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Dietary soya allergen β-conglycinin induces intestinal inflammatory reactions, serum-specific antibody response and growth reduction in a carnivorous fish species, turbot *Scophthalmus maximus* L.

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Abstract

This study was conducted to investigate the effects of dietary β-conglycinin on the growth performance, digestion, gut morphology and immune responses of juvenile turbot (*Scophthalmus maximus* L.). Four diets were formulated to contain 0%, 2%, 4% and 8% purified β-conglycinin. Triplicate groups of 30 fish were fed to apparent satiation twice daily for 12 weeks. Fish fed 4% and 8% dietary β-conglycinin showed significantly reduced specific growth rate, feed efficiency ratio, apparent digestibility coefficient of nutrients and whole-body lipid contents, as well as a profound infiltration of mixed leucocytes in the lamina propria and a significant decrease in the absorptive surface of distal intestine. The expression of pro-inflammatory cytokines, TNF-α and IL-1β, in the distal intestine was significantly upregulated by 4% dietary β-conglycinin, whereas a significantly lower expression level of IgM and anti-inflammatory cytokine TGF-β1 was observed in fish fed 8% dietary β-conglycinin. Serum lysozyme and alternative complement pathway activity were first significantly enhanced by 2% dietary β-conglycinin and then rapidly declined by 4% and 8% dietary β-conglycinin. Respiratory burst activity of head kidney macrophages and serum superoxide dismutase activity were significantly suppressed by 4% and 8% dietary β-conglycinin. Dietary β-conglycinin (2–8%) significantly increased the level of specific antibody against β-conglycinin in serum. Collectively, these results suggested that higher levels of dietary β-conglycinin (4–8%) induced a variety of non-specific and specific immune responses and intestinal mucosal lesions in turbot, resulting in inferior feed utilization and poor growth performance.

Keywords: β-conglycinin, growth performance, digestibility, gut morphology, immune responses, turbot

Introduction

The global wild fish catch has reached a plateau in the past years yet aquaculture industry continues to grow steadily, giving rise to shortages in the supply of key feed ingredients for aquaculture, that is fish meal and fish oil. Turbot is an economically important carnivorous fish species widely farmed in Europe and East Asia for its delicious meat and rapid growth. It has high dietary protein requirement (ranging from more than 50% to 65% of the diet) that was largely met by the fish meal in commercial diets (Peres & Oliva-Teles 2005). Therefore, it is of interest to replace fish meal with more accessible and sustainable protein sources in turbot diets. Soya beans are widely used by the human and animal nutrition industry as dietary protein feedstuffs due to their exceptional protein content and favourable amino acid profile. They are used in various forms, such as soya bean meal (SBM), soya flour and/or flakes, soya protein concentrate (SPC) and soya protein isolate (SPI)

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(Nishinari, Fang, Guo & Phillips 2014). Previous studies showed that up to 30% of fish meal could be replaced by SBM without compromising the growth performance of juvenile turbot; however, substitution level at 45% or higher significantly deteriorated the fish growth and caused lesions to the digestive tract (Zhao 2008; Peng, Xu, Ai, Mai, Liufu & Zhang 2013). This is partially due to the presence of antinutrients, such as allergens, in the soya bean that are used by the plant to defence itself from animal consumptions.

Soya bean is one of the 'big eight' allergenic foods that are responsible for 90% of all food allergies, and allergic reactions of soya ingestion may range from skin, gastrointestinal or respiratory reactions to anaphylaxis (Hefle, Nordlee & Taylor 1996; Holzhauser, Wackermann, Ballmer-Weber, Bindslev-Jensen, Scibilia, Perono-Garoffo, Utsumi, Poulsen & Vieths 2009). So far, at least 16 allergenic proteins have been identified from soya bean, among which β-conglycinin is one of the major allergenic proteins that accounts for ~30% of the total seed proteins (Mujoo, Trinh & Ng 2003; L'Hocine & Boye 2007). β-conglycinin is a trimeric protein made up of three subunits, α (MW, 57~76 kDa), α' (MW, 57~83 kDa) and β (MW, 42~53 kDa)(Garcia, Torre, Marina, Laborda & Rodriquez 1997). All of the β-conglycinin subunits are glycoproteins and contain 4–5% carbohydrate, which may contribute to their immunoreactivity (Amigo-Benavent, Clemente, Ferranti, Caira & del Castillo 2011). All three subunits of β-conglycinin have been identified to bind IgE antibodies of soya-allergic patients, and sensitization to β-conglycinin is likely to result in severe reactions to soya bean (Holzhauser *et al.* 2009; Krishnan, Kim, Jang & Kerley 2009). Meanwhile, β-conglycinin is also one of the main antigenic soya bean proteins that favour the development of gut hypersensitivity reactions in preruminant calves and post-weaning piglets fed insufficiently processed soya bean products as milk replacers (Pedersen 1986; Li, Nelssen, Reddy, Blecha, Hancock, Allee, Goodband & Klemm 1990). The gut hypersensitivity reactions in these young animals are featured by reduced villus height associated or not with increased crypt depth, maldigestion, malabsorption, decreased intestinal transit time, hypermotility and diarrhoea, and result in decreased food intake, nitrogen utilization as well as severe lag in growth (Dréau & Lallès 1999).

In contrast to other extensively studied soya bean-derived antinutrients, effects of soya bean allergenic proteins on fish health remain underinvestigated. As early as 1994, Rumsey, Siwicki, Anderson and Bowser suggested that high levels of antigenic proteins β-conglycinin and glycinin (another primary soya allergenic protein) in conventional SBM, where protease inhibitors and lectins have been largely inactivated, seemed to be responsible for the inferior growth performance of rainbow trout (*Oncorhynchus mykiss*) compared to those fish fed on the diet using SPC as the main protein source. However, no supporting evidence was provided until recently when Zhang, Guo, Feng, Jiang, Kuang, Liu, Hu, Jiang, Li, Tang and Zhou (2013) showed that 8% dietary β-conglycinin (purity, ~80%) induced oxidation and inflammation in the digestive tract of Jian carp (*Cyprinus carpio* var. Jian) and resulted in dysfunction of intestinal digestion and absorption. Gu, Bai, Xu, Zhou, Zhang and Mai (2014) also reported that turbot (*Scophthalmus maximus* L.) fed a diet supplemented with 6% dietary β-conglycinin (purity, ~76%) showed significantly lower digestive and absorptive enzyme activities concomitantly with reduced villi height and increased mucosal fold fusion. These findings in fish suggested that the presence of immunoreactive β-conglycinin in SBM may play a role in limiting its use in the fish diets by inducing the development of intestinal disorders.

While the study by Gu *et al.* (2014) showed detrimental effects of dietary β-conglycinin on the gut function of turbot and its amelioration by heat treatments of β-conglycinin, this study investigated the effects of different doses of dietary β-conglycinin on turbot's feed utilization, growth performance and immune responses, in an attempt to elucidate the role of β-conglycinin in limiting the use of soya bean meal in turbot diets.

