon cancer cell lines (Yang et al., 2015), which finally suppress cell growth via the regulation of protein synthesis (Jastrzebski, Hannan, Tchoubrieva, Hannan, & Pearson, 2007). With the increasing levels of the dietary CSM, the free gossypol, which would damage the liver, accumulated in liver obviously in flounder (Pham, Lee, Lim, & Park, 2007), channel catfish (Robinson, 1991), common carp (Wang et al., 2014) and turbot (Table 8). In this experiment, there was a larger than expected difference in liver free gossypol between SC15 and SC25. It was possible that the tolerance threshold of turbots for SC might lie between SC15 and SC25. Over dosage could cause pathological damages to liver and its function, which resulted in accumulation of liver gossypol. Similar results were reported in previous studies. For example, there was a large difference in free liver gossypol content between 18% and 27% cottonseed meal level in an experiment conducted by Wang et al. (2014). Liver damage could consequently lead to declined CF and reduced lipid synthesis in this study.

TABLE 8 Free gossypol content (mg/kg dry matter) in liver of juvenile turbot fed the experimental diets*

Treatments	Free gossypol
FM	ND
XC15	11.87 ± 1.29^{a}
XC25	20.45 ± 1.53^{a}
XC35	45.37 ± 2.94^{b}
XC45	62.54 ± 2.89^{c}
SC15	13.74 ± 1.80^{a}
SC25	41.48 ± 3.41^{b}
SC35	47.61 ± 1.17^{b}
SC45	90.62 ± 0.91^{d}
ANOVA	
F	173.46
p	.00

*Values show mean ± standard error, n = 3; values in the same column with different superscripted small letters mean significant difference (p < .05). ND, not detected.

TABLE 9 Distal intestinal and liver histology indexes of juvenile turbot fed the experimental diets*

Treatments	R_1 (10^{-1})	R_2 (10^{-3})	Hepatocyte area (μm^2)
FM	4.45 ± 0.15^{a}	1.66 ± 0.10^{a}	99.11 ± 8.26^{a}
XC15	4.26 ± 0.12^{abc}	1.45 ± 0.08^{ab}	99.12 ± 3.22^{a}
XC25	4.37 ± 0.07^{ab}	1.63 ± 0.08^{a}	86.80 ± 4.63^{ab}
XC35	4.32 ± 0.15^{abc}	1.18 ± 0.06^{bc}	72.37 ± 5.12^{abc}
XC45	3.69 ± 0.17 ^{cd}	0.92 ± 0.09^{c}	52.49 ± 6.66 ^{cd}
SC15	3.69 ± 0.18 ^{cd}	1.12 ± 0.07^{bc}	61.96 ± 7.92^{bcd}
SC25	3.67 ± 0.12 ^{cd}	0.94 ± 0.05^{c}	56.62 ± 9.97^{bcd}
SC35	3.74 ± 0.09^{bcd}	1.04 ± 0.06^{c}	42.93 ± 2.37 ^{cd}
SC45	3.25 ± 0.11^{d}	1.13 ± 0.09^{bc}	40.77 ± 4.45^{d}
ANOVA			
F	9.87	12.68	12.83
p	.00	.00	.00

*Values show mean ± standard error, n = 3; values in the same column with different superscripted small letters mean significant difference (p < .05). R_1 = villi height/lumen diameter; R_2 = microvilli height/lumen diameter.

The damaged liver by the increasing addition amounts of CSM in the present study was also featured by the reduced hepatosomatic index, structural damage and the serum indexes. The designed 45% level of CSM-included diet led to smaller liver cell and structural damage of liver in turbot. Wang et al. (2014) reported that the common carp liver cell showed volume shrinkage and increased number with the increased levels of dietary CSM. A significant increase in the activities of serum AST and ALT might further indicate a damage of liver function. In this experiment, high CSM-included diets improved the activities of serum ALT and AST significantly. The significantly increased AST activity of common carp fed diets containing CSM was observed by Wang et al. (2014). All these data indicated that high levels of dietary cottonseed meal might damage the liver structure and function.

