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Soya-saponins induce intestinal inflammation and barrier dysfunction in juvenile turbot (*Scophthalmus maximus*)

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ABSTRACT

Soybean meal-induced enteritis (SBMIE) is a well-described condition in the distal intestine (DI) of several cultured fish species, but the exact cause is still unclear. The work on Atlantic salmon and zebrafish suggested soya-saponins, as heat-stable anti-nutritional factors in soybean meal, are the major causal agents. However, this conclusion was not supported by the research on some other fish, such as gilthead sea bream and European sea bass. Our previous work proved that soybean could induce SBMIE on turbot and the present work aimed to investigate whether soya-saponins alone could cause SBMIE and the effects of soya-saponins on the intestinal barrier function in juvenile turbot. Turbots with initial weight 11.4 ± 0.02 g were fed one of four fishmeal-based diets containing graded levels of soya-saponins (0, 2.5, 7.5, 15 g kg^{-1}) for 8 weeks. At the end of the trial, all fish were weighed and plasma was obtained for diamine oxidase (DAO) activity and D-lactate level analysis and DI was sampled for histological evaluation and quantification of antioxidant parameters and inflammatory marker genes. The activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and intestinal glutathione level were selected to evaluated intestinal antioxidant system. The distal intestinal epithelial cell (IEC) proliferation and apoptosis were investigated by proliferating cell nuclear antigen (PCNA) labelling and TdT-mediated dUTP nick end labeling (TUNEL), respectively. The results showed that soya-saponins caused significantly dose-dependent decrease in the growth performance and nutrient utilization ($p < 0.05$). Enteritis developed in DI of the fish fed diet containing soya-saponins. Significantly dose-dependent increases in severity of the inflammation concomitant with up-regulated expression of *il-1β*, *il-8*, and *tnf-α*, increased IEC proliferation and apoptosis, and decreases in selected antioxidant parameters were detected ($p < 0.05$). The epithelial permeability (evaluated by the plasma DAO activity and D-lactate level) was significantly increased with the increasing of dietary level of soya-saponins ($p < 0.05$), which was concomitant with the destroyed the intracellular junctions. In conclusion, the present work proved that soya-saponins induced enteritis and compromised the intestinal barrier functions. Based on the present work, strategies focus on regulation of cell apoptosis, epithelial permeability, intracellular junctions and redox homeostasis worth further investigating to develop new and efficient ways for SBMIE alleviation.

1. Introduction

Fish meal is the primary protein source in feed of farmed fish, especially for carnivorous fish [1,2]. However, the production of fish meal has reached its maximum level over the last decade and price is increasing due to the increased demand [3–5]. The unbalance between the fish meal supply and demand has stimulated exploration of dietary alternative protein sources for the aquaculture industry [6,7]. Among the ingredients that are being investigated as alternatives to fish meal in fish diet, products derived from soybeans are some of the most promising because of the security of supply, price and protein/amino acid composition [8]. However, soybean meal (SBM) of standard quality can be used in carnivorous fish diets only at relatively low levels due to its negative effects on gut health in several fish species [9,10].

Specifically, SBM has been observed to cause proliferative or inflammatory conditions in the distal intestinal mucosa of cultured fish species such as Atlantic salmon, rainbow trout, common carp and zebrafish [11–16]. The histopathological changes, commonly referred to as soybean meal-induced enteritis (SBMIE), have been extensively studied [17]. SBMIE are characterized by a shortening of the mucosal folds, a swelling of the lamina propria and subepithelial mucosa, a strong infiltration of various inflammatory cells, and decreased numbers of absorptive vacuoles in the enterocytes [11,18–20]. Our previous study has shown that inclusion level of SBM in the range of

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260–540 g kg^{-1} caused the similar inflammatory changes in turbot as seen in other fish species [20].

The exact cause and mechanisms behind the inflammation and other negative effects are not fully understood. Work on the Atlantic salmon indicated that soya-saponins in soybean meal were the vital factors to induce SBMIE [21–26]. Saponins are heat-stable, triterpenoid or steroid, amphipathic glycosides [27]. The amphiphilic property provides saponins the ability to bind and form non-absorbable complexes with cholesterol [28,29]. In mammals, saponins apparently have the ability to bind to membrane cholesterol of intestinal epithelial cells (IEC) and thus form holes and alter membrane permeability, possibly facilitating the uptake of molecules, including antigens and potential toxins that normally are not absorbed by the enterocytes [30]. The previous study in Atlantic salmon clearly demonstrated that soya-saponins alone, supplemented at levels of 2–10 g kg^{-1}, caused a dose-dependent increase in the severity of inflammatory changes in the distal intestine tissue as described for SBMIE [25]. Soya-saponins were also proved to be responsible for the SBMIE in Zebrafish [16]. However, dietary soya-saponins at the level of 2–3 g kg^{-1} had no clear negative effects on channel fish [31], European sea bass [32,33] and gilthead sea bream [34,35] and rainbow trout [36]. The differences might be due to the differences in fish feeding habit, size and age, but also the basal diet, culture condition and experimental duration.