Materials and methods

Experimental diets

Purified β-conglycinin fraction was offered by Prof. Shuntang Guo at China Agricultural University (the purified protein was made by fractional salting-out method with sodium and potassium salts of different pH, Patent No. 200410029589.4, China). The crude protein content of β-conglycinin fraction was 96.2% (determined by the Kjeldahl method), and the protein profile of β-conglycinin

fraction was revealed by the SDS-PAGE analysis (Fig. 1). The purity of β-conglycinin fraction, determined using quantitation software Quantity One (Bio-Rad, Hercules, CA, USA), is 80.6% β-conglycinin, the rest being 5.3% glycinin and 14.1% non-allergenic proteins.

Four isonitrogenous and isolipidic practical diets were formulated to contain 51% crude protein and 12% crude lipid (Table 1). The basal diet used fish meal, casein and gelatin as the main protein sources. Then graded levels of soya bean β-conglycinin were included into the basal diet to replace 2%, 4% and 8% casein and gelatin (4:1). Crystalline amino acids were supplemented to obtain the same essential amino acid profile among all the diets. Additionally, 1 g kg^{-1} yttrium oxide (Y_2O_3) was used as an inert tracer in each diet for determining apparent digestibility of nutrients. The level of immunologically active β-conglycinin in diets 2, 3 and 4 was determined by a commercial ELISA kit developed by You, Li, Qiao, Wang, He, Ou and Dong (2008), and it was found to be 1.55%, 3.18% and 6.14% respectively.

Dietary ingredients were ground into fine powder to pass through 320-μm mesh. All ingredients were thoroughly mixed with fish oil and soya bean

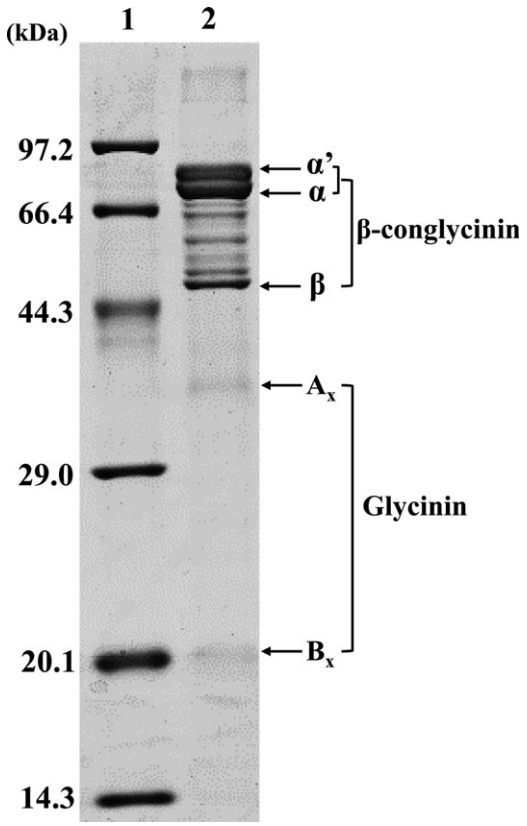

Figure 1 SDS-PAGE profile of purified β-conglycinin fraction. Lane 1: protein marker; Lane 2: β-conglycinin fraction. α', α and β are subunits of β-conglycinin, and A_x and B_x are acidic and basic subunits of glycinin.

Table 1 Formulations and chemical analyses of the experimental diets (% dry matter)

	Diet No./dietary β-conglycinin inclusion level			
Ingredients	**Diet 1/0%**	**Diet 2/2%**	**Diet 3/4%**	**Diet 4/8%**
Fish meal*	43.00	43.00	43.00	43.00
Casein*	12.00	10.40	8.80	5.56
Gelatin*	3.00	2.60	2.20	1.39
β-conglycinin	0.00	2.00	4.00	8.00
Wheat flour*	31.03	30.95	30.87	30.74
Fish oil	4.00	4.00	4.00	4.00
Soya bean oil	2.00	2.00	2.00	2.00
Soya bean Lecithin	1.00	1.00	1.00	1.00
Vitamin premix†	1.80	1.80	1.80	1.80
Mineral premix‡	1.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20
$Ca(H_2PO_4)_2$	0.50	0.50	0.50	0.50
Calcium propionate	0.10	0.10	0.10	0.10
Ethoxyquin	0.05	0.05	0.05	0.05
Yttrium oxide	0.10	0.10	0.10	0.10
Arginine	0.23	0.16	0.11	0.00
Leucine	0.00	0.02	0.05	0.11
Lysine	0.00	0.02	0.03	0.07
Methionine	0.00	0.03	0.05	0.10
Threonine	0.00	0.03	0.06	0.12
Valine	0.00	0.03	0.07	0.15
Nutrient composition (% dry matter)				
Crude protein	51.30	51.44	51.19	51.34
Crude lipid	11.94	11.81	11.63	11.37
Ash	8.66	8.56	8.69	8.65
β-conglycinin§	–	1.55	3.18	6.14

*Fish meal (dry matter, %): crude protein 73.38, crude lipid 10.42; casein (dry matter, %): crude protein 96.90, crude lipid 0.53; gelatin (dry matter, %): crude protein 99.30, crude lipid 0.21; wheat flour (dry matter, %): crude protein 17.05, crude lipid 2.29.

†Vitamin premix (mg kg^{-1} diet): retinyl acetate, 32; vitamin D_3, 5; DL-α-tocopheryl acetate, 240; vitamin K_3, 10; thiamine, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitamin B_{12} (1%), 10; L-ascorbyl-2-monophosphate-Na (35%), 2000; calcium pantothenate, 60; nicotinic acid, 200; inositol, 800; biotin (2%), 60; folic acid, 20; choline chloride (50%), 2500; cellulose, 2473.

‡Mineral premix (mg kg^{-1} diet): $FeSO_4{\cdot}H_2O$, 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; zeolite, 2485.

§Determined by a commercial ELISA kit developed by You *et al.* (2008).

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oil, and water was added to produce stiff dough. The dough was then pelleted with an experimental single-screw feed mill. The die diameter was 3 mm, and the screw speed was 90 rpm. Product temperature at the end of the barrel was 60.5–63.5°C. After being pelleted, the feeds were dried for about 12 h in a ventilated oven at 45°C and stored in a freezer at −20°C.

Fish, experimental procedure and conditions

A 12-week feeding trial was conducted with disease-free juvenile turbot obtained from a commercial farm in Laizhou, Shandong Province, China. Prior to the start of the experiment, fish were acclimatized to the experimental tanks for 2 weeks where maintained on a commercial diet (Qingdao Great Seven Bio-Tech Co. Ltd, Qingdao, Shandong, China) for juvenile turbot. Then, a total of 360 fish with a mean initial body weight of 9.98 ± 0.01 g (mean ± SEM) were randomly assigned into 12 fibreglass tanks (300 L, 30 fish per tank). Each of the four experimental diets was randomly allocated to three tanks. The water flow rates were adjusted to 2 L min^{-1}. The fish were fed by hand to apparent satiation twice daily (7:00 am and 7:00 pm) and maintained under a photoperiod regime of 12 h day light and 12 h darkness. Uneaten feeds were collected from the tank outlets for the calculation of feed intake.

