

Effects of dietary glycinin on the growth performance, digestion, intestinal morphology and bacterial community of juvenile turbot, *Scophthalmus maximus* L.



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ABSTRACT

This study was run to investigate effects of dietary glycinin on the growth performance, digestion, intestinal morphology and bacterial community of juvenile turbot (*Scophthalmus maximus* L.). Graded levels (0%, 3%, 6% and 12%) of purified glycinin were added to the basal diet to formulate four experimental diets containing 0%, 2.18%, 4.17% and 8.31% immunologically active glycinin, respectively. Triplicate groups of 30 fish were fed to apparent satiation twice daily for 12 weeks. Dietary inclusion of glycinin did not affect feed intake, feed efficiency ratio or weight gain of turbot, however, fish fed 12% glycinin showed a significant decrease in the apparent digestibility of dry matter and protein, as well as the whole-body lipid content. Meanwhile, slightly increased infiltration of mixed leukocytes in the lamina propria was observed in turbot fed 12% glycinin, so was specific IgM against glycinin in the serum. Sequencing of bacterial 16S rRNA V₄ region showed that Proteobacteria (90.64%), Bacteroidetes (6.01%) and Firmicutes (1.73%) were the dominant bacteria in the intestine of turbot. Dietary inclusion of glycinin had no significant effect on the overall structure of bacterial community but dramatically reduced the relative abundance of the *Vibrio* genus, one of the core microbes, in a dose-independent manner. Our results suggest that turbot can tolerate a relatively high level of immunologically active glycinin (8.31%) in the diet without showing notable impairments of growth performance and gut function.

1. Introduction

Due to constraints on the availability of fish meal (FM) and fish oil (FO), the diet composition of carnivorous fish species has been experiencing a major shift from the domination of marine-origin ingredients to the increasing use of vegetable feedstuffs. In particular, soybean protein products, such as soybean meal (SBM), soybean protein concentrate (SPC) and soybean protein isolate (SPI), have been widely used as a substitute for FM due to their exceptional protein content and favorable amino acid profile. The presence of anti-nutrients, however, has limited the use of low-cost soy proteins like SBM in the diets of carnivorous species. Research on identifying anti-nutrients in soybeans and their biological effects on fish physiology and health has been going on for decades and tremendous progress has been made. Yet, some anti-nutritional factors remain under-investigated, such as soy allergenic

proteins.

Accounting for roughly 40% of the total soy seed protein, glycinin is also a food allergen (Mujoo et al., 2003). It is a hexamer consisting of 6 subunits, each made up of an acidic (A) polypeptide chain (MW, 37–40 kDa) and a basic (B) polypeptide chain (MW, 19.9–20 kDa) linked together by a disulfide bond (García et al., 1997). The allergenic effects of glycinin were well documented in humans and farmed land animals. Its subunits have been identified to bind IgE antibodies from soy allergic patients (Krishnan et al., 2009) and sensitization to glycinin is likely to result in severe allergic reactions to soybean (Holzhauser et al., 2009). Pre-ruminant calves and post-weaning piglets fed insufficiently processed soybean products as milk replacers showed depressed weight gain coincidental with reduced nitrogen utilization and increased plasma antibodies specific for glycinin and β -conglycinin, another major soy allergenic protein (Pedersen, 1986; Li et al., 1990).

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Conversely, when given feeds containing soybean proteins that have been processed to denature these allergens, these young animals manifested better digestion processes and growth performance (Sissons et al., 1982; Li et al., 1991; Lalles et al., 1997). Thus, measuring the concentrations of immunoreactive glycinin and β -conglycinin in soybean protein products was suggested to be useful in predicting the digestibility and suitability of soy products for piglets and preruminant calves. More recent studies in murine and porcine models have also showed that glycinin alone was able to provoke type-I hypersensitivity reactions by oral administration without adjuvant (Liu et al., 2008; Huang et al., 2010).

In contrast with other extensively studied soybean-derived anti-nutrients, effects of glycinin on fish health are less clear. Early in 1994, Rumsey et al. (1994) suggested that high levels of glycinin and β -conglycinin in the conventional SBM seemed to be responsible for the inferior growth performance of rainbow trout (*Oncorhynchus mykiss*) compared to those fed the FM-based diet, as protease inhibitors and lectins have been largely inactivated. However, their conclusions were not convincing since SBM contains other anti-nutritional substances that would adversely affect the growth of fish. More recently, Jiang et al. (2015) showed that 8% dietary glycinin (purity not reported) induced growth reduction in Jian carp (*Cyprinus carpio* var. Jian), concomitant with cellular oxidative damage in the intestine and seemingly disrupted tight junction between enterocytes. Gu et al. (2014), on the other hand, reported that turbot (*S. maximus* L.) fed a diet supplemented with 6% glycinin (purity, ~85.2%) did not show lesser performance in terms of intestinal morphology and digestive functions.

