Taurocholate supplementation attenuates the changes in growth performance, feed utilization, lipid digestion, liver abnormality and sterol metabolism in turbot (*Scophthalmus maximus*) fed high level of plant protein

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Abstract

Inclusion of plant ingredients in carnivorous fish feeds is often associated with decreased lipid digestibility, reduced bile acid levels and hypocholesterolaemia, which may result in lower growth performance and feed utilization. The present study aimed at evaluating the effects of dietary taurocholate supplementation in turbot (*Scophthalmus maximus*) fed high levels of plant protein and gaining further insight into how cholesterol and bile acid metabolism is modulated by taurocholate. A 56 day feeding trial was conducted using juvenile turbot (initial weight 8.5 g) fed one of three isonitrogenous and isolipid diets: a fish meal diet (FM), a plant protein meal based diet (a mixture of 40% soybean meal and 16% wheat gluten meal, named as PP) and a taurocholate supplemented diet (0.5% supplemented in PP, named as C-tau). Compared with fish fed the FM diet, the fish fed the PP diet had significantly lower growth performance, feed utilization, hepatosomatic index (HSI) and more atrophied hepatocytes. PP diet also induced enteritis in the distal intestine. Lipid digestion and metabolism was depressed as indicated by decreased whole body lipid content, lipid digestibility, pancreatic lipase activity and cholesterol and total bile acids (TBA) levels. Except the distal intestinal histology, all these tested parameters of fish fed the C-tau diet were significantly higher than those of fish fed the PP diet. Furthermore, the HSI, whole body lipid content, lipid digestibility and TBA levels of C-tau fed fish were similar to those of FM fed fish. Regardless of taurocholate, dietary plant protein could significantly increase the expression of HMG-CoA reductase (*hmgcr*) and decrease the hepatic cholesterol 7 α -hydroxylase (*cyp7a1*). Compared with the fish fed PP diet, taurocholate supplementation significantly increased the expression of *cyp7a1*, but had no effect on *hmgcr* expression. In conclusion, dietary supplementation with taurocholate attenuated the plant protein meal induced negative effects on growth performance, feed utilization and lipid digestion and metabolism. In addition, the cholesterol and TBA-increasing effects of taurocholate could possibly be attributed to an improved digestion and absorption of cholesterol and TBA synthesis due to the up-regulation of *cyp7a1* expression.

Keywords: Turbot, Taurocholate, Plant protein, Cholesterol, Bile acid

1 Introduction

Turbot (*Scophthalmus maximus*) has become the most important cultured flatfish in Europe and Asia because of its high quality flesh and rapid growth, with a global production of around 70 000 t per year (FAO, 2013). Global aquaculture has continued to grow while the production of fish meal is stable. Therefore, replacing some of the fish meal with plant protein ingredients has become a focus (Hardy, 2010; Tacon and Metian, 2008). However, the use of plant protein sources for carnivorous fish species is still met with challenges as certain plant components may interfere with fish health and welfare (Krogdahl et al., 2010). Besides the negative effect on growth performance and intestinal function (Baeverfjord and Krogdahl, 1996; Gu et al., 2016; Hedrera et al., 2013; Urán, 2008; van den Ingh et al., 1991), inclusion of less refined plant protein ingredients such as soybean meal (SBM) in fish feeds has been reported to be associated with decreased lipid digestibility, reduced bile salt levels and hypocholesterolaemia, suggesting an altered regulation of lipid metabolic pathways (Iwashita et al., 2008; Kortner et al., 2013; Krogdahl et al., 2003; Murashita et al., 2013; Nguyen et al., 2015; Regost et al., 1999; Romarheim et al., 2008, 2006; Yamamoto et al., 2007; Yun et al., 2011) and paralleling with reductions in feed efficiency, fish growth and pathogen resistance (Maita et al., 1998, 2006; Yun et al., 2011). The increased economical and quantitative importance of dietary plant protein ingredients in commercial fish feeds clearly warrants research to counteract the observed alterations in lipid metabolism apparently caused by the use of plant protein sources, to improve utilization and production cost optimization, as well as fish health and welfare.