During the feeding period, water temperature ranged from 15 to 18°C, pH 7.5–8.0, salinity 30–33‰, ammonia nitrogen lower than 0.4 mg L^{-1}, nitrite lower than 0.1 mg L^{-1} and dissolved oxygen higher than 7.0 mg L^{-1}.

Sample collection

At the termination of the experiment, fish were fasted for 24 h before harvest. Total number and body weight of fish in each tank were counted and measured. Five fish per tank were randomly collected and stored frozen at −20°C for whole-body proximate composition analysis. The middle part (about 1 cm in length) of midgut and distal gut from three fish per tank was dissected and divided into two equal parts: one part was fixed at Bouin's fixative solution for histological evaluation, and the other was frozen in liquid nitrogen immediately and then stored at −80°C for gene express analysis. Another 10 fish from each tank were anesthetized with eugenol (1:10 000), and blood was withdrawn using a syringe from the caudal vein and stored at 4°C for 4 h. Serum was collected after centrifugation (4000 *g*, 10 min) and stored at −80°C as separate aliquots until analysis. Head-kidney macrophages from three fish in each tank were isolated as described by Secombes (1990) with some modifications. Briefly, the head kidney was removed under aseptic conditions, pushed through a 100-μm nylon mesh and suspended in L-15 medium (Gibco, Breda, the Netherlands) supplemented with 2% foetal bovine serum (FBS) (Gibco), 100 IU mL^{-1} penicillin (Gibco), 100 IU mL^{-1} streptomycin (Gibco) and 20 IU mL^{-1} heparin (Gibco). The resultant cell suspensions were enriched by centrifugation (836 *g*, 25 min, 4 °C) on 34%/51% Percoll (Sigma-Aldrich Co., St Louis, MO, USA) density gradient. The band of cells lying at the interface of the Percoll gradient was collected and washed twice. The viable cell concentration was determined by the trypan blue exclusion test. Cells were then counted in a haemocytometer and adjusted to 1×10^7 cells per mL L-15 medium for respiratory burst activity assay.

Faecal collection was performed a week after the above sampling procedure, during which the remaining fish from the feeding trial were reared under the same feeding regime before sampling. As watery faeces of fish fed the diet with 8% β-conglycinin could not be used for analysis, faeces were collected manually as described by Cheng, Ai, Mai, Xu, Ma, Li and Zhang (2010). Briefly, fish from each tank were anesthetized and gently stripped of faecal matter by hand 6 h after feeding twice a week, until sufficient faeces were collected for digestibility analysis (about 2 g, dry matter). Pooled faeces from each replicate were stored at −20°C and frozen-dried with a vacuum freeze drier (ALPHA 1–4 LD; Christ, Osterode, Germany) as soon as the faeces samples were transported to the laboratory.

Analysis and measurements

Diets, faeces and fish body composition analysis

Analyses of the chemical compositions of feed ingredients, experimental diets, faeces and fish bodies were performed following standard protocols of AOAC (1995): dry matter was measured by drying samples to a constant weight at 105°C; crude protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method (2300 Auto-analyzer; FOSS Tecator, Höganäs, Sweden);

crude lipid was determined by ether extraction using Soxhlet method (B-811; BUCHI, Flawil, Switzerland); and ash was measured by combustion at 550°C. Essential amino acids profiles of ingredients and experimental diets were determined as described by Men, Ai, Mai, Xu, Zhang and Zhou (2014). For the determination of the apparent digestibility coefficients (ADCs) of dry matter, protein and lipid of experimental diets, the concentration of yttrium oxide in the diets and faeces was measured following the method of Cheng *et al.* (2010).

Functional immune assays

Lysozyme (LYZ) activity. The LYZ activity in serum was determined by the turbidimetric method of Ellis (1990). Briefly, 50 μL of serum was added to 1.4 mL of a suspension of *Micrococcus lysodeikticus* (Sigma) (0.2 mg mL^{-1}, 0.05 M sodium phosphate buffer, pH 6.8). Absorbance of the mixed reacting solution was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer at room temperature. Each unit is defined as the amount of sample causing a decrease in absorbance of 0.001 per minute.

Alternative complement pathway (ACP) activity. The activity of serum ACP was determined using rabbit red blood cells (RaRBC) as targets. RaRBC were washed three times by centrifugation at 400 *g* for 5 min with isotonic veronal-buffered saline (GVB, pH 7.3, containing 0.1% gelatin) and resuspended in the same buffer to a concentration of 2.0×10^8 cells mL^{-1}. Then, 0.1 mL of RaRBC suspension was added to 0.25 mL of serially diluted serum in Mg-EGTA-GVB buffer (GVB with 10 mM Mg^{2+} and 10 mM EGTA). Samples were incubated at 22°C for 90 min with occasional shaking. The reaction was stopped by adding 3.15 mL cold 0.9% NaCl. Samples were then centrifuged at 836 *g* for 5 min at 4°C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at 414 nm. The ACP haemolytic activity (ACH) was reported as the reciprocal of the serum dilution causing 50% lysis of RaRBC (ACH50) established by Yano (1992).

Respiratory burst activity. Respiratory burst activity of head-kidney macrophages was measured according to the method of Secombes (1990) with some modifications. In brief, aliquots of 100 μL macrophages suspension were dispensed into 1.5-mL sterile microcentrifuge tubes (Axygen, Corning, NY, USA). The tubes were centrifuged at 1500 *g* for 10 min, and the supernatant was removed. Then aliquots of 200 μL 0.2% nitroblue tetrazolium (NBT; Sigma), which was dissolved in L-15 and contained 1 mg mL^{-1} phorbol myristate acetate (PMA; Sigma), were added to each tube and incubated for 40 min at 18°C. After incubation, the NBT solution was removed and the cells were fixed with 200 μL absolute methanol for 10 min, followed by cells washed with 70% methanol for three times and air-dried. The induced formazan was solubilized by adding 120 μL 2 M KOH and 140 μL dimethyl sulfoxide (DMSO; Sigma). The production of superoxide anion was expressed as the absorption value at 630 nm.

Superoxide dismutase (SOD) activity. Serum SOD activity was measured by its ability to inhibit superoxide anion generated by xanthine and xanthine oxidase reaction system according to Ma, Hou, Mai, Bharadwaj, Ji and Zhang (2014) using a commercial kit (Nanjing Jiancheng Bioengineering, Nanjing, Jiangsu, China). One unit of SOD was defined as the amount of enzyme required inhibiting xanthine reduction rate by 50% measured in the reaction system. Enzyme activity was expressed as units per mL serum (U mL^{-1}).