Gut microbiota has become an integral component of the host and received increasing attentions. However, for many animals, especially for fish, information on the intestinal microbiota composition and its modulation by external factors is relatively limited and more efforts need to be made to increase our knowledge in this active field. Understanding the factors that influence the composition of microbial community in the host alimentary canal is essential to manipulate the microbial community composition to benefit the host (Wu et al., 2012). Many studies have suggested that dietary composition is one of the most important factors shaping the fish intestinal microbial community and altering the metabolism and population sizes of key symbiont species, causing biological changes to the host (Ringø et al., 2006; Sullam et al., 2012; Askarian et al., 2013; Ringø et al., 2014). Though a myriad of studies have investigated modulating effects of plant proteins, soya proteins in particular, on fish intestinal microbiota (Zhou et al., 2017), there is little focus on the impacts of plant-derived anti-nutritional factors so far.

Previous studies showed that up to 30% of fish meal could be replaced by soybean meal without impairing the growth performance of juvenile turbot while a substitution level at 45% or higher significantly reduced the fish growth and caused disrupted intestinal morphology (Zhao, 2008; Peng et al., 2013). The present study investigated effects of increasing doses of purified glycinin fraction (3%, 6% and 12%) on turbot's growth performance, feed utilization, intestinal morphology and bacterial community, in an attempt to elucidate the role of glycinin in limiting the use of SBM in turbot's diet.

2. Materials and methods

2.1. Experimental diets

Purified glycinin 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 protein content of glycinin fraction is 93.6% (determined by the Kjeldahl method) and its SDS-PAGE profile is shown in Fig. 1. Quantitative analysis of the gel image was performed using Quantity One (Bio-Rad, Hercules, CA, USA)

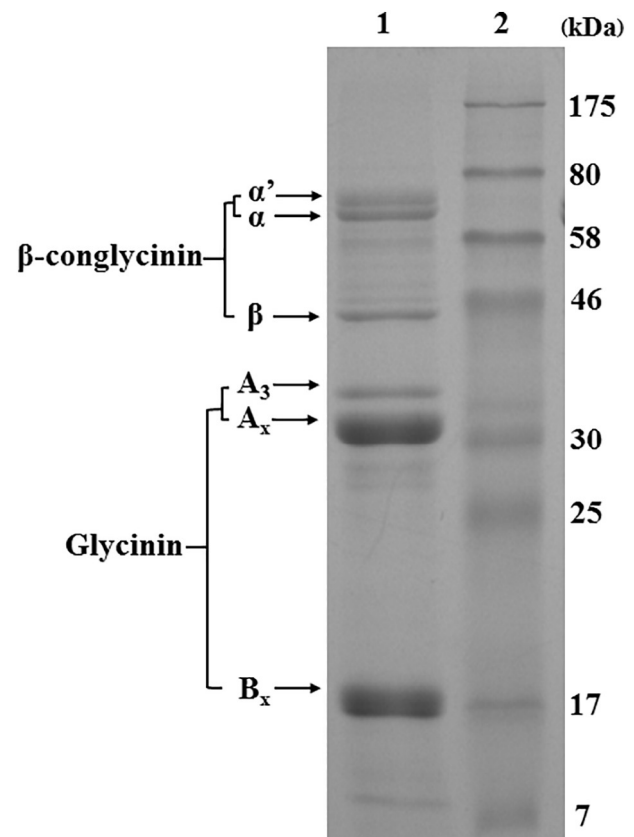


Fig. 1. SDS-PAGE profile of purified glycinin fraction. Lane 1: glycinin fraction; Lane 2: protein marker. A_x and B_x stand for acidic and basic subunits of glycinin.

and the result showed that glycinin accounted for 80.4% of the total protein, the rest being 11.1% β -conglycinin and 8.5% non-allergenic proteins.