Bile acids have a variety of physiological roles, including micellar solubilization of dietary lipids, acting as cofactors of bile salt-dependent lipase, and accelerating proteolysis (Hofmann, 2009). They are synthesized in the liver from the precursor cholesterol, conjugated with glycine or taurine, and are released into the intestine upon ingestion of a meal. **Cholesterol 7 α hydroxylase (CYP7A1) is the rate-limiting enzyme in hepatic bile acid synthesis from cholesterol (Lu et al., 2000) and cholesterol could be obtained from diets (exogenous) and self-synthesis (endogenous), in which HMG-CoA reductase (HMGCR) is the rate limiting enzyme (Maita et al., 2006).** The proportion between conjugated (often called bile salts) and unconjugated bile acids may vary between different body compartments. The term bile acid is used consistently throughout the present study for the sum of free and conjugated bile acids. After being secreted into the intestine, most of the secreted bile acids are reabsorbed and transported back to the liver via the enterohepatic circulation system (Russell, 2003). In **mammals**, it is clear that bile acids have many functions other than as emulsifiers and excreting waste products of cholesterol metabolism (Hylemon et al., 2009). They increase protein hydrolysis and modulate mucus secretion as well as fluid and electrolyte absorption (Hofmann and Hagey, 2008). Bile acids are also signaling molecules and inflammatory agents that rapidly activate nuclear receptors and cell signaling pathways for lipid, glucose, and energy metabolism regulating (Chiang, 2009).

The growth suppressing effect of certain plant protein meals and soya-saponins have been reported to be accompanied by a depletion of bile acids in several fish species (Gu et al., 2014; Iwashita et al., 2009; Krogdahl et al., 2015; Romarheim et al., 2006, 2008; Yamamoto et al., 2007; Yun et al., 2011). In rainbow trout, dietary supplementation with bovine bile acids or synthetic taurocholate, which is the major bile salt composition in gallbladder bile of fish (Goto et al., 1996; Kortner et al., 2014), has been proved to prevent the decreased growth performance, feed utilization, lipid digestibility, bile acid levels and distal intestine and liver abnormalities caused by

soybean meal or soy antinutrients (Iwashita et al., 2008, 2009; Yamamoto et al., 2007). However, the situation is sometimes different in the Atlantic salmon. Kortner et al. (2016) stated that supplementing bovine bile salt could increase the plasma bile salt concentration, but neither purified taurocholate nor bovine bile salt could diminish the poor growth performance, lipid digestibility and intestinal inflammation caused by dietary plant protein. The effects of bile acid supplementation to plant protein based diets in turbot are currently unknown. It is also unclear how dietary bile acids may affect cholesterol metabolism in fish fed high levels of plant proteins. Therefore, the objectives of this study were to investigate effects of taurocholate supplementation on growth performance, feed utilization, liver and intestine histology, lipid digestion in turbot fed a diet with high levels of plant protein.

2 Materials and methods

2.1 Ethics statement

The experiment was performed according to the regulation of local and central government and was approved by the Institutional Animal Care and Use committee of the Institute of Oceanology, Chinese Academy of Sciences.

2.2 Experiment feed and animals

A fish meal based diet (diet FM) was formulated with 60% FM, and this diet was used as control. The other two isonitrogenous and isolipid diets were formulated to contain 15% FM and 56% plant protein meal (a mixture of 40% SBM and 16% wheat gluten meal). The two experimental diets were supplemented without or with 0.5% taurocholate (purity=95%, CAS: 145-42-6, Aladdin Co. Shanghai, China), respectively (Table 1). They were named as PP and C-tau, respectively. All ingredients (except fish oil, soybean oil and soybean lecithin) were first ground into fine powder through a 180 μ m mesh. And then, the ingredients (except fish oil, soybean oil and soybean lecithin) of each diet were blended thoroughly by hand. Fish oil, soybean oil and soybean lecithin were mixed thoroughly and this mixed oil was mixed with other ingredients thoroughly. At last, water was added into the mixture to produce stiff dough. Then the dough was pelleted by experimental feed mill (SKJ120, Shandong Minglun machinery factory) at the length and diameter of 2mm and dried for about 12 h at 45 $^{\circ}$ C. The dried pellets stored in freezer at -20 $^{\circ}$ C until used. All the experimental feed was prepared two weeks before the feeding trial.

Apparent disease-free juvenile turbot were obtained from a commercial farm in Haiyang, China and transferred to an indoor flow-through water system in the Haiyang Yellow Sea Aquatic Product Co., Ltd. Fish were acclimated to the system and fed with the FM diet for 2 weeks. After that, turbot with initial body weight of about 8.5 g were randomly distributed into 9 tanks, 30 fish per tank (filled with 300 L seawater). The seawater was pumped from coast adjacent, filtered through a sand filter and distributed to each tank at approximate 2.0 L/min. Each diet was fed to fish in three tanks. Fish were fed with the experimental diets to apparent satiation twice daily at 07:00 and 18:00. Any uneaten feed was collected 1 h after each meal, dried to constant weight at 70 $^{\circ}$ C and reweighed. Leaching loss from the uneaten feed was estimated by leaving five pellets of each diet in tanks without fish for 1 h, recovering, drying and reweighing. During the 56 day feeding trial, water temperature was 12-16 $^{\circ}$ C, pH 7.8-8.2 and salinity 28-30. All rearing tanks were provided with continuous aeration and maintained under natural photoperiod (14 h light/10 h

dark at the end of August and 11 h light/13 h dark at the end of October).

