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Effects of tributyrin supplemented in a high-soybean meal diet on the growth performance and intestinal histopathology of juvenile *Scophthal musmaximus* L.

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Key words: turbot, tributyrin, growth performance, intestinal histopathology

Abstract

This study was conducted to evaluate the influence of tributyrin (TB) supplemented in diet on the growth, feed utilization and intestinal health of turbot (*Scophthalmus maximus* L.). Four experimental diets were formulated with TB at 0 mg/kg, 100 mg/kg, 200 mg/kg and 300 mg/kg levels respectively. A total of 2016 turbot (initial body weight: 31.22±0.05g) were randomly distributed into 12 tanks and fed the corresponding diets for 70d. Results showed that fish in 100 mg/kg group had significantly higher weight gain rate (WGR) and specific growth rate (SGR) than that in other groups ($P<0.05$). No significant differences were observed in survival rate (SR), feeding rate (FR), feed conversion ratio (FCR), condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI) among all groups ($P>0.05$). There was no significant difference in intestinal superoxide dismutase (SOD) activities and intestinal malonaldehyde (MDA) contents among all treatments ($P>0.05$), while the highest SOD activity and the lowest MDA content were found in 100 mg/kg group. The height of intestinal fold in 100 mg/kg treatment was significantly higher than that in other groups ($P<0.05$). However, no significant difference existed in intestinal wall thickness among all groups ($P>0.05$). The present study suggested that 100 mg/kg TB inclusion level was recommended in the diets of turbot to promote the growth and improve the intestinal morphology structure and health in turbot.

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Introduction

Turbot (*Scophthalmus maximus* L.) is an important aquaculture species in Europe and Asia, and its rapid growth mainly relay on the high protein provided by fishmeal in diet (Liu et al., 2014). But it is not sustainable to use a large number of fishmeal in diet due to its scarce resource and high price (Hardy, 2010). Many plant protein feedstuffs including soybean meal were applied in the formula of turbot to replace the fishmeal. However, compared with fishmeal, plant protein sources have some disadvantages, such as defect in amino acid profile, low palatability and low digestibility, especially the anti-nutritional factors (ANF), which directly cause the intestinal health problems in fish (Francis et al., 2001; Hardy, 2010). Turbot was induced the typical symptoms of intestinal inflammation and enteritis when fed diets with excess soybean meal (Gu et al., 2016; Wang et al., 2016; Li et al., 2020). And in fact, enteritis often breaks out in turbot aquaculture nowadays and leads to numerous economic losses. Therefore, the search for safe feed additives that could improve the intestinal health and promote the growth of turbot has become a feasible solution and a research hotspot in fish nutrition study.

Short-chain fatty acids including acetic acid, propionic acid and butyric acid were the end-products of anaerobic bacterial fermentation of undigested carbohydrates and proved to have multiple beneficial effects on modulation intestinal health in aquatic animals (Hoseinifar et al., 2017; Bedford and Gong, 2018). Butyric acid is the main energy substance in intestinal tract, which can promote intestinal cell proliferation, improve intestinal morphology and enhance antioxidant capacity (Antongiovanni et al., 2007; Abdel-Latif et al., 2020). However, butyric acid is volatile with some inherent drawbacks including short half-life and strong odour, which seriously restricted its application in feed. Tributyrin (TB) is a kind of butyrate derivate, consisting of 3 molecule butyric acids and 1 molecule glycerol, it is stable when passing through the stomach and without strong odour. In intestinal tract TB is decomposed by pancreatic lipase to release butyric acid, which better exerts the function of butyric acid in the intestine (Li et al., 2009). It was reported recently in aquatic animal that the diet supplemented with TB could promote the intestinal healthy development, enhance the immune function and improve the growth performance of tawny puffer (*Takifugu flavindus*) (Zhai et al., 2014), black sea bream (*Acanthopagrus schlegelii*)(Volatiana et al., 2020), yellow drum (*Nibea albiflora*)(Tan et al., 2020), common carp (*Cyprinus carpio*)(Xie et al., 2020) and blunt snout bream (*Megalobrama amblycephala*) (Liang et al., 2021). However, the research on TB in the culture of turbot has not been reported yet. Therefore, this experiment will preliminarily study the effects of TB on growth performance and intestinal health of turbot.