Four isonitrogenous and isolipidic 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 (CNT). Then graded levels of glycinin were included into the basal diet to replace 3%, 6% and 12% casein and gelatin (4:1) (G-3, G-6 and G-12). Crystalline amino acids were supplemented to obtain the same essential amino acid profile across all the diets. Additionally, 1 g kg^{-1} yttrium oxide (Y_2O_3) was used as an inert tracer in each diet to determine apparent digestibility of nutrients. The level of immunologically active glycinin in the diet G-3, G-6 and G-12 was determined by a commercial ELISA kit developed by Ma et al. (2010) and it was found to be 2.18%, 4.17% and 8.31%, respectively.

Dietary ingredients were ground into fine powder to pass through 320 μm mesh. All ingredients were thoroughly mixed with fish oil and soybean oil, then water was added to produce stiff dough. The dough was pelleted with an experimental single-screw feed mill. The die diameter was 3 mm and the screw speed 90 rpm. Product temperature at the end of the barrel ranged from 60.5 to 63.5 $^\circ\text{C}$. After being pelleted, the feeds were dried for about 12 h in a ventilated oven at 45 $^\circ\text{C}$ and stored in a freezer at $-20 \text{ }^\circ\text{C}$.

2.2. Fish husbandry

A 12-week feeding trial was carried out using disease-free juvenile turbot provided by a commercial fish farm in Laizhou, Shandong, China. Prior to the start of the experiment, fish were acclimatized to the experimental tanks for two weeks during which a commercial diet for juvenile turbot (Qingdao Great Seven Bio-Tech Co. Ltd., Shandong, China) was given. Then a total of 360 fish with a mean initial body

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

Ingredients	CNT	G-3	G-6	G-12
Fish meal ^a	43	43	43	43
Casein ^a	12.00	9.68	7.32	2.64
Gelatin ^a	3.00	2.42	1.83	0.66
Glycinin	0.00	3.00	6.00	12.00
Wheat flour ^a	30.94	30.79	30.67	30.39
Fish oil	4.00	4.00	4.00	4.00
Soybean oil	2.00	2.00	2.00	2.00
Soybean lecithin	1.00	1.00	1.00	1.00
Vitamin premix ^b	1.80	1.80	1.80	1.80
Mineral premix ^c	1.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20
Ca(H ₂ PO 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	0.32	0.23	0.16	0.00
Leucine	0.00	0.02	0.05	0.11
Lysine	0.00	0.04	0.08	0.16
Methionine	0.00	0.04	0.07	0.13
Threonine	0.00	0.01	0.02	0.05
Valine	0.00	0.02	0.05	0.10
Nutrient composition (% dry matter)				
Crude protein	51.28	51.60	51.77	51.65
Crude lipid	11.51	11.39	11.48	11.77
Ash	8.75	8.81	8.79	8.88
Glycinin ^d	0	2.18	4.17	8.31

^a 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.

^b Vitamin premix (mg kg⁻¹ diet): retinyl acetate, 32; vitamin D₃, 5; DL- α -tocopherol acetate, 240; vitamin K₃, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitamin B₁₂ (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.

^c Mineral premix (mg kg⁻¹ diet): 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, 2485.

^d Determined by a commercial ELISA kit developed by Ma et al. (2010).

weight of 9.98 ± 0.01 g were randomly assigned into 12 fiberglass tanks (300 L, 30 fish per tank). Each of the four experimental diets was randomly allocated to 3 tanks. The fish were fed by hand to apparent satiation twice daily (7 a.m. and 7 p.m.) and maintained under a photoperiod regimen of 12 h daylight and 12 h darkness. Uneaten feeds were collected from the tank outlets and dried for the calculation of feed intake.

During the feeding trial, water temperature ranged from 15 °C 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.

2.3. Sample collection

The sampling started 24 h after the last feeding when the turbot's digestive tract was empty according to our previous observations. All fish were anesthetized with eugenol (1:10,000) and euthanized by a sharp blow to the head before they were sampled. Five fish per tank were randomly collected and stored frozen at -20 °C for the whole-body proximate composition analysis. Blood was withdrawn from the caudal vein of 10 fish per tank 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. The middle part (about 1 cm in length) of mid and distal intestine from 3 blood-withdrawn fish per tank were dissected and fixed in Bouin's fixative solution for the histological evaluation. Another 3 fish were used for the collection of microbial samples. Briefly, after decontamination of the exterior surface with 70% ethanol, the abdomen was opened and the whole gastrointestinal tract was taken

out aseptically. Then the whole intestine (rectum excluded) was dissected and frozen immediately in liquid N₂ in a 2 mL sterile tube. The operations were performed near a flame and all the tools were sprayed with 70% ethanol and burnt before they were used for the next sampling.