Specific IgM detection by ELISA. Serum β-conglycinin-specific IgM was determined by an indirect sandwich-type enzyme-linked immunosorbent assay (ELISA). The β-conglycinin (>90.0% purity) used to coat the plate was kindly donated by Prof. Shuntang Guo. This purified product was previously used in a pig experiment as a substrate for ELISA assay (Wang, Qin, Zhao & Sun 2009). In brief, wells of flat bottom microtitre plates (96 well; Costar, Corning, NY, USA) were coated with β-conglycinin (50 μg mL^{-1}) in 100 μL of carbonate–bicarbonate buffer (CB buffer, 35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6) and incubated overnight at 4°C. The wells were washed three times with PBST (PBS containing 0.05% Tween-20) and blocked with 200 mL PBS containing 3% BSA for 1 h at 37°C. Then, the plate was washed as above, and 100 μL turbot sera per well (1:100 diluted in PBS buffer) was added as first antibody. After incubation for 1.5 h at 37°C, the plate was washed again and 100 μL of rabbit antiturbot polyclonal antibody diluted 1:1000 in PBS was added as second antibody and incubated for 1.5 h at 37°C. Following a further washing, 100 μL of goat anti-rabbit Ig-alkaline phosphatase conjugate diluted 1:4000 in PBS was added as third antibody and incubated for 1 h at 37°C. After the last

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washing, 100 μL of 0.1% (w/v) p-nitrophenyl phosphate (pNPP; Sigma) in 50 mM CB buffer (pH 9.8) containing 0.5 mM $MgCl_2$ was added to each well of the plate and incubated for 30 min at room temperature in dark. The reaction was stopped with 50 μL per well of 2 mol L^{-1} NaOH, and absorbance at 405 nm was measured by a microplate reader (Synergy HT; Biotek, Winooski, VT, USA). As negative controls, incubation with PBS instead of turbot serum as first antibody was carried out. The test was repeated in triplicate.

Histology

Bouin's solution-fixed midgut and distal gut (Fig. 2 illustrates the location of these two compartments along the GI tract) were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological techniques. Sections of approximately 7 μm were cut and stained with haematoxylin and eosin (H&E). The slides were examined under a light microscope (DP72; Olympus, Tokyo, Japan) equipped with a camera (E600; Nikon, Tokyo, Japan) and CellSens Standard Software (Olympus) for image acquisition for the presence of degenerative changes in epithelial cells or inflammation according to the criteria suggested by Krogdahl, Bakke-McKellep and Baeverfjord (2003), namely (i) widening and shortening of the intestinal folds; (ii) loss of the supranuclear vacuolization in the absorptive cells (enterocytes) in the intestinal epithelium; (iii) widening of the central lamina propria within the intestinal folds, with increased amounts of connective tissue; and (iv) infiltration of a mixed leucocyte population in the lamina propria and submucosa. Additionally, micrographs from light microscopy were analysed with an image analySIS® software package (Image Pro Plus®; Media Cybernetics, Silver Spring, MD, USA) to determine the perimeter ratio (PR) between the internal perimeter (IP) of the intestine lumen and the external perimeter (EP) of the intestine (PR = IP/EP, arbitrary units AU) (Dimitroglou, Merrifield, Moate, Davies, Spring, Sweetman & Bradley 2009). A high PR value indicates high absorptive surface area brought about by high villi length and/or increased mucosal folding. At least three images from each sample were analysed.

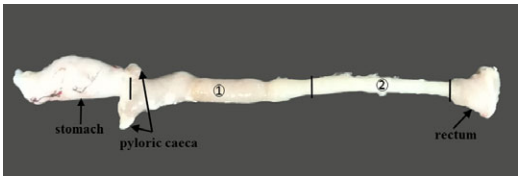

Figure 2 Illustration of gastrointestinal tract of turbot. ① The midgut section, ② the hindgut section. [Colour figure can be viewed at wileyonlinelibrary.com]

Real-time quantitative PCR

Total RNA was extracted from distal intestine using RNAiso Plus (9108; Takara Biotech, Dalian, China) and then electrophoresed on a 1.2% agarose gel to test the integrity. The quality of RNA was assessed by spectrophotometric analysis (A260: A280 nm ratio). Subsequently, purified 1 μg total RNA was reversely transcribed to cDNA using PrimeScript RT reagent kit with gDNA Eraser (RR047A; Takara Biotech) according to the instructions.

Marker genes for pro- and anti-inflammatory reaction (IL-1β, TNF-α and TGF-β1) and B-cell immune response (IgM) were profiled. Primers for these target genes were designed using Primer Premier 5 Software and synthesized by Sangon (Shanghai, China). RPSD (RNA polymerase II subunit D), and β-actin were chosen as reference genes due to their stable expression profile across all the experimental groups. To determine the application efficiency, standard curves were made with five different dilutions (in triplicate) of the cDNA samples, and the results were presented in Table 2.

The first strand cDNA product was diluted eight times using sterilized double-distilled water. Real-time PCR was performed using a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany), and a final volume of 25 μL was used, which contained 12.5 μL 2 × TransStart Green qPCR SuperMix (AQ101-03; Transgen Biotech, Beijing, China), 9.5 μL nuclease-free water, 1 μL diluted cDNA template and 1 μL of each primer (10 mM). The real-time PCR reactions were run employing the following conditions: 95°C for 2 min and then 40 cycles of 95°C for 10 s, 58°C for 20 s and 72°C for 20 s. Melting curve analysis was performed at the end of each PCR reaction to confirm that only one PCR product was present in each of these reactions. The gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), and the relative expression level of gene in the control group was used as a calibrator.

Table 2 Primers used for real-time PCR analysis

Gene	5′–3′ primer sequence		Amplicon size (bp)	Annealing temperature (°C)	Primer efficiency	GenBank Accession no.
	Forward	Reverse				
TNF-α	GGACAGGGCTGGTACAACAC	TTCAATTAGTGCCACGACAAAGAG	170	60	1.00	FJ654645.1
IL-1β	CGCTTCCCCAACTGGTACAT	ACCTTCCACTTTGGGTCGTC	144	60	1.14	AJ295836.2
TGF-β1	TCAGCATTCCAGATGTAGGTG	GGAGAGTGGCTTCAGTTTTTC	312	60	1.01	KU238187
IgM	CTGGATTGGAATGAGATCTACTGGAG	GCTCTTGGCACAGTAATACACAGC	154	60	0.97	AJ296096.1
β-actin	CGTGCGTGACATCAAGGAG	AGGAAGGAAGGCTGGAAGAG	177	60	1.09	EU686692.1
RPSD	CCTCATGTCGCGGATGCT	CCTCGGAAAGTTCCTGCTC	157	60	1.10	DQ848899.1

RPSD, RNA polymerase II subunit D.

Calculations and Statistical analysis

The following variables were calculated:

Survival (%) = 100 × final amount of fish/initial amount of fish

Specific growth rate (SGR, % day^{-1}) = $100 \times (\ln W_t - \ln W_O)/t$

Feed intake (FI, % day^{-1}) = $100 \times$ feed consumed $\times\ 2/(W_0 + W_t)/t$

Feed efficiency ratio (FER) = $(W_t - W_0)$/feed consumed

Apparent digestibility coefficients (ADC, %) = $100 \times [1 -$ (% tracer in diet × % nutrient in faeces)/(% tracer in faeces × % nutrient in diet)]

where W_t and W_0 are final and initial fish weight, respectively; t is duration of experimental days; feed and protein consumed are calculated on a dry matter basis.