2.3 Sampling

The fish were weighed at start and at the end of the experiment. All experimental fish were anesthetized with eugenol (1:10,000) (Shanghai Reagent Co., Shanghai, China) before sampling. After 56 days of feeding, four fish of similar weights per tank were sampled and stored at -20 °C for whole-body composition analysis. Another six fish from each tank were randomly selected and blood samples were collected from the caudal vein using heparinized syringes to obtain plasma samples after centrifugation (4000 × g for 10 min) at 4 °C and immediately stored at -80 °C until analysis. Subsequently, the fish were killed with a blow to the head and sampled for biometric parameters. Individual body weight, body length and liver weight were recorded to calculate condition factor and hepatosomatic index. From these six fish, four were selected and their livers and distal intestines were removed individually and divided into two parts with nearly same weight. For each fish, one part of the liver and distal intestine was placed in 4% phosphate-buffered formaldehyde solution for 24 h, and subsequently stored in 70% ethanol until further processing for histology analysis. Another part was placed in RNA later (Ambion), stored at 4 °C for 24 h and then stored at -20 °C for gene expression analysis. In these two analyses, the liver and distal intestine from one fish was treated and stored individually and given specific code. For the liver and intestine samples from the fish in one tank, all four liver and intestine samples were used for histology analysis and two of four samples were used for gene expression analysis.

The remaining fish continued to be fed the experimental diets, and feces were filtered and collected by water siphons every 30 min from 6 h to 12 h after feeding. After collecting sufficient fecal matter for determination of lipid digestibility, twelve fish from each tank were dissected 2 h after feeding to collect intestinal digesta from proximal to mid intestine. The digesta samples were frozen in liquid nitrogen, stored at -80 °C until freeze-drying and subsequent homogenization. Homogenized samples were stored at -80 °C until analyzed for bile acid levels and pancreatic lipase activity.

2.4 Chemical analysis

Standard methods (AOAC, 1995) were used for analyzing experimental diets and sampled fish whole body composition. Moisture and ash content were determined gravimetrically to constant weight in an oven at 105 °C and 550 °C, respectively. Crude lipid was determined gravimetrically after extraction with ethyl ether (Extraction System B-811, BUCHI, Switzerland). Crude protein was determined by the Kjeldahl method with a FOSS Kjeltac System (2300, Sweden) using boric acid to trap released ammonia. Before analysis, the fish were first cut into small pieces (about 0.5 g one piece) and were used for moisture and ash content analysis. After the moisture analysis, the dried fish were grounded with a grinder (LFP-800T, Zhongguo Laifu machine factory, Shandong, China) and were used for protein and lipid content analysis. The feces samples were freeze-dried first and analyzed for the lipid content with the same method described above. Yttrium oxide levels in diets and feces were determined using Inductively Coupled Plasma-Atomic Emission Spectrometry (Perkin Elmer, USA) as described by Refstie et al. (1997).

2.5 Histology

Fixed liver and intestine tissue samples were processed according to standard histological

techniques and stained with hematoxylin and eosin (H&E). The liver sections were examined blindly with a light microscope and the cell diameters (60 measurements per individual) were recorded. For the intestine section, examination was conducted blindly with a light microscope using a continuous scoring scale from 0 to 10 as described by Penn et al. (2011). The following histological characteristics were evaluated: length and fusion of mucosal folds, cellular infiltration and width of the lamina propria and submucosa, enterocyte vacuolization and nucleus position within the enterocytes.

2.6 Cholesterol and total bile acid assays

The concentration of total cholesterol in plasma was measured by peroxidase–anti-peroxidase (PAP) complex method using commercial kits (Nanjing Jiancheng Bioengineering Co., Nanjing, China) according to the protocols of the manufacturer at a wavelength of 500 nm. Bile acid in the intestinal digesta powder was extracted with 90% ethanol, followed by extraction with chloroform:methanol (1:1, v/v), according to a method described by (Setchell et al., 1983). Total bile acid (TBA) levels in plasma and intestinal digesta were determined using commercial kits (Nanjing Jiancheng Bioengineering Co., Nanjing, China) according to the protocols of the manufacturer at a wavelength of 505 nm.