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 [37]. The turbot faming is facing the problems of fish meal shortage. Our previous work proved soybean meal induced SBMIE on turbot [20]. However, the mechanisms behind SBMIE on turbot need to be further exploited. The purpose of this study was to investigate whether soya-saponins alone may induce enteritis and their effects on intestinal barrier function in turbot.

2. Materials and methods

2.1. Feed ingredients and diet formulation

Based on our previous work [20], a fish meal based diet (FM diet) was formulated to contain 48% crude protein and 12% crude lipid with fish meal as the primary protein source, fish oil and soybean oil as lipid sources, and wheat flour as the carbohydrate source (Table 1). This diet was used as the control diet. Based on previous work on the soya-saponins on fish [25,38], another three isonitrogenous and isolipidic diets were formulated with the addition of 2.5, 7.5 and 15 g kg^{-1} soya-saponins (98% in purity, obtained from Xi'an Chukang Biotechnology Co., Ltd, Shaanxi, China) to FM diet, named as SAP2.5, SAP7.5 and SAP15 respectively. The level of soya-saponins in soybean meal ranges in 5–7 g kg^{-1} [39,40], the soya-saponin concentration of diet SAP2.5 was similar with that of the diet supplemented with 40% soybean meal, which was proved to induce SBMIE in turbot [20]. All four diet formulations were designed to meet the essential amino acid (EAA) requirements of juvenile turbot based on the whole body amino acid profile [41,42]. The diet preparation and storage were as described by our previous work [43]. Standard methods were used to analyze the experimental diets [44]. 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 ethylether (Extraction System B-811, BUCHI, Switzerland). Crude protein was determined by the Kjeldahl method with a FOSS Kjeltec System (2300, Sweden) using boric acid to trap released ammonia. Gross energy was determined by calorimetric bomb (Parr, Moline, IL, USA).

2.2. Experimental procedures

Apparent disease-free juvenile turbots 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. The fish were acclimated to the system and fed with the FM diet for 2 weeks. Next, turbot with initial body weight of about 11.4 g were randomly distributed into 12 tanks, 30 fish per tank (filled with 300 L seawater). The seawater was pumped from the adjacent coastal water, filtered through a sand filter, and distributed to each tank at approximately 2.0 L min^{-1}. 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 and the feed consumption was recorded. During the 8-week feeding trial, the water temperature was 12–16 °C, pH 7.8–8.2, and the salinity was 28–30.

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

	Experimental diet[a]			
	FM	SAP2.5	SAP7.5	SAP15
Ingredients (g kg^{-1})				
Fish meal[b]	610	610	610	610
Wheat meal	269	269	269	269
Fish oil	17	17	17	17
Soybean oil	17	17	17	17
Soybean lecithin	20	20	20	20
Vitamin and mineral premix[c]	35	35	35	35
Choline chloride	5	5	5	5
Yttrium premix	1	1	1	1
Calcium propionic acid	1	1	1	1
Ethoxyquin	0.5	0.5	0.5	0.5
Phaseomannite	0.5	0.5	0.5	0.5
Cellulose	24	21.5	16.5	9
Soya-saponins[d]	0	2.5	7.5	15
Proximate composition (%)				
Dry matter	95.1	94.9	95.0	95.0
Crude protein	48.5	48.7	48.5	48.6
Crude lipid	12.2	12.3	12.1	12.3
Ash	12.8	12.7	12.9	12.7
Gross energy (KJ/g)	20.1	20.2	20.2	20.1

[a] FM: a basal diet; SAP: soya-saponins included to the FM diet and 2.5, 7.5, and 15 indicate the levels of soya-saponins inclusion.

[b] Fish meal: steam dried fish meal (COPENCA Group, Lima, Peru).

[c] Vitamin premix supplied the diet with (mg kg^{-1} diet) the following compounds: retinyl acetate, 32; vitamin D_3, 5; DL-α-tocopherol acetate, 240; vitamin K_3, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitaminB_{12} (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: $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $CuSO_4 \cdot 5H_2O$, 10; $MnSO_4 \cdot H_2O$, 45; KI, 60; $CoCl_2 \cdot 6H_2O$ (1%), 50; Na_2SeO_3 (1%), 20; $MgSO_4 \cdot 7H_2O$, 1200; calcium propionate, 1000; zoelite, 17485.

[d] Soybean saponin (98% in purity) was obtained from Xi'an Chu-kang Biotechnology Co., Ltd (Shaanxi, China).

2.3. Sampling

After 8 weeks of feeding, all experimental fish were anesthetized with eugenol (1: 10000, Shanghai Reagent Co., Shanghai, China) and the body weight was recorded before sampling. And then, eight fish from each tank were randomly selected and blood samples were collected from the caudal vein using heparinized syringes. Plasma samples were obtained by 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 samples for body length were determined. The intestine was removed, cleared of any mesenteric, adipose tissue, and rinsed with ice-cold PBS to remove the eventual remaining gut contents. Only fish with food in the intestinal tract were sampled to ensure recent intestinal exposure to the diets. Four of the eight sampled fish were randomly selected and their distal intestines