Tank means were used as the statistical unit in the analyses. All data were subjected to a one-way analysis of variance using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) followed by the Tukey's multiple-range test. Differences were regarded as significance when $P < 0.05$, and the results are presented as means ± SEM.

Result

Growth performance and feed utilization

The survival of experimental fish was greater than 95% in each group, and no treatment-related difference in survival was observed among all groups ($P > 0.05$). The SGR, FER and FI of turbot were not affected by 2% dietary β-conglycinin ($P > 0.05$), whereas higher inclusion levels of β-conglycinin at 4% and 8% both significantly reduced the SGR and FER, in spite of a significant increase in the FI ($P < 0.05$) (Table 3).

The ADCs of dry matter, protein and lipid were all significantly depressed by higher levels of dietary β-conglycinin (4% and 8%) compared with the control group ($P < 0.05$) (Table 4).

The moisture and ash content of whole fish significantly increased by the addition of 4% and 8% β-conglycinin in the diets ($P < 0.05$). However, the whole-body lipid content declined greatly as dietary β-conglycinin increased from 2% to 8% ($P < 0.05$). The whole-body protein content was affected only at the highest inclusion level of

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Table 3 Growth performance and survival of turbot fed the diets with graded levels of β-conglycinin (Means ± SEM, $n = 3$)*

Growth performance	Diet No./dietary β-conglycinin inclusion level			
	Diet 1/0%	Diet 2/2%	Diet 3/4%	Diet 4/8%
Initial weight (g)	9.9 ± 0.04	10.0 ± 0.01	10.0 ± 0.01	10.0 ± 0.01
Final weight (g)	92.6 ± 3.19^{a}	92.5 ± 1.38^{a}	56.4 ± 6.15^{b}	21.5 ± 0.63^{c}
Survival (%)	100.0 ± 0.00	97.8 ± 1.11	97.8 ± 1.11	96.7 ± 1.93
SGR (% day^{-1})	2.7 ± 0.04^{a}	2.7 ± 0.02^{a}	2.1 ± 0.13^{b}	0.9 ± 0.03^{c}
FI (% day^{-1})	1.5 ± 0.06^{a}	1.5 ± 0.02^{a}	2.1 ± 0.17^{b}	3.4 ± 0.14^{c}
FER	1.3 ± 0.05^{a}	1.3 ± 0.01^{a}	0.8 ± 0.10^{b}	0.3 ± 0.02^{c}

*Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).
SGR, specific growth rate; FI, feed intake; FER, feed efficiency ratio.

Table 4 Apparent digestibility coefficients of nutrients by turbot fed the diets with graded levels of β-conglycinin (Means ± SEM, $n = 3$)*

	Diet No./dietary β-conglycinin inclusion level			
	Diet 1/0%	Diet 2/2%	Diet 3/4%	Diet 4/8%
ADC of dry matter (%)	64.5 ± 0.59^{a}	62.1 ± 0.77^{a}	48.4 ± 0.84^{b}	36.3 ± 0.94^{c}
ADC of crude protein (%)	83.6 ± 0.91^{a}	81.7 ± 0.44^{a}	62.4 ± 0.84^{b}	40.7 ± 0.69^{c}
ADC of crude lipid (%)	85.6 ± 0.74^{a}	83.7 ± 0.57^{a}	66.0 ± 0.90^{b}	42. 9 ± 0.63^{c}

*Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).
ADC, apparent digestibility coefficients.

β-conglycinin (8%) with a minor reduction ($P < 0.05$) (Table 5).

Systematic immune responses

Compared with the control group, serum LYZ and ACP activities were significantly enhanced by 2% dietary β-conglycinin supplementation. However, these activities declined significantly as dietary β-conglycinin levels increased at 4% and 8% ($P < 0.05$). The respiratory burst activity of head-kidney macrophages and serum SOD activity were not affected by dietary inclusion of 2% β-conglycinin ($P > 0.05$), but declined significantly when dietary β-conglycinin levels reached 4% and 8% of dietary supplementation ($P < 0.05$) (Table 6). Serum-specific IgM was detected in all the treatment groups and mounted as dietary β-conglycinin increased. The specific antibody level of fish fed the diet with 8% β-conglycinin was significantly higher than those of fish fed the diets with lower β-conglycinin (Fig. 3).

Intestinal morphology

Degenerative or inflammatory changes were not observed in the mid-intestine of any fish from all diet groups (data not shown). However, the distal

Table 5 Whole-body composition of turbot fed the diets with graded levels of β-conglycinin (Means ± SEM, $n = 3$)*

	Diet No./dietary β-conglycinin inclusion level			
	Diet 1/0%	Diet 2/2%	Diet 3/4%	Diet 4/8%
Moisture (%)	77.7 ± 0.20^{a}	77.9 ± 0.45^{a}	79.6 ± 0.19^{b}	81.5 ± 0.34^{c}
Crude protein (% w.w.)	15.2 ± 0.11^{a}	15.2 ± 0.09^{a}	15.1 ± 0.06^{ab}	14.8 ± 0.05^{b}
Crude lipid (% w.w.)	2.8 ± 0.10^{a}	2.7 ± 0.02^{a}	2.1 ± 0.01^{b}	1.1 ± 0.09^{c}
Ash (% w.w.)	3.3 ± 0.04^{a}	3.4 ± 0.03^{a}	3.9 ± 0.05^{b}	5.4 ± 0.06^{c}

*Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).
w.w., wet weight.

Table 6 Immune responses of turbot fed the diets with graded levels of β-conglycinin (Means ± SEM, $n = 3$)*

	Diet No./dietary β-conglycinin inclusion level			
	Diet 1/0%	Diet 2/2%	Diet 3/4%	Diet 4/8%
Respiratory burst activity (OD_{630})	1.3 ± 0.06^{a}	1.3 ± 0.04^{a}	1.0 ± 0.04^{b}	0.8 ± 0.03^{b}
Lysozyme activity (units mL^{-1})	566.8 ± 27.68^{b}	939.6 ± 53.57^{a}	624.3 ± 27.28^{b}	181.8 ± 31.56^{c}
ACP activity (ACH_{50}, units mL^{-1})	130.0 ± 3.67^{b}	189.9 ± 7.09^{a}	112.4 ± 4.61^{b}	59.7 ± 4.63^{c}
SOD activity (units mL^{-1})	92.5 ± 1.84^{a}	88.8 ± 2.06^{a}	75.8 ± 0.70^{b}	74.4 ± 0.70^{b}

*Mean values in the same row with different superscript letters are significantly different ($P < 0.05$); respiratory burst activity was measured using head-kidney macrophages; lysozyme activity, ACP activity and SOD activity were determined using serum.
ACP, alternative complement pathway; ACH_{50}, 50% haemolysis; SOD, superoxide dismutase.