Feces collection was performed a week after the above sampling procedure, during which the remaining fish were kept under the same feeding regimen. Feces were collected manually. Briefly, fish from each tank were anesthetized and gently stripped of fecal matter from rectal intestine by hand 6 h after the morning feeding. To prevent causing injuries to the rectum, the feces collection was performed every 5 days for 3 times, which gave sufficient feces for the digestibility analysis. Pooled feces from each tank were stored at -20 °C and frozen-dried with a vacuum freeze drier (ALPHA 1–4 LD, Christ, Osterode, Germany) as soon as samples were transferred to the lab.

2.4. Diets, feces and fish body composition analysis

Analyses of chemical compositions of feed ingredients, experimental diets, feces 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 \times 6.25) using the Kjeldahl method (2300 Auto-analyzer, FOSS Tecator, Höganäs, Sweden); crude lipid by ether extraction using Soxhlet method (B-811, BUCHI, Flawil, Switzerland) and ash by combustion at 550 °C. The essential amino acid profile of feed ingredients and experimental diets was determined as described by Men et al. (2014). For the determination of apparent digestibility coefficients (ADCs) of dry matter, protein and lipid of experimental diets, the concentration of Yttrium oxide in the diets and feces were measured following the method of Cheng et al. (2010).

2.5. Histology

After fixation in the Bouin's solution for 24 h, mid and distal gut were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological techniques. Sections of 7 μ m were cut and stained with hematoxylin and eosin (H & E). The slides were examined under a light microscope (DP 72, Olympus, Tokyo, Japan) equipped with a camera (E 600, Nikon, Tokyo, Japan) and an image acquisition software (CellSens Standard, Olympus, Tokyo, Japan) for the presence of degenerative changes of epithelial cells or inflammation according to the criteria suggested by Kroghdal et al. (2003), namely: (1) widening and shortening of the intestinal folds; (2) loss of the supranuclear vacuolization in the absorptive cells (enterocytes) in the intestinal epithelium; (3) widening of the central lamina propria within the intestinal folds, with increased amounts of connective tissue and (4) infiltration of a mixed leucocyte population in the lamina propria and submucosa. Additionally, micrographs from light microscopy were analyzed using 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 et al., 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 analyzed.

2.6. ELISA

Serum glycinin specific IgM was determined by an indirect sandwich-type enzyme-linked immunosorbent assay (ELISA). The glycinin (purity > 90%) used to coat the plate was kindly donated by Prof. Shuntang Guo. In brief, wells of flat bottom microtiter plates (96-well, Costar, Corning, NY, USA) were coated with glycinin (50 μ g mL⁻¹) in 100 μ L of carbonate-bicarbonate buffer (CB buffer, 35 mM NaHCO₃,

15 mM Na₂CO₃, 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-anti-turbot 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 washing, 100 µL of 0.1% (w/v) p-nitrophenyl phosphate (pNPP, Sigma-Aldrich Co., St Louis, MO, USA) in 50 mM CB buffer (pH 9.8) containing 0.5 mM MgCl₂ 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.

2.7. Bacterial DNA extraction and 16s rRNA V₄ region sequencing

Bacterial DNA was extracted using a QIAamp DNA Stool minikit (Qiagen, Hilden, Germany) with some modifications. In brief, the intestinal samples were transferred from -80 °C to ice for a short while. Then the intestine was opened longitudinally on a sterile petri dish on ice before thawing, and the mucosal layer was scraped and transferred immediately to a 5 mL sterile tube containing sufficient InhibitEX buffer (proportional to the tissue weight). The tube was subjected to vortex at maximum speed for 1 min and 1 mL of the homogenate was used for the downstream DNA extraction according to the manufacturer's instructions. The concentration and purity of the extracted genomic DNA were measured using a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). The integrity of the extracted genomic DNA was determined by electrophoresis on a 1% (w/v) agarose gel. DNA samples from fish in one tank were pooled for the intestinal bacterial community profiling to reduce variations between individual fish (Hovda et al., 2012; Li et al., 2014). The library construction and sequencing of bacterial 16 s rRNA V₄ region were performed by Beijing Genomics Institute (BGI) (Wuhan, China) as described by Yao et al. (2016).