Materials and Methods

Ethics statement

All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of China. The protocol of this study was reviewed and approved by the Ethical Committee of Hebei Normal University.

Diet preparation

The tributyrin (TB, >70% active ingredient) in the form of a white powder was provided by Hubei Horwath Biotechnology Co., Ltd. (Wuhan, China). Four experimental diets were formulated by adding 0 mg/kg, 100 mg/kg, 200 mg/kg and 300 mg/kg levels of TB in the basal diet respectively. Soybean meal, fish meal and poultry meat meal were the main protein sources. The ingredients and chemical composition of the basal diet were presented in **Table 1**. All ingredients were mixed and ground through 175-μm mesh. The 3.0 mm soft pellets were made by extruded pelletizer (EL-260, Youyi Machinery Factory, Weihai, China) and stored in refrigerator at -20°C until using.

Fish and culture conditions

Juvenile turbots were obtained from Shengyi Farm (Tianjin, China). After 2 weeks' acclimation, turbots were deprived of diets for 24 h and 2016 turbot (initial body weight: 31.22±0.05g) were randomly assigned to 12 tanks (2m×1.2m×1m) with 3 tanks per group.

All fish were manually fed with the corresponding diets to apparent satiation twice daily at 6:00 and 18:00. Feces and uneaten food was siphoned out after feeding. The water temperature was ranged from 12°C to 16°C, salinity was 27.5 ±1.1‰ and dissolved oxygen was more than 5 mg/L, ammonia-N was less than 0.1 mg/L. The experiment period was 70d.

Table1 Ingredients and proximate composition of basal diet of turbot (%, dry matter basis)

Ingredients	*Contents (%)*
Soybean meal	29.25
Fish meal	21.50
Poultry meat meal	12.00
Squid liver meal	3.50
Wheat gluten	8.50
α-starch	10.30
Soybean oil	3.50
Fish oil	2.50
Calcium carbonate	0.60
Calcium hydrophosphate	4.42
Potassium chloride	0.41
Zeolite powder	0.52
Vitamin-mineral Premix [a]	3.00
Proximate composition (%)	
Crude protein	42.60
Crude lipid	8.15
Ash	17.44
Gross energy/(MJ/kg)	18.57

[a] Vitamin - mineral premix was according to Zhang et al. (2015).

Sample collection and chemical analysis

At the end of the trial, all fish were starved for 24 h and anaesthetized with MS-222 before sampling. Fish were counted and batch weighted in each tank. Four fish in each tank were selected randomly and pooled into one sample to analyze the proximate composition of while body. One fish midgut tissue (1cm length) per tank was fixed in 10% buffered formalin for histological measure. Another two fish per tank were weighted individually and dissected out the viscera and liver to weight respectively. The intestinal tissues were also sampled to determine the Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content.

Proximate composition analysis of diets and whole body were conducted following Zhang et al. (2015). The intestinal SOD activities and MDA contents were measured using commercial kits (A001, A003; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by a spectrophotometry (TU-1901; Purkinje General Ltd., Beijing, China). Total protein concentration was determined with bovine serum albumin as the standard. The slices were made by haematoxylin-eosin staining and paraffin embedding. The thickness of intestinal wall and the height of the intestinal fold were observed under ZEISS microscope (Imager A1m).

Data calculation

Survival rate (SR, %) = 100 × (final number of fish/initial number of fish).