2.7 Pancreatic lipase activity assays

Pancreatic lipase (EC 3.1.1.3) in the intestinal digesta powder was extracted by homogenization into 4 volumes (v/w) of ice-cold distilled water. The homogenates were then centrifuged at 20 000 rpm for 15 min. The supernatant was further diluted tenfold with cold distilled water. Lipase activity was measured with external sodium deoxycholate added to the buffer as described by (Murashita et al., 2008). The unit of activity for the lipase was defined as the difference in optical density (OD_{405} ; $\Delta OD = U$ [units]) per mg dried digesta.

2.8 Quantitative real-time PCR

Total RNA was extracted and purified from liver tissue samples (approximately 50 mg) of FM, PP and C-tau fed fish (6 samples/diet treatment) using RNeasy protect Mini Kit (Qiagen, 74126) according to manufacturer's instructions. Quality and integrity of RNA were quantified using NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc.) and 2100 BioAnalyzer (Agilent Technologies, USA), respectively. The cDNA synthesis was performed with the QuantiTect Reverse Transcription Kit (Qiagen, 205311) using 1.0 µg RNA following the manufacturer indications. The expression of *hmgcr* gene and *cyp7a1* gene were analysed using the primers designed by Zhu et al. (2014) and Yun et al. (2012), respectively. The constitutively expressed ribosomal protein S4 (RPS4), demonstrated to be stably expressed in turbot (Robledo et al., 2014), was chosen as the reference gene for sample normalization. Individual real-time PCR reactions were carried out in 25 µl reaction volume using 12.5 µl of SYBR Green PCR Master Mix (QuantiTect SYBR Green RT-PCR Kit, Qiagen, 204243), 9.5 µl of ultrapure water (Sigma-Aldrich), 1.0 µl of each specific primer (10 µM) and 1.0 µl of cDNA template. The thermal profile for SYBR Green RT-PCR was 95 °C for 20 s, followed by 40 cycles of 95 °C for 5 s, 56 °C for 30 s, and 72 °C for 30 s. Fluorescence readings at the end of each cycle were used to estimate quantification cycle values (Cq). Raw Cq values were normalized to RPS4 using a relative quantitative method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001) and expressed as fold change.

2.9 Calculations

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

Specific growth rate, SGR (% body weight / day) = $100 \times (\ln(\text{final body weight}) - \ln(\text{initial body weight})) / \text{time (day)}$

Feed intake, FI (%/day) = $100 \times \text{total amount of the feed consumed} / ((\text{initial body weight} + \text{final body weight}) / 2) / \text{days}$

Feed efficiency ratio, FER = $\text{weight gained} / \text{total amount of the feed consumed}$

The biometric parameters were calculated as follows:

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

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

The apparent lipid digestibility was estimated indirectly using Y_2O_3 and calculated as follows:

Apparent lipid digestibility = $100 - 100 \times (M_{\text{feed}} / M_{\text{feces}})(N_{\text{feces}} / N_{\text{feed}})$

where M_{feed} and M_{feces} are the concentrations (%) of marker (Y_2O_3) in the feed and feces and N_{feed} and N_{feces} are the concentrations (%) of lipid in the feed and feces.

2.10 Statistics

JMP (version 10) were used for statistics. Data were subjected to one way ANOVA (analysis of variance) and differences between the means were tested by Tukey's multiple range tests. The level of significance was chosen at $P < 0.05$.

3 Results

3.1 Growth performance and feed utilization

During the feeding trial no mortality was recorded. The effects of the experimental diets on turbot growth performance and feed utilization are shown in Table 2. The growth performance (FBW, WG and SGR) of fish fed diet PP was markedly lower than that of fish fed diet FM ($P < 0.05$). The taurocholate supplementation significantly increased the growth performance ($P < 0.05$), although it was still significantly lower than that of fish fed diet FM ($P < 0.05$). Similar results were seen in FI. The FER of fish fed the PP diet was significantly inferior to that of fish fed the FM diet, while fish fed the C-tau diet attained the similar level of FER as fish fed the FM diet.