2.8. Bioinformatics and data mining

QIIME pipeline (v. 1.8) was used for the data mining (Caporaso et al., 2010). In brief, clean reads were obtained by filtering the primer sequence, truncating sequence reads not having an average quality score of 20 over a 30-bp sliding window based on the phred algorithm and removing trimmed reads having < 75% of their original length (as well as its paired read). These high quality paired-end reads were merged to tags using FLASH (v. 1.2.11), allowing no mismatches in the

primer or barcode regions. The tags were then clustered to OTU (operational taxonomic unit) using USEARCH (v. 7.0.1090) based on 97% sequence similarity and taxonomic assignment was performed using RDP Classifier (v. 2.2) (reference database, Greengenes database 13.8). Chimeras and tags with ambiguous bases (N) were excluded for the OTU picking. Finally, an OTU table (given in the Supplemental information) and a phylogenetic tree were produced for α and β diversity analysis. Alpha diversity analysis included Observed species, Chao 1, ACE (abundance-based coverage estimator), Shannon index and Simpson index. PCoA (principal coordinate analysis) based on weighted or unweighted UniFrac was used for β diversity analysis. To assess the changes in microbial community structure brought by the glycinin inclusion, PERMANOVA (permutational multivariate analysis of variance) was performed using the R package “vegan”. The permuted *P*-value was obtained by 999 times of permutations using the weighted UniFrac distance matrix generated from the β diversity analysis. Differentially abundant taxa between the control group and the treatment groups were identified by the LefSe analysis (linear discriminant analysis effect size) (Segata et al., 2011). The alpha level for the factorial Kruskal-Wallis rank sum test and pairwise Wilcoxon test was set at 0.05 and a threshold of 2.0 was used for the linear discriminant analysis. The approach was all-against-all multi-class.

2.9. Calculations and statistical analysis

The following variables were calculated:

$$\text{Survival (\%)} = 100 \times \text{final amount of fish}/\text{initial amount of fish}$$

$$\text{Specific growth rate (SGR, \%day}^{-1}\text{)} = 100 \times (\ln W_t - \ln W_0)/t$$

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

$$\text{Feed efficiency ratio (FER)} = (W_t - W_0)/\text{feed consumed}$$

$$\text{Apparent digestibility coefficients (ADC, \%)}$$

$$= 100 \times [1 - (\% \text{tracer in diet} \times \% \text{nutrient in feces})/(\% \text{tracer in feces} \times \% \text{nutrient in diet})]$$

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.

For data not analyzed in the previous section, one-way ANOVA was performed using SPSS 17.0 for Windows, followed by the Tukey's multiple-range test. Tank means were used as the statistical unit in the analyses. Differences were regarded as significance when $P < 0.05$ and the results are presented as means ± SEM.

3. Result

3.1. Growth performance and feed utilization

The survival of experimental fish was > 95% in each group and no

Table 2

Growth performance and survival of turbot fed the diets with graded levels of glycinin (means ± SEM, n = 3)¹.

	CNT	G-3	G-6	G-12
Initial weight (g)	10.02 ± 0.02	9.96 ± 0.03	9.97 ± 0.03	10.00 ± 0.01
Final weight (g)	95.71 ± 6.67 ^{ab}	99.31 ± 4.87 ^b	93.05 ± 1.20 ^{ab}	76.17 ± 4.26 ^b
Survival (%)	96.67 ± 1.93	97.78 ± 1.11	97.78 ± 1.11	98.89 ± 1.11
SGR ² (% day ⁻¹)	2.68 ± 0.09 ^{ab}	2.73 ± 0.06 ^a	2.66 ± 0.02 ^{ab}	2.42 ± 0.07 ^b
FI ³ (% day ⁻¹)	1.60 ± 0.02 ^{ab}	1.42 ± 0.03 ^a	1.47 ± 0.04 ^{ab}	1.64 ± 0.08 ^b
FER ⁴	1.25 ± 0.03 ^{ab}	1.37 ± 0.04 ^a	1.31 ± 0.03 ^{ab}	1.12 ± 0.02 ^b

¹ Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).

² SGR: specific growth rate.

³ FI: feed intake.

⁴ FER: feed efficiency ratio.

Table 3
Apparent digestibility coefficients of nutrients by turbot fed the diets with graded levels of glycine (means \pm SEM, n = 3)¹.