Weight gain rate (WGR, %) = 100 × [FBW (g) - IBW (g)]/ IBW (g);

Specific growth rate (SGR, %/d) =100 × (LnFBW - LnIBW)/days;

Feed conversion ratio (FCR) =dry feed intake (g)/ wet weight gain (g);
Feeding rate (FR, %/d) = 100×dry feed intake (g)/ [(IBW (g) + FBW (g))/2]/days;
Condition factor (%) = 100× (body weight, g)/ (body length, cm)^3
Hepatosomatic index (HSI, %) = 100× (liver weight, g) / (body weight, g);
Viscerosomatic index (VSI, %) =100× (viscera weight, g) / (body weight, g).

Statistical analysis

All Data were expressed as means ± SD and analyzed using STATISTICA 10.0 software (Statsoft, Inc., Tulsa, OK, USA). Before analysis, the normality of data distribution and the homogeneity of variance were assessed by Shapiro-Wilk test and Levene's test. One-way analysis of variances (ANOVA) was employed in this study. The significant level was set at P <0.05. Whenever the significant level achieved, Duncan's multiple range test was performed to compare the differences among all treatments.

Results

Growth performance

Table2 showed that the diet supplemented with 100 mg/kg tributyrin significantly increased the FBW, WGR and SGR of turbot compared with the other groups (P < 0.05), no significant difference were observed among 0 mg/kg, 200 mg/kg and 300 mg/kg groups (P > 0.05).There were no significant differences in FR, FCR, SR, CF, HSI and VSI among all groups (P > 0.05).

Table2 Effects of tributyrin supplemented in diets on growth performance of juvenile turbot

	Dietary tributyrin levels (mg/kg)			
	0	*100*	*200*	*300*
FBW (g)	71.37±0.99^b	78.38±0.65^a	69.76±0.41^b	70.46±0.78^b
SR (%)	99.20±0.91	99.40±0.84	99.60±0.35	100.00±0.00
WGR (%)	128.76±3.11^b	151.10±2.17^a	123.38±1.17^b	125.75±2.50^b
SGR (%/d)	1.18±0.02^b	1.32±0.01^a	1.15±0.01^b	1.16±0.02^b
FR (%/d)	1.63±0.08	1.72±0.16	1.62±0.08	1.60±0.05
FCR	0.87±0.06	0.84±0.07	0.89±0.05	0.86±0.04
CF (%)	3.85±0.36	3.69±0.51	3.71±0.36	3.51±0.39
HSI (%)	0.85±0.21	1.08±0.25	0.99±0.23	0.90±0.35
VSI (%)	4.64±0.30	4.89±0.52	4.57±0.35	4.53±0.50

Note: Values within the same row with different letters are significantly different (P<0.05).

Intestinal histopathology and antioxidant activity

The supplementation of TB in diet did not significantly influence the intestinal SOD activities and MDA contents (P > 0.05) (**Table3**), while the highest SOD activity and the lowest MDA content were found in 100 mg/kg group. As seen from **Figure 1**, the height of intestinal fold in 100 mg/kg group was significantly higher than that in other groups (P<0.05). No significant difference existed in intestinal wall thickness among all groups (P>0.05) (**Table4**).

Table3 Effects of tributyrin supplemented in diets on intestinal SOD activities and MDA contents of juvenile turbot

	Dietary tributyrin levels (mg/kg)			
	0	*100*	*200*	*300*
SOD (U/mg prot.)	25.13±6.19	28.90±5.46	24.19±6.25	22.38±3.20
MDA (nmol/mg prot.)	9.14±3.09	8.80±2.87	12.40±5.95	12.44±5.05

Note: Values within the same row with different letters are significantly different (*P*<0.05).