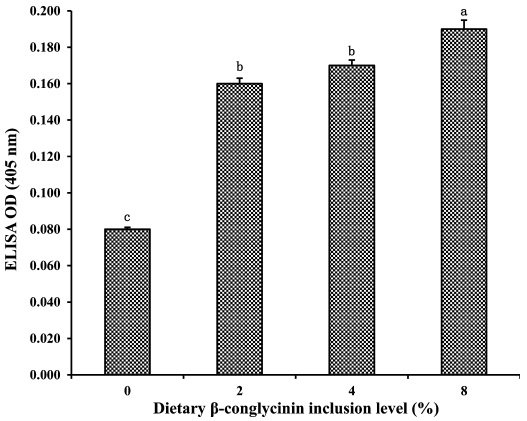

Figure 3 Specific antibody level against β-conglycinin in serum from turbot fed 0–8% purified β-conglycinin fraction. Error bars of columns denote standard error of means ($n = 3$), and columns with different letters above are significantly different ($P < 0.05$).

intestine of fish fed the diets supplemented with 4% and 8% β-conglycinin showed some typical morphological changes in SBM-induced enteritis, such as widening and shortening of the intestinal folds, widening of the lamina propria within the intestinal folds and a profound infiltration of mixed leucocytes (eosinophil was abundant; neutrophil, macrophages and lymphocytes were also present) in the lamina propria (Fig. 4). Nevertheless, the infiltration of inflammatory cells in the submucosa was not found, nor the loss of the supranuclear vacuolization in the absorptive cells (enterocytes) was observed in this study. Indeed, the absorptive cells of the distal intestine showed various degrees of supranuclear vacuolization, and the presence of vacuolated and non-vacuolated cells was seen within all experimental groups and was not related to the treatment.

No significant differences in the absorptive surface area (PR, AU) were observed in the mid-intestine ($P > 0.05$) (data not shown). The PR of the distal intestine of turbot fed 4% (4.4 ± 0.10) and 8% (3.4 ± 0.14) dietary β-conglycinin, however, was significantly lower than that of the control group (6.5 ± 0.11) ($P < 0.05$), due to the decreased number and height of mucosal folds. Fish fed 2% dietary β-conglycinin showed no significant changes in the PR of distal intestine (6.2 ± 0.13) when compared with the control group ($P > 0.05$).

Expression of immune-relevant genes in the distal gut

Compared with the control group, the expression of TNF-α and IL-1β was significantly higher in fish fed 4% dietary β-conglycinin, whereas TGF-β1 showed a significantly higher expression level in fish fed 2% dietary β-conglycinin and a significantly lower expression level in fish fed 8% dietary β-conglycinin ($P < 0.05$) (Fig. 5). The expression of IgM was significantly lower in fish fed 4% and 8% dietary β-conglycinin ($P < 0.05$) (Fig. 5).

Discussion

In the present study, the feed intake (FI) of turbot increased greatly with increasing dietary β-conglycinin; however, the growth (SGR) of fish was significantly impaired by 4% and 8% dietary β-conglycinin. This indicates that higher levels of dietary β-conglycinin impair the growth performance of turbot by reducing the feed utilization, which is supported by the significantly lower feed efficiency ratio (FER) and apparent digestibility coefficients (ADC) of nutrients. Similar results were also reported in Jian carp that dietary β-conglycinin (8%) induced a dysfunction in intestinal digestion and absorption and resulted in reduced fish growth

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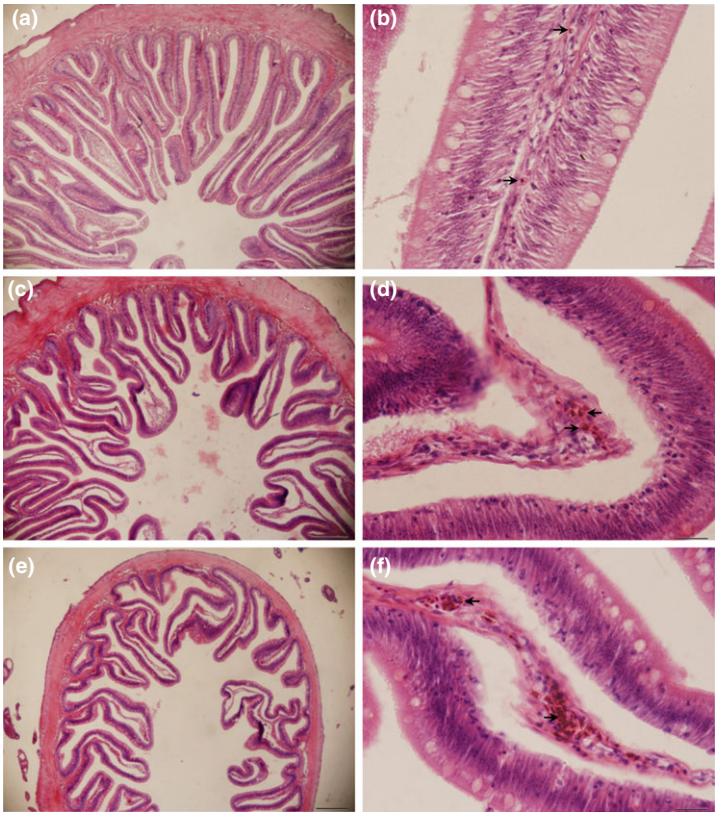

Figure 4 Histological sections of the distal intestine from turbot fed 0% (a, b), 4% (c, d) and 8% (e, f) dietary β-conglycinin. Note the morphological changes brought about by higher levels of dietary β-conglycinin: decreased number and height of mucosal folds and widening of the lamina propria within the intestinal folds (a, c, e) (scale bar = 200 μm); a profound infiltration of mixed leucocytes (arrow) in the lamina propria (b, d, f) (scale bar = 20 μm). Staining: h & e. [Colour figure can be viewed at wileyonlinelibrary.com]

(Zhang *et al.* 2013). Meanwhile, growth reduction was also observed in murine and porcine models of oral allergy induced by β-conglycinin (Guo, Piao, Ou, Li & Hao 2007; Hao, Zhan, Guo, Piao & Li 2009; Wang, Geng, Wu, Kou, Xu, Sun, Feng, Ma & Luo 2014). However, the growth reduction of fish fed 4% dietary β-conglycinin (of which 3.18% are immunologically active) seems to be contradictory with the results from previous studies by Peng *et al.* (2013) and Zhao (2008) that up to 30% of fish meal can be replaced by soya bean meal, which contains approximately 4% β-conglycinin, in the turbot diet without compromising fish growth. This discrepancy could be caused by (i) the different concentration of immunoreactive β-conglycinin in the β-conglycinin fraction used in the present study and in the SBM used in the previous study, due to their differences in processing and storage conditions (ii) and the variations in the β-conglycinin content in different soya bean varieties, which may range from 17.2% to 44.1% depending on the genetic and environmental differences (Murphy & Resurreccion 1984; Cai & Chang 1999; Mujoo *et al.* 2003; Zarkadas, Gagnon, Gleddie, Khanizadeh, Cober & Guillemette 2007).