	CNT	G-3	G-6	G-12
ADC ² of dry matter (%)	64.90 \pm 0.70 ^a	63.04 \pm 0.93 ^a	62.06 \pm 1.13 ^{ab}	58.25 \pm 1.18 ^b
ADC of crude protein (%)	84.15 \pm 0.93 ^a	83.11 \pm 0.75 ^a	80.86 \pm 0.70 ^{ab}	78.34 \pm 1.08 ^b
ADC of crude lipid (%)	85.19 \pm 0.66	83.77 \pm 0.53	82.88 \pm 0.81	83.54 \pm 0.93

¹ Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).

² ADC: apparent digestibility coefficients.

treatment-related difference in survival was observed ($P > 0.05$). The SGR, FER and FI of turbot were not affected by dietary inclusion of glycine ($P > 0.05$) (Table 2).

Turbot fed the diet G-12 showed a significantly lower ADC value of dry matter and protein ($P < 0.05$). Lipid digestion was not influenced by the inclusion of glycine in the diets ($P < 0.05$) (Table 3).

3.2. Body composition

The moisture and protein content of whole fish were not affected by the inclusion of glycine in the diets ($P > 0.05$). However, turbot fed the diet G-12 showed a significantly lower whole-body lipid content and a significantly higher ash content ($P < 0.05$) (Table 4).

3.3. Intestinal morphology

No degenerative or inflammatory changes were observed in the mid intestine of any fish from all the experimental groups (data not shown). However, the distal intestine of turbot fed the diet G-12 showed a slightly increased infiltration of mixed leucocytes (eosinophil was abundant, neutrophil, macrophages and lymphocytes were also present) (Fig. 2). 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.

No significant change in the absorptive surface area (PR, AU) was observed in the mid or distal intestine ($P > 0.05$) (Table 5).

3.4. Serum specific antibody against glycine

Specific IgM against glycine was only detected in the serum from turbot fed the diet G-12 ($P < 0.05$) (Fig. 3).

3.5. Intestinal bacterial community

High-throughput sequencing generated a total number of 3,162,360 raw reads. After quality-filtering, the number of clean reads was 2,760,720. After merging, 1,351,342 tags were obtained from 12 samples, resulting in identification of 1335 non-singleton OTUs clus-

Table 4
Whole body composition of turbot fed the diets with graded levels of glycine (means \pm SEM, n = 3)¹.

	CNT	G-3	G-6	G-12
Moisture (%)	77.22 \pm 0.37	77.75 \pm 0.31	78.51 \pm 0.36	78.36 \pm 0.61
Crude protein (% w.w. ²)	15.27 \pm 0.09	15.12 \pm 0.02	15.16 \pm 0.06	15.08 \pm 0.02
Crude lipid (% w.w.)	2.92 \pm 0.08 ^a	2.91 \pm 0.04 ^a	2.84 \pm 0.05 ^a	2.42 \pm 0.08 ^b
Ash (% w.w.)	3.32 \pm 0.03 ^b	3.52 \pm 0.01 ^{ab}	3.51 \pm 0.06 ^{ab}	3.72 \pm 0.11 ^a

¹ Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).

² w.w.: wet weight.

tered under 97% sequence similarity. Rarefaction curves showed that all samples reached the saturation phase, indicating adequate sequencing depth (Fig. S1).

At phylum level, the bacterial composition of the control group (CNT) was dominated by Proteobacteria (90.64%), followed by Bacteroidetes (6.01%) and Firmicutes (1.73%). At genus level, *Halomonas* accounted for 45.97% of the total bacterial population, followed by *Shewanella* (20.14%), *Vibrio* (17.46%), *Prevotella* (2.22%) and *Enterovibrio* (1.88%) (Fig. 4). Dietary inclusion of glycine showed no significant effect on the alpha diversity of intestinal microbiota ($P > 0.05$) (Table S1). Neither weighted nor unweighted principal coordinate analysis showed a clear separation of microbial samples from different experimental groups, indicating that dietary inclusion of glycine may not have a significant effect on the overall structure of intestinal microbial community (Fig. S2). This was confirmed by the PERMANOVA analysis, giving > 0.05 permuted P values for all the pair-wise test (Table S2). Fig. S3 shows differentially abundant taxa in turbot fed the glycine supplemented diets compared with those fed the control diet. To identify bacteria sensitive to the glycine inclusion, we searched for genera showing consistent response to glycine exposure regardless of dosage. Three genera were identified: *Vibrio* (negative), *Cellulosimicrobium* (negative) and *Staphylococcus* (positive) (Fig. 5). It is noteworthy that *Cellulosimicrobium* and *Staphylococcus* identified in the present study consist of sole species, i.e., *C. funkei* and *S. capitis*.