As the biggest difference between the two CSM was protein quality, therefore we only focused on the ADC of dry matter and crude protein. Li and Robinson (2006) reported that the apparent digestibility coefficients of dry matter and crude protein of cottonseed meal-included

diets were between 34%–70% and 74%–85%. The present results, dry matter ranging from 31% to 71% and protein ranging from 82% to 93%, agreed with them. CSM was less digestible by fish and crustaceans than soybean meal. This was probably attributed to the high fibre content in CSM, because carnivorous fishes possess limited ability to digest starch or fibre in plant materials. Studies had revealed that apparent dry matter digestibility varied significantly among ingredients of both plant and animal origin and appeared to be negatively related to fibre and starch content of the ingredient in turbot (Wei et al., 2015) and hybrid striped bass (Sullivan & Reigh, 1995). Another reason might be the existence of gossypol, for apparent digestibility coefficients appeared to be negatively related to gossypol content in the diets of rainbow trout (Cheng & Hardy, 2002), tilapia (Mbahinzireki et al., 2001) and turbot (Table 6). In this trial, there was a large drop in dry matter digestibility between SC15 and SC25, a similar phenomenon with free gossypol accumulation in the liver as mentioned above. Over dosage of SC could also cause pathological damages to intestine and resulted in markedly reduced diet digestibility. In an experiment conducted by Yue and Zhou (2008), there was also a large drop in DM digestibility between CSM45 and CSM60. Our results were consistent with this. Antioxidant enzymes are components of an organism for combating oxidative stress which may be a reaction in response to external stimuli (Johnson, 2002). Zheng, Wen, Han, Li, and Xie (2012) reported an increase in activity and gene expression levels of antioxidant enzymes in fish fed with high CSM-included diets. In the present study, the activity of SOD, which can reduce the damage to the body from the oxygen free radicals, increased significantly at the highest substitution levels. It indicated that oxidative stress occurred when 45% fishmeal protein was replaced by CSMs in turbots.

The distal intestine histology indexes, villi height, microvilli height and microvilli density decreased significantly when fish meal protein was replaced at high levels by CSM. Consequently, the protective barrier of the intestinal epithelium would diminish and the enteritis-like effect was induced. Literature regarding the effect of cottonseed meal on intestine histology is limited. However, many researchers have illuminated that the structure of distal intestine of fish would be damaged when fed high plant protein-contained diets in Atlantic salmon (Baeverfjord & Krogdahl, 1996), Asian seabass (Boonyaratpalin, Suraneiranat, & Tunpibal, 1998), rainbow trout (Merrifield, Dimitroglou, Bradley, Baker, & Davies, 2009) and turbot (Peng et al., 2013).

The gossypol concentration in muscle was tested in the present study. However, it was too low to be detected. Lee et al. (2006) also showed that <1.0 μg gossypol/g wet basis of muscle was found in the highest dietary cottonseed meal group after 3 years of feeding. These results indicated that the fillet produced by fish fed cottonseed meal-included diets should be safe for customers.