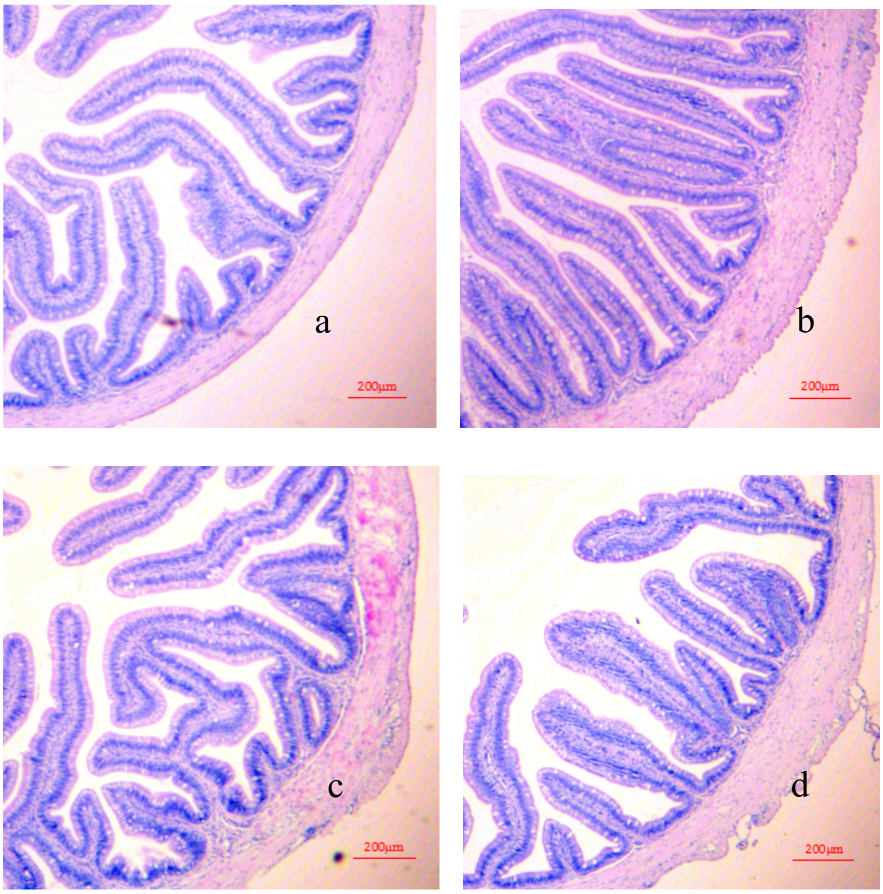

Figure 1 Hematoxylin-eosin staining of intestine in juvenile turbot fed with graded levels of dietary tributyrin.
Note: **a**: 0mg/kg tributyrin; **b**: 100 mg/kg tributyrin; **c**: 200 mg/kg tributyrin, **d**: 300 mg/kg tributyrin.

Table 4 Effects of tributyrin supplemented in diets on intestinal structure of juvenile turbot

	Dietary tributyrin levels (mg/kg)			
	0	*100*	*200*	*300*
Intestinal fold length (μm)	581.58±33.49[b]	689.58±43.58[a]	636.96±42.94[b]	564.42±61.14[b]
Intestinal wall thickness (μm)	179.11±20.13	217.47±43.92	190.93±27.37	178.90±42.03

Note: Values within the same row with different letters are significantly different (*P<0.05*).