4. Discussion

A previous study by Gu et al. (2014) found that young turbot (initial body weight, 6.80 g) fed 6% glycine (purity, ~85.2%) for 4 weeks showed normal intestinal morphology and digestive functions. Similar results were reproduced in the present study where juvenile turbot (initial body weight, 9.98 g) was fed 6% glycine (purity, ~75.3%) for a longer period (12 weeks). Interestingly, while Jian carp (initial body weight, 5.37 g) fed 8% glycine for 6 weeks manifested a marked decrease of weight gain (Jiang et al., 2015), inclusion of glycine as high as 12% in turbot's diet only resulted in a trend of growth reduction (SGR) ($P = 0.072$). The discrepancy might be due to their difference in the digestive physiology with regard to the presence of stomach. The low pH environment in the stomach and the preliminary digestion by pepsin may have helped to degrade glycine to a much greater extent than it was in the agastric digestive tract of Jian carp, since glycine was known to be susceptible to the gastric digestion (Astwood et al., 1996).

The digestion of lipids showed no significant changes regardless of inclusion level of glycine, indicating that the digestive function of turbot was probably not affected. This was supported by the similar absorptive surface (PR) of mid and distal intestine across all the experimental groups. The decline in the apparent digestibility of dry matter and protein were most likely due to the digestion-resistant nature of glycine, which is a common feature of food allergens. In vivo digestion experiments showed that the residual rate of immunoreactive glycine in ileum was 10.3% in preruminant calves (Tukur et al., 1993) and 9.4% in piglets (Wang et al., 2010). The flow of glycine in ileum was suggested to be a main contributor to the decreased apparent ileal protein digestibility in preruminant calves fed soy flour. In vitro digestion experiments also showed that the basic subunits of glycine

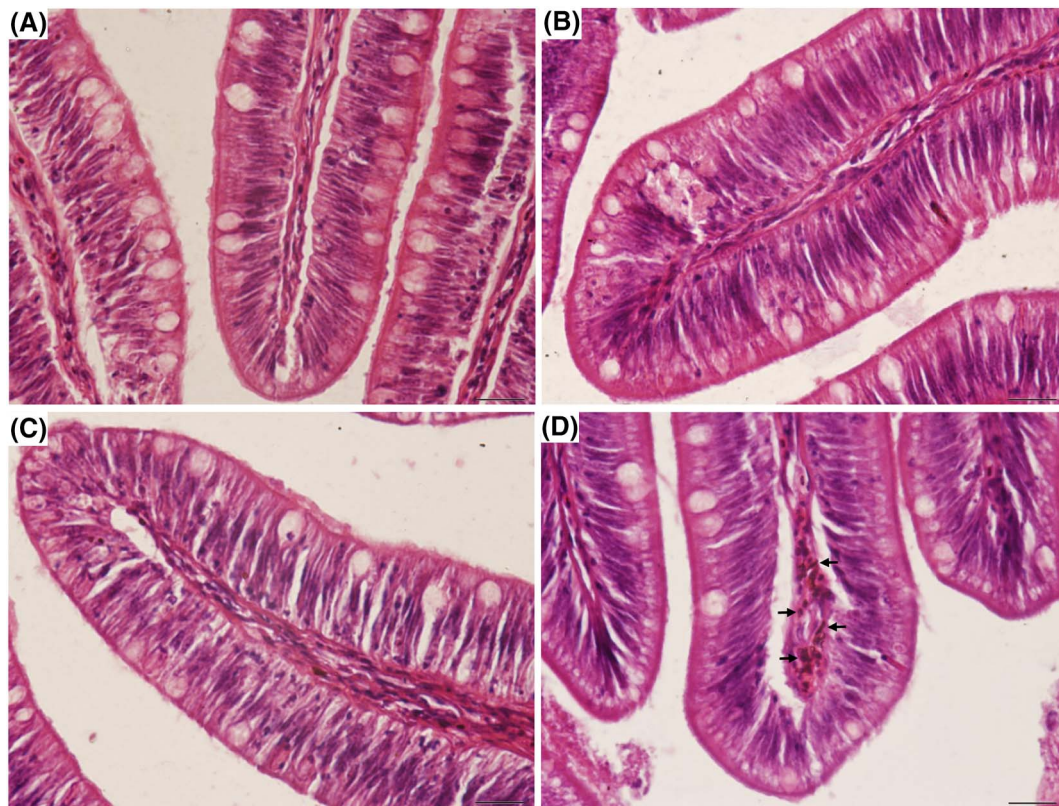


Fig. 2. Representative histological sections of distal intestine from turbot fed the CNT (A), G-3 (B), G-6 (C) and G-12 (D) diet. Note the mildly increased infiltration of leucocytes (arrows) in the lamina propria of turbot fed 12% glycine. Staining: H & E. Scale bar = 20 μ m.