3.2 Biometric parameters and histology of liver and distal intestine

As presented in Table 3, the condition factor of fish fed the diets PP and C-tau was significantly lower than that of fish diet FM. The HSI of fish fed PP diet was significantly lower than that of fish fed diet FM, but was increased by taurocholate supplementation to comparable level of fish fed diet FM. The effects of the experimental diets on turbot liver histology and hepatocyte diameters are shown in Figure 1 and Table 3. The hepatocytes of fish fed FM had clearly eosinophilic cytoplasm filled with round or elliptic vacuoles. However, in fish fed PP diet, the hepatocytes were stained darkly, atrophied with significantly decreased diameters and vacuoles compared to the fish fed the FM diet. The histological appearance of the livers of fish fed C-tau diet was somewhat between those of fish fed FM diet and fish fed PP diet. The diameter of hepatocytes of fish fed C-tau diet was significantly higher than that of fish fed PP diet, but significantly lower than for fish fed the FM diet ($P < 0.05$). The effects of the experimental diets on turbot distal intestine histology are shown in Table 4. Confirming with our previous work, soybean

meal at the level of 40% significantly decreased height and increased fusion of the mucosal folds, increased width and cellular (leucocyte) infiltration of the lamina propria and submucosa, reduced numbers of supra-nuclear absorptive vacuoles in enterocytes and displaced nuclei toward the apexes of the cells, which are the characteristics of the SBM-induced enteritis. Dietary taurocholate could not help to solve the soybean meal induced enteritis. No significant difference was observed in all parameters among the three treatments ($P>0.05$).

3.3 Whole body composition and lipid digestibility

As shown in Table 5, the whole-body lipid content and lipid digestibility of fish fed the PP diet were significantly lower than those of fish fed the FM diet ($P<0.05$). The fish fed the C-tau diet showed similar levels of crude lipid whole-body composition and lipid digestibility with the fish fed the FM diet. No significant difference was observed in crude protein and ash content among the three treatments ($P>0.05$).

3.4 Pancreatic lipase activity in digesta

The pancreatic lipase activity in digesta of fish fed the PP diet was significantly lower than those of fish fed FM diet (Figure 2, $P<0.05$). The lipase activity of the fish fed C-tau diet was significantly higher than those fed PP diet ($P<0.05$), although the levels were still significantly lower than fish fed the FM diet ($P<0.05$).

3.5 Lipids profiles in plasma and total bile acids in digesta

The total cholesterol (T-CHO) in plasma and total bile acid (TBA) levels in plasma and digesta in fish fed PP diet were significantly lower than those fed FM diet (Table 6, $P<0.05$). The taurocholate supplemented group showed significantly higher levels of T-CHO than the PP group and similar levels as FM group in TBA level both in plasma and digesta ($P<0.05$). No significant difference was observed in plasma triacylglycerides among the three groups.

3.6 Expression of *hmgcr* and *cyp7a1* genes in liver and distal intestine

In liver, the mRNA expression of *hmgcr* was significantly up-regulated in fish fed PP diet (3.3 fold up) and C-tau (3.6 fold up) compared to fish fed the FM diet ($P<0.05$). No significant difference was observed between PP group and C-tau group. The mRNA expression of *cyp7a1* was significantly down-regulated in fish fed PP diet (3.2 fold down) and the changing trend was significantly alleviated by dietary taurocholate to 2.0-fold down compared to fish fed the FM diet ($P<0.05$). The situation in the distal intestine is same as in the liver.

4 Discussion

In the present study, growth performance, FI and FER of fish fed PP diet were significantly inferior to those of fish fed FM diet, which are consistent with several previous studies conducted on carnivorous fish species (Bowyer et al., 2013). The decreased growth performance of fish fed the PP diet was partly prevented by taurocholate supplementation. Positive effects of taurocholate on growth performance and FER have been also reported in rainbow trout fed with SBM based and casein-based diets (Iwashita et al., 2008, 2009). However, different with Iwashita et al. (2008), taurocholate in the present work could significantly increase the feed intake of turbot compared with those fed PP diet. This difference could be attributed to the different fish and dose of

311 taurocholate. Condition factor was decreased in turbot fed with PP diet and this phenomenon was
312 also observed by Bonaldo et al. (2011), who suggested that the lower CF could be due to a
313 reduced development of muscle in fish, leading to a lower thickness of fillets. The taurocholate
314 supplementation prevented the decreased HSI caused by plant protein meal and this result is in
315 accordance with the observation in rainbow trout (Iwashita et al., 2008). Plant ingredients could
316 also cause atrophied hepatocytes with decreased vacuole accumulation in rainbow trout fed the
317 diet with SBM (Iwashita et al., 2008, 2009; Ostaszewska et al., 2005; Yamamoto et al., 2007) and
318 in Atlantic salmon fed the diet supplemented with soya isoflavones (Gu et al., 2015). Iwashita et al.
319 (2008) reported that taurocholate supplementation prevented SBM-induced hepatocyte atrophy,
320 and similar results were also observed in the present study. The histology results also illustrated
321 that main content of the vacuoles in the hepatocytes was glucose, as suggested by periodic acid
322 Schiff (PAS) staining (Gu et al., 2015). Bakke et al. (2014) reported that bile could enhance the
323 baseline glucose transporter affinity, absorption and transport in Atlantic salmon intestinal tissue
324 ex vivo and suggested that this was at least partially due to taurocholate. Increased liver glycogen
325 stores could increase the primary source of blood glucose, which is another possible reason for the
326 growth promotion in C-tau fed fish compared to PP fed fish. However, increased HSI and
327 glycogen stores in hepatocytes may simply be caused by increased digestive function and/or feed
328 intake in C-tau fed fish.