Discussion

Supplementation of butyrate products in diet has positive impact on the growth of fish (reviewed in Abdel-Latif et al., 2020). TB could be decomposed into three butyric acid and glycerol under the action of pancreatic lipase in the intestine. Its main active component is butyric acid, which can directly provide energy to the enterocyte, maintain intestinal health and promote animal growth (Li et al., 2009). Previous studies have reported that an appropriate dietary TB level significantly increased the growth of some teleost fish. 0.1g/kg dietary TB supplementation produced the greatest value of growth performance in tawny puffer (Zhai et al., 2014); optimum tributyrin supplementation for juvenile black sea bream is 2.24 g/kg in the 45% soya bean meal diet (Volatiana et al., 2020); dietary 1.0 g/kg tributyrin supplementation in high-soya bean meal diet has significantly improved growth performance of yellow drum (Tan et al., 2020); 2.0 g/kg–4.0 g/kg dietary TB supplementation led to high growth performance in common carp fed all-plant diets (Xie et al., 2020); 0.3-0.6 g/kg TB supplementation in diet significantly increased the growth of blunt snout bream fed with high-plant diets (Liang et al., 2021). In the present study, it is also found that dietary TB supplementation improved the growth of turbot. The appropriate TB level in diet for turbot is 100mg/kg, which is similar with tawny puffer, but lower than that in other teleost fish. This may be related to the fish species and the diet formulation with plant-based resources, especially the soybean meal contents in the diet. Excess soybean meal or soybean meal derivatives induced intestinal inflammation and growth retardation on fish due to the ANF in the soybean meal (Wang et al., 2017). TB could improve the growth performance by attenuating soybean meal-induced intestinal inflammation. The more soybean meal was added in diet, the more serious damage was in intestine of fish, and the more TB will be required. The dietary soybean meal level in the present study was 29.25%, which is lower than that in the diets of black sea bream (45%) and yellow drum (36%). Maybe this is the main cause that the appropriate TB level in the present study was lower than that in these fish. Moreover, it is showed that TB has not influenced the feeding rate of the turbot in this study, which demonstrated that TB was friendly to turbot without inducing appetite inhibition. So, in this study, dietary 100mg/kg TB supplementation improved the growth performance of turbot fed with high-soybean meal diets and had no negative impact on the feeding rate.

Intestinal health is an important guarantee for rapid growth of fish and healthy maintenance of fish (Hu et al., 2015).The height of intestinal folds, villus and the thickness of intestinal wall are important indexes for evaluating intestinal health. Intestinal folds and villus are served as the physical barriers to protect the intestinal integrity and nutrient absorption (Bedford and Gong, 2018). For turbot, high-soybean meal (about 40%) based diets have damaged the intestinal structure by decreasing the height of villus and induced the typical intestinal inflammation and enteritis (Yu et al., 2021). In the present study, no typical enteritis symptom was observed from the intestinal histology due to the relative low soybean meal content (29.2%). It is noteworthy that adding 100mg/kg TB to the basal diet increased the intestinal fold length of turbot, the longer fold length increased surface area for nutrient absorption, which could explain the better growth in this group. This result is consistent with the studies in tawny puffer (Zhai et al., 2014), yellow drum (Tan et al., 2020) and common carp (Xie et al., 2020). Butyric acid acts as a signaling molecule in regulating proliferation, differentiation and improving intestinal barrier function of epithelial cells (Rimoldi et al., 2016). As the preferred metabolic fuel for intestinal cells, it can quickly provide energy for epithelial cells and stimulate the proliferation of intestinal cells. In addition, butyric acid can also dilate intestinal blood vessels, increase intestinal blood flow, improve intestinal microcirculation, and thus play a nutritional role in intestinal mucosa (Pan et al., 2017).

Furthermore, antioxidant status plays a vital role in the intestinal health maintenance. The activities of SOD are crucial for scavenging excess amounts of free radicals in fish (Martínez-Alvarez et al., 2005), and Malondialdehyde (MDA) is a product of lipid peroxidation and associated with the oxidative damage (Tsikas, 2017). In the present study, the highest intestinal SOD activity and the lowest MDA content were found in 100 mg/kg TB group, suggesting that 100 mg/kg TB can alleviate or reduce the intestinal oxidative stress of turbot. TB supplementation also effectively decreased the MDA content and improved the SOD activities in black sea bream (Volatiana et al., 2020) and common carp (Xie et al., 2020) fed with high-plant diets. Therefore, dietary 100mg/kg TB addition improved the intestinal antioxidant capacity to maintain the intestinal health.

In summary, supplementation of 100 mg/kg tributyrin in diets significantly increased the weight gain rate, specific growth rate and the height of intestinal fold of turbot in this study. The recommended supplemental level of tributyrin in the diet with 29.2% soybean meal was 100 mg/kg for juvenile turbot.

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