In general, the protein content of fish remains relatively constant across life stage and can be hardly affected by dietary factors (provided the dietary essential amino acids are adequate); however, lipid content of fish is highly variable and affected by both endogenous (fish size, growth rate) and exogenous (dietary, environmental) factors (Shearer 1994; Bureau, Hua & Cho 2006). In addition, protein deposition greatly exceeds lipid deposition under severe feed restriction (Bureau, Kaushik & Cho 2002). In the present study, fish fed the diets with increasing levels of β-conglycinin showed significant reduction in body lipid content, whereas the body protein content was much less influenced. This indicated that the fish's body lipid reserve was mobilized to support protein deposition in response to the limited metabolic energy obtained from the diets due to the poor digestion of nutrients, as similarly observed in other fish species (Shearer 1994) and farmed animals (Kyriazakis & Emmans 1992a,b; Boekholt, Van Der Grinten, Schreurs, Los & Leffering 1994) under feed restriction. As observed in previous studies, the moisture content of fish increased with increasing dietary β-conglycinin, showing an opposite pattern to the

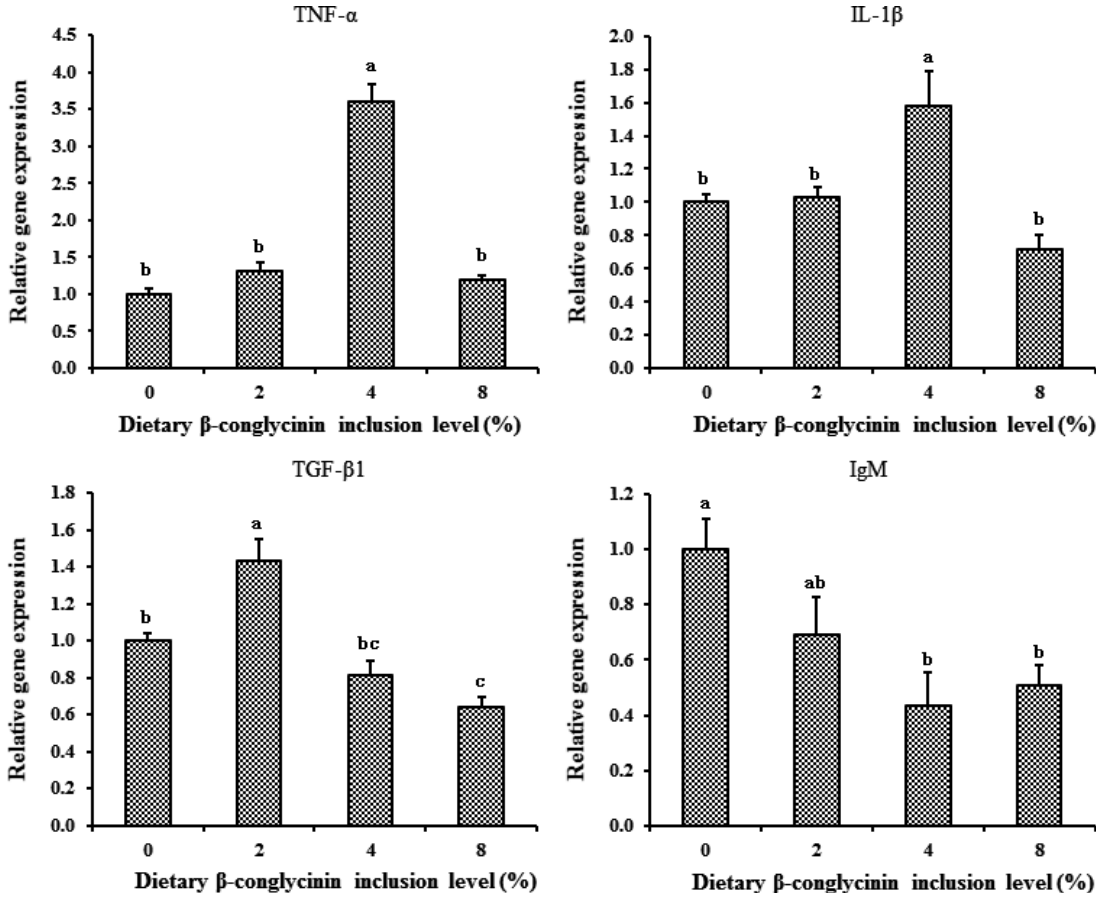

Figure 5 Gene expression of TNF-α, IL-1β, TGF-β1 and IgM in the distal intestine of turbot fed 0-8% purified β-conglycinin fraction. Error bars of columns denote standard error of means ($n = 3$), and columns with different letters above are significantly different ($P < 0.05$).

lipid content (Bureau *et al.* 2002). The increased ash content may be a result of less soft tissue mass and a greater bone mass of fish because of the mobilization of lipids and even proteins to produce energy for maintenance requirement under the circumstance that metabolic energy obtained from the diets was greatly limited by the higher levels of dietary β-conglycinin (4-8%) (Bureau *et al.* 2006).

Significantly lower ADC values of nutrients observed in fish fed 4% and 8% dietary β-conglycinin and diarrhoea-like symptoms found in fish fed 8% dietary β-conglycinin are indicative of intestinal disorders caused by higher levels of dietary β-conglycinin. This is supported by the intestinal morphological study showing inflammatory reactions and villi atrophies in the distal intestine of fish fed diets supplemented with 4% and 8% β-conglycinin. The present finding that dietary β-conglycinin (4–8%) compromises gut function in turbot is in line with previous results of Gu *et al.* (2014) that turbot fed a diet supplemented with 6% dietary β-conglycinin for 4 weeks showed significantly lower digestive and absorptive enzyme activities concomitant with reduced villi heights and increased mucosal fold fusion. Similar results were also reported in Jian carp where dietary β-conglycinin (8%) induced oxidation and inflammation of the carp's digestive tract and subsequently resulted in dysfunction of intestinal digestion and absorption (Zhang *et al.* 2013). In mammalian studies, oral administration of β-conglycinin without adjuvant was able to provoke hypersensitivity reactions that led to intestinal damage and increased occurrence of diarrhoea (Liu, Feng, Xu, Wang & Liu 2008; Huang, Xu, Yu, Gao & Liu 2010; Sun, Liu, Wang, Liu & Feng 2013). Although several hypersensitivity reactions

have been reported in fish, their mechanisms were more like analogies rather than strict homologies to mammalian hypersensitivities (Jurd 1987). The mechanism by which β-conglycinin induced intestinal disorders in turbot warrants further investigation.