329 The present work confirmed that soybean meal inclusion level of 40% could induce enteritis in
330 turbot (Gu et al., 2016) and taurocholate could not eliminate this distal intestine morphology
331 abnormal. Our observation is consistent with Kortner et al. (2016). However, Iwashita et al. (2008)
332 stated that taurocholate could normalize the intestinal morphology of rainbow trout fed the
333 SBM-based diet. As bile acids are natural endogenous ligands of farnesoid X receptor, which was
334 proved to relate with inflammatory response in murine intestinal mucosa (Gadaleta et al., 2011),
335 whether the similar mechanism exists in fish with different feeding habit need further work to be
336 cleared.

337 In the present study, the reduction of total bile acid level in the intestinal digesta of fish fed
338 plant protein meal is in accordance with results reported in Atlantic salmon (Kortner et al., 2013),
339 rainbow trout (Iwashita et al., 2008; Romarheim et al., 2008; Yamamoto et al., 2007, 2010) and
340 yellow-tail (Nguyen et al., 2011). Bile acids are important for lipid digestion and absorption as
341 they are essential for emulsification of lipids and micelle formation. Thus, the reduced lipid
342 digestibility in fish fed PP diet could, at least partly, be related to the insufficient supply of bile
343 acids in the intestine, which was modulated by the taurocholate supplementation in the present
344 study. Similar positive effect of dietary bile acids on lipid digestibility was also observed in
345 rainbow trout in previous studies (Iwashita et al., 2008; Yamamoto et al., 2007). Besides the role
346 in emulsifying ingested lipids, bile acids are also needed for the activation of lipases (Romarheim
347 et al., 2006). The decreased pancreatic lipase activity in fish fed PP diet suggested that insufficient
348 secretion of lipase may also be responsible for low lipid digestibility in PP fed fish and that
349 taurocholate supplementation may attenuate this situation. In the present study, the decrease of the
350 whole-body lipid content caused by plant protein meal inclusion is in accordance with
351 observations in many fish species, such as turbot (Regost et al., 1999), Japanese flounder (Deng et
352 al., 2006), Atlantic salmon (Espe et al., 2006) and Senegalese sole (Silva et al., 2009), and this was
353 modulated by the taurocholate supplementation. These results suggest that, in the present study,
354 taurocholate supplementation might enhance the utilization of dietary lipid (digestion and

absorption), and then increased lipid storage in juvenile turbot fed PP diet.

In the present work, dietary plant protein could decrease the cholesterol level in turbot, which was also observed by Yun et al. (2011), but the reason of this hypocholesterolemia effect was still not clear. Dietary bile acids could help to resolve this problem in two ways, cholesterol absorption and self-synthesis, as there are two sources of body cholesterol, diet (exogenous) and self-synthesis (endogenous) (Friedman et al., 1962). Cholesterol absorption is a complicate and step-by-step process. Before absorption into enterocytes, cholesterol traverses the unstirred water layer and reaches the brush border (Westergaard and Dietschy, 1974, 1976). This process is carried out by cholesterol carriers, predominantly micelles. The formation of micelles and cholesterol incorporation into micelles are therefore considered importantly for cholesterol absorption. As bile acids are essential for micelle formation, they are obligatory for cholesterol absorption (Westergaard and Dietschy, 1976). Positive effects of individual bile acid on cholesterol absorption have been observed in human (Leiss et al., 1984; Wang et al., 2006) and mice (Jones et al., 2012). For the cholesterol self-synthesis, HMG-CoA reductase (HMGCR) is the rate limiting enzyme (Maita et al., 2006). In the present study, the mRNA expression of *hmgcr* in fish fed the diet with plant protein sources, whether supplemented taurocholate or not, was significantly higher than those fed FM diet, but no significant difference was observed between the fish fed C-tau diet and PP diet. These results indicated that the fish fed the diet with plant protein sources may try to cover the dietary cholesterol deficiency by enhancing the self-synthesis, but this process was not further enhanced by taurocholate supplementation. Thus, the increased cholesterol body pool in fish fed C-tau diet compared with fish fed PP diet could be a result of increased cholesterol absorption by taurocholate. The down-regulation of *cyp7a1* after plant protein meal feeding observed in the present study is in accordance with the findings in turbot (Yun et al., 2012) and Atlantic salmon (Gu et al., 2014). Therefore, fish fed plant protein based diets may have lower conversion of cholesterol into bile acids. Taurocholate supplementation increased the expression of *cyp7a1*, which is similar to the result in rats (Chen et al., 2005). The increased TBA synthesis due to up-regulation of *cyp7a1* expression is possibly one reason for the increased TBA body pool of fish fed C-tau diet compared with those fed PP diet.