COLOUR

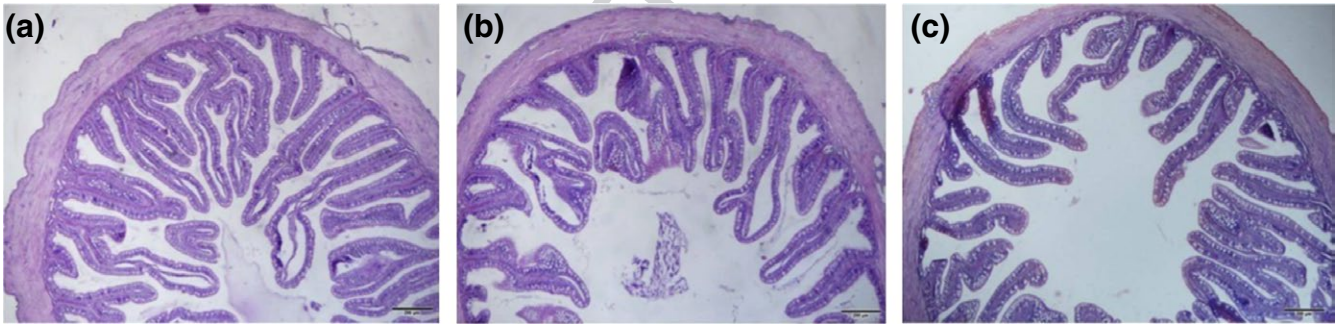

FIGURE 1 Intestine sections of turbots fed different experimental diets. Sections were stained by haematoxylin and eosin. a, b and c were sections from fish fed diet FM, XC45 and SC45, respectively. Scale bars, 200 μm

COLOUR

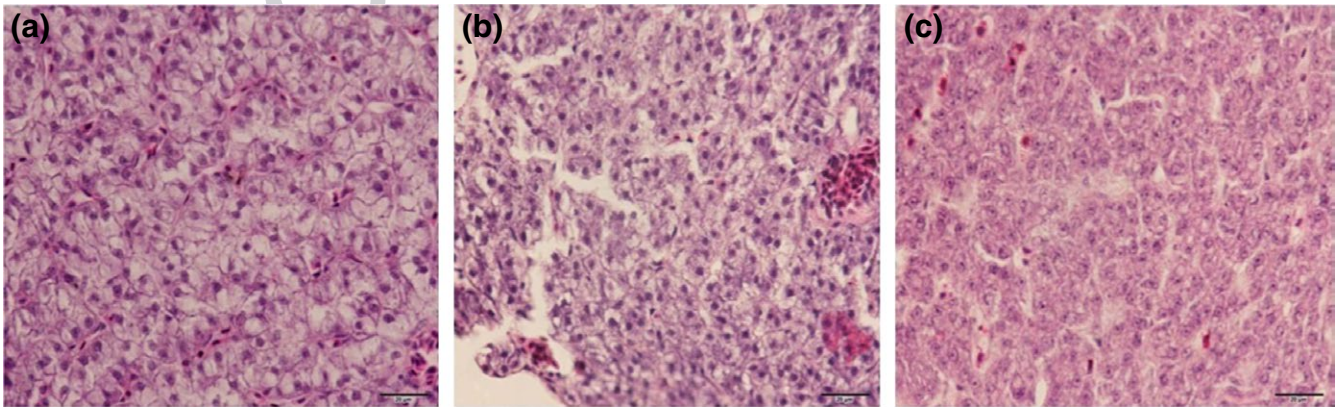

FIGURE 2 Liver sections of turbots fed different experimental diets. Sections were stained by haematoxylin and eosin. a, b and c were sections from fish fed diet FM, XC45 and SC45, respectively. Scale bars, 20 μm

In conclusion, it has been shown that different processing methods affected the nutritional value of CSM, which will finally affect its effect on turbot growth. A safe level of the cottonseed meal processed in Xinjiang in the diet of turbot was 249.0 g/kg of dry matter, which would replace 35% of fish meal, without having a significant negative effect on growth and feed utilization, while adding cottonseed meal processed in Shandong at 118.4 g/kg of dry matter significantly affected the growth of turbot. Although high levels of dietary cottonseed meal may reduce growth, damage the liver function and structure, CSM with high nutritional value (high protein content, high protein solubility and low fibre content) should be a promising plant protein resource for turbot with its relatively lower price and high yield.

ACKNOWLEDGEMENT

We thank the Xinjiang Tycoon Group for providing Xinjiang cottonseed meals. This study was supported by Special Fund for Agro-scientific Research in the Public Interest (grant number 201303053) and Shandong Provincial Natural Science Foundation (grant number JQ201206) to G.H.

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