IL-1β and TNF-α are hallmark genes for inflammatory reactions. IL-1β can induce growth and activation of macrophages and lymphocytes, and its induction following viral, bacterial and parasitic infection has been characterized in teleosts (Mulder, Wadsworth & Secombes 2007; Picchietti, Fausto, Randelli, Carnevali, Taddei, Buonocore, Scapigliati & Abelli 2009). TNF-α, predominantly secreted by macrophages and T lymphocytes, plays an important role in the regulation of inflammation and cellular immune responses such as the stimulation of respiratory burst activity and phagocytosis (Mulder *et al.* 2007). TGF-β1 is a pleiotropic cytokine with anti-inflammatory functions and plays a critical part in inducing host immune tolerance to intestinal microbes and food antigens (Macdonald & Monteleone 2005; Wang & Secombes 2013). The higher expression of TGF-β1 in the distal intestine of turbot fed 2% dietary β-conglycinin might explain the lack of intestinal inflammatory reactions in fish when specific antibodies against β-conglycinin were detected in serum. The rise in the expression of IL-1β and TNF-α in fish fed 4% dietary β-conglycinin is consistent with the morphological changes in the distal intestine. Meanwhile, IgM showed lower transcript level in fish fed 4% and 8% β-conglycinin, indicating that B-cell immune responses could be suppressed. Interestingly, transcript level of IL-1β and TNF-α showed no significant changes at the highest β-conglycinin inclusion level, which seemingly contradicts results from the histological study. To get a better picture of the intestinal immune responses to dietary β-conglycinin, further studies that investigate the changes in the expression of immune cell markers and cytokines involved in different immune pathways at different time points after oral exposure to various doses of β-conglycinin are needed.

As an allergen, β-conglycinin can survive the process of digestion by the digestive enzymes when passing through the digestive tract and be absorbed in an immunologically active form by mammalian gut cells (Lallès, Tukur, Salgado, Mills, Morgan, Quillien, Levieux & Toullec 1999; Zhao, Qin, Sun, Zhang, Bao, Wang, Zhang, Zhang, Zhu & Sun 2008; Adachi, Horikawa, Shimizu, Sarayama, Ogawa, Sjolander, Tanaka & Moriyama 2009; *Wang et al.* 2009). Distal intestine is the active compartment of teleost GI tract in response to immune stimuli from gut lumen. Uptake of intact macromolecules such as horseradish peroxidase (HRP; MW ~40 KDa) and ferritin (MW ~500 KDa) by epithelial cells of distal intestine was well documented in several stomachless and stomach-containing fish species (Rombout, Abelli, Picchietti, Scapigliati & Kiron 2011). Using SBM as an alternative protein source to substitute 15%, 30%, 45% and 60% fish meal in the diet of carp (*Cyprinus carpio* L.), Zhu (2008) reported that immunoreactive β-conglycinin was detected in the lamina propria within mucosal folds along the digestive tract of carp, with the highest accumulation of β-conglycinin recorded in the posterior gut segment. Hence, the detection of β-conglycinin-specific antibodies in the serum of fish from all the treatment groups indicates that uptake of β-conglycinin in an immunologically active form by intestinal epithelial cells may have taken place in turbot as well. Subsequently, the engulfed β-conglycinin may be transported to the local as well as systematic immune system, inducing intestinal inflammatory reactions and the production of serum-specific antibodies (Rombout, Lamers, Helfrich, Dekker & Taverne-Thiele 1985; McLean & Ash 1987; Rombout, Berg, Berg, Witte & Egberts 1989; O'donnell, Reilly, Davidson & Ellis 1996). β-conglycinin-specific IgM detected in the serum of fish fed diets containing 2–8% β-conglycinin is in line with previous work by Rumsey, Siwicki, Anderson and Bowser (1994), who detected increased level of total serum immunoglobulins in rainbow trout fed SBM-based diet. Burrells, Williams, Southgate and Crampton (1999), however, failed to detect antibodies to soya proteins in serum samples from the same fish species fed diets containing dehulled solvent-extracted soya bean meal (DSSM) at levels from 0 to 89% (with an interval of 10%). They suggested that fish may be immunosuppressed or soya proteins are not exposed to the circulatory immune system.

In fish, the innate immune response has been considered as an essential component in combating disease incidents due to the constraints placed on the adaptive immune response by their poikilothermic nature plus the limited antibody repertoires, affinity maturation and memory and lastly the relatively slow lymphocyte proliferation

(Whyte 2007). Serum lysozyme and complement activity are two important humoral parameters of the non-specific immune system. Moreover, respiratory burst and superoxide dismutase (SOD) are critical in controlling the balance of production and clear of oxidative radicals in phagocytic cells and hence are indicative parameters of immune functions (Holmblad & Söderhäll 1999; Campa-Córdova, Hernández-Saavedra & Ascencio 2002). The enhanced serum lysozyme activity and complement activity observed in turbot fed the diet containing 2% β-conglycinin indicated that β-conglycinin may act in a way similar to immunostimulants when included at a low level in the diet. When higher levels of β-conglycinin (4–8%) were included, however, nearly all the non-specific systematic immune responses examined in turbot were suppressed. This may be a result of long-term insufficient intake of energy from diets, which can lead to the suppression of some of the immune functions (Lochmiller & Deerenberg 2000), or more likely a result of recruitment of immune cells from the systematic compartments to the gut as indicated by the increase in the number of immune cells in the lamina propria of mucosal folds.

Glycinin is another major allergenic protein present in soya bean protein products. It has been shown to induce hypersensitivity reactions in young animals such as piglets (Sun, Li, Li, Dong & Wang 2008) and preruminant calves (Lallès, Dreau, Salmon & Toullec 1996), causing digestive disorders and reduced performance. However, Gu *et al.* (2014) demonstrated that turbot fed a diet supplemented with 6% purified glycinin (90.5% purity) showed normal intestinal function relative to fish fed the fish meal-based diet. Accordingly, it is not likely that the presence of 5.3% glycinin in the purified β-conglycinin fraction would contribute to the intestinal inflammatory reactions and the reduced growth observed in fish fed the diet 3 and 4, containing ~0.20% and ~0.41% glycinin, respectively.

Beta-conglycinin is heat stable and cannot be denatured by the heat treatment during the production of soya bean meal. ELISA analyses of different soya bean varieties reveal β-conglycinin levels in a broad range of 39.9-147.8 g kg^{-1} (dry matter base), and the concentration of β-conglycinin in SBM is even higher (Hei, Li, Ma & He 2012). Despite the fact that refined soya protein products such as SPC and SPI show low antigenicity and excellent nutritional values for animals, the costs of manufacturing such products are high. While extruded and fermented soya bean protein products could exhibit reduced antigenicity, they can still induce the production of specific antibodies to β-conglycinin in animals, however, to a less extend (Mir, Burton, Wilkie & Burnside 1993; Song, Pérez, Pettigrew, Martinez-Villaluenga & de Mejia 2010). Additionally, the contents of immune-reactive β-conglycinin in these soya products can vary greatly due to the different processing conditions employed. The results of the present study suggest that it may be necessary to measure and secure the level of immunologically active β-conglycinin in turbot feeds when less refined soya protein products are included in the diet.

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