In conclusion, the present study showed that taurocholate supplementation partly prevented the decreases of growth performance, feed utilization, biometric parameters and the occurrence of histological abnormalities in liver of turbot fed the plant protein meal. Furthermore, the lipid digestion was improved by taurocholate supplementation, as proved by the increased lipid apparent digestibility and pancreatic lipase activity. In addition, the cholesterol and TBA-increasing effects of taurocholate could possibly be attributed to the improved digestion, absorption and conversion of cholesterol to bile acid.

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586 Table 1 Ingredients and compositions of experimental diets (dry-matter basis)

	Experimental diets ¹		
	FM	PP	C-tau0.5
<i>Ingredients (g kg⁻¹)</i>			
Fish meal ^a	600	150	150
Soybean meal ^b	0	400	400
Wheat gluten meal ^b	0	160	160
Wheat meal ^b	280	145	145
Fish oil ^c	20	60	60
Soybean oil ^b	20	10	10
Soybean lecithin ^b	20	20	20
Taurocholate ^d	0	0	5
Vitamin and mineral premix ^e	25	25	25
Monocalcium phosphate ^d	0	5	5
Choline chloride ^d	5	5	5
Yttrium premix ^d	1	1	1
Calcium propionic acid ^d	1	1	1
Ethoxyquin ^d	0.5	0.5	0.5
Cellulose ^d	27.5	17.5	12.5
<i>Proximate composition (%)</i>			
Crude protein	48.8	48.3	48.4
Crude lipid	12.5	12.4	12.6
Ash	12.5	11.2	11.5

¹ FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

^a Fish meal: steam dried fish meal prepared from anchovy and provided by COPENCA Group, Lima, Peru.

^b provided by QINGDAO GREAT SEVEN BIO-TECH. CO. LTD, Qingdao, China

^c provided by QINGDAO GREAT SEVEN BIO-TECH. CO. LTD, Qingdao, China and prepared from anchovy.

^d provided by Aladdin Co. Shanghai, China

^e provided by Qingdao Master Biotechnology Co. Ltd, Qingdao, China. Vitamin premix supplied the diet with (mg kg⁻¹ diet) the following compounds: retinyl acetate, 32; vitamin D₃, 5; DL- α -tocopherol acetate, 240; vitamin K₃, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitaminB₁₂ (1%), 10; Lascorbyl-2-monophosphate-Na (35%), 2000; calcium pantothenate, 60; nicotinic acid, 200; inositol, 800; biotin (2%), 60; folic acid, 20; cellulose, 11473. Mineral premix consisted of (mg kg⁻¹ diet) the following ingredients: FeSO₄ H₂O, 80; ZnSO₄ H₂O, 50; CuSO₄ 5H₂O, 10; MnSO₄ H₂O, 45; KI, 60; CoCl₂ 6H₂O (1%), 50; Na₂SeO₃ (1%), 20; MgSO₄ 7H₂O, 1200; calcium propionate, 1000; zeolite, 17485.

Table 2 Growth performance and feed utilization of turbot fed the experimental diets (n=3).

	FM	PP	C-tau	Pooled S.E.	<i>P</i> value
IBW (g)	8.50	8.51	8.50	0.03	0.931
FBW (g)	40.2 ^a	31.9 ^c	35.3 ^b	0.43	<0.001
SGR (% body weight / day)	2.77 ^a	2.36 ^c	2.54 ^b	0.07	<0.001
WG (%)	373 ^a	280 ^c	325 ^b	5.1	<0.001
FI	2.59 ^a	2.42 ^c	2.49 ^b	0.02	<0.001
FER	1.41 ^a	1.31 ^b	1.37 ^a	0.02	0.002

Mean values in the same row with different superscript letters are significantly different ($P<0.05$).

Abbreviations: IBW, initial body weight; FBW, final body weight; SGR, specific growth rate; WG, weight gain; FI, feed intake; FER, feed efficiency ratio; FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

Table 3 Condition factor, hepatosomatic index and diameters of the hepatocytes of turbot fed the experimental diets (n=3 or n=12).

	FM	PP	C-tau	Pooled S.E.	<i>P</i> value
Condition factor	1.92 ^a	1.75 ^b	1.79 ^b	0.02	0.015
Hepatosomatic index (%)	1.27 ^a	0.94 ^b	1.17 ^a	0.04	<0.001
Diameters of the hepatocytes (μm) *	14.6 ^a	12.1 ^c	13.4 ^b	0.22	<0.001

Mean values in the same row with different superscript letters are significantly different ($P<0.05$).

* Values are means (n=12, mean of 60 cells $\times$ 4 fish $\times$ 3 tanks).

Abbreviations: FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

Table 4 Distal intestine tissue variable scores of the turbot fed the experimental diets (n=12).

	FM	PP	C-tau	Pooled S.E.	<i>P</i> value
MF height	7.79 ^a	5.29 ^b	5.63 ^b	0.24	<0.001
MF fusion	2.41 ^a	4.25 ^b	4.41 ^b	0.18	<0.001
LP width	1.58 ^b	3.50 ^a	2.95 ^a	0.17	<0.001
LP cellular infiltration	1.46 ^b	3.29 ^a	2.96 ^a	0.15	<0.001
LP width	1.83 ^b	2.96 ^a	2.67 ^a	0.17	<0.001
LP cellular infiltration	1.54 ^b	3.12 ^a	2.92 ^a	0.19	<0.001
ent vacuolization	8.08 ^a	5.75 ^b	6.08 ^b	0.21	<0.001
ent nucleus position	2.04 ^b	2.95 ^b	3.16 ^a	0.16	<0.001

Mean values in the same row with different superscript letters are significantly different ($P<0.05$)

Abbreviations: MF, mucosal fold; LP, lamina propria; SM, submucosa; ent, enterocyte; FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

624

625 Table 5 Whole body composition and lipid digestibility of turbot fed the experimental diets (n=3).

	FM	PP	C-tau	Pooled S.E.	<i>P</i> value
Moisture	76.8	77.4	77.0	0.27	0.121
Crude protein	15.2	14.6	15.0	0.22	0.116
Crude lipid	4.47 ^a	3.83 ^b	4.37 ^a	0.17	0.020
Ash	3.77	4.27	3.77	0.21	0.081
Lipid digestibility (%)	88.9 ^a	86.6 ^b	89.1 ^a	0.48	0.003

626 Mean values in the same row with different superscript letters are significantly different ($P<0.05$).

627 Abbreviations: FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75%

628 fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

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Table 6 Lipid profiles in plasma and total bile acids level in digesta of turbot fed the experimental diets (n=3).

	FM	PP	C-tau	Pooled S.E.	<i>P</i> value
Plasma triacilglycerides (mmol/L)	3.92	4.03	3.05	0.41	0.103
Plasma total cholesterol (mmol/L)	4.92 ^a	3.02 ^c	3.84 ^b	0.27	0.001
Plasma total bile acids (μmol/L)	17.0 ^a	10.7 ^b	16.1 ^a	1.0	0.002
Digesta total bile acids (μmol/g dried digesta)	74 ^a	56 ^b	69 ^a	3.9	0.009

Mean values in the same row with different superscript letters are significantly different ($P<0.05$).

Abbreviations: FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

Fig. 1 Representative histomorphological images from hematoxylin and eosin-stained sections of the liver of turbot depicting the different degree of accumulation of vacuoles in hepatocytes in turbot fed the FM, PP and C-tau diets (bar = 50 μ m). Abbreviations: FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

Fig. 2 Lipase activity in digesta of turbot fed the experimental diets. Data are expressed as mean and standard error (n=3). Mean values with unlike letters are significantly different ($P<0.05$). Abbreviations: FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

Fig. 3 Relative mRNA expression of HMG-CoA reductase (*hmgcr*) and cholesterol 7 α hydroxylase (*cyp7a1*) of liver of the turbot fed the experimental diets. Data are expressed as mean and standard error (n=6). Mean values for the same gene with unlike letters are significantly different ($P<0.05$). Abbreviations: FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

Fig. 4 Relative mRNA expression of HMG-CoA reductase (*hmgcr*) and cholesterol 7 α hydroxylase (*cyp7a1*) of intestine of the turbot fed the experimental diets. Data are expressed as mean and standard error (n=6). Mean values for the same gene with unlike letters are significantly different ($P<0.05$). Abbreviations: FM, fish meal diet; PP, 56% of the plant protein meal inclusion level to replace 75% fish meal in basal diet; C-tau, 0.5% of the taurocholate inclusion level to PP diet.

Figure 1

Figure 2

Figure 3

Figure 4