Table 5

Perimeter ration of mid and distal intestine of turbot fed the diets with graded levels of glycine (means \pm SEM, n = 3).

	CNT	G-3	G-6	G-12
PR ¹ of midgut	6.32 \pm 0.07	6.33 \pm 0.11	6.32 \pm 0.05	6.31 \pm 0.05
PR of hindgut	6.01 \pm 0.12	6.07 \pm 0.03	6.01 \pm 0.15	6.00 \pm 0.06

¹ PR: perimeter ration.

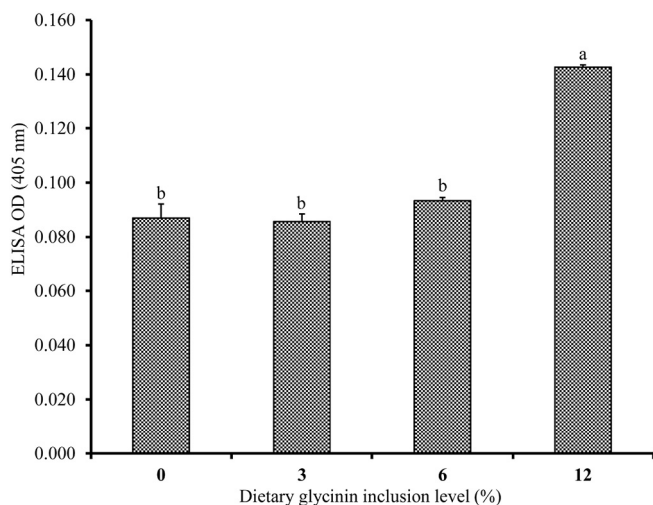


Fig. 3. Glycine-specific antibody titer in serum from turbot fed increasing doses of glycine. Error bars of columns indicate standard error of means (n = 3) and columns with different letters above are significantly different ($P < 0.05$).

were hardly hydrolyzed by trypsin (Zhao et al., 2010).

Despite having a similar digestibility of lipids to the control group, turbot fed the diet G-12 showed lower whole-body lipid content. This might be related to the hypocholesterolemic effect of glycine. Peptides IAVPGEVA, IAVPTGVA, and LPYP derived from the hydrolysis of glycine were found to be hypocholesterolemic. They were shown to increase the low density lipoprotein receptor (LDLR) activity in HepG2 cells, a human hepatic cell line, via three possible pathways that would result in the increased binding of extracellular LDL and the final hypocholesterolemic effects (Lammi et al., 2015). However, the involvement of other components in soybeans that also possess hypocholesterolemic effects, such as saponins, phytic acid, fiber, and isoflavones, cannot be excluded. Though most of these phytochemicals are poorly soluble in water, they may marginally co-participate with glycine during the extraction process.

As is aforementioned, parts of glycine can survive the digestion by mammalian digestive enzymes when passing through the digestive tract. Subsequently, it's taken up by gut cells in an immunologically active form (Zhao et al., 2013). Teleost distal intestine is active in sampling antigens from the gut lumen. Uptake of intact macromolecules like horseradish peroxidase (HRP; MW ~40 kDa) and ferritin (MW ~500 kDa) by epithelial cells in the distal intestine was documented in several stomachless and stomach-containing fish species (Rombout et al., 2011). Using SBM as an alternative protein source to substitute 15%, 30%, 45% and 60% FM in the diet for common carp (*Cyprinus carpio* L.), Zhu (2008) reported that immunoreactive glycine was detected in the lamina propria within mucosal folds along the digestive tract of carp, with the highest accumulation of glycine recorded in the posterior gut. Hence, the detection of glycine-specific IgM in turbot fed 12% glycine indicated that uptake of glycine in an immunologically active form by intestinal epithelial cells may have taken place in turbot as well. Subsequently, the engulfed glycine might be transported to the local as well as systematic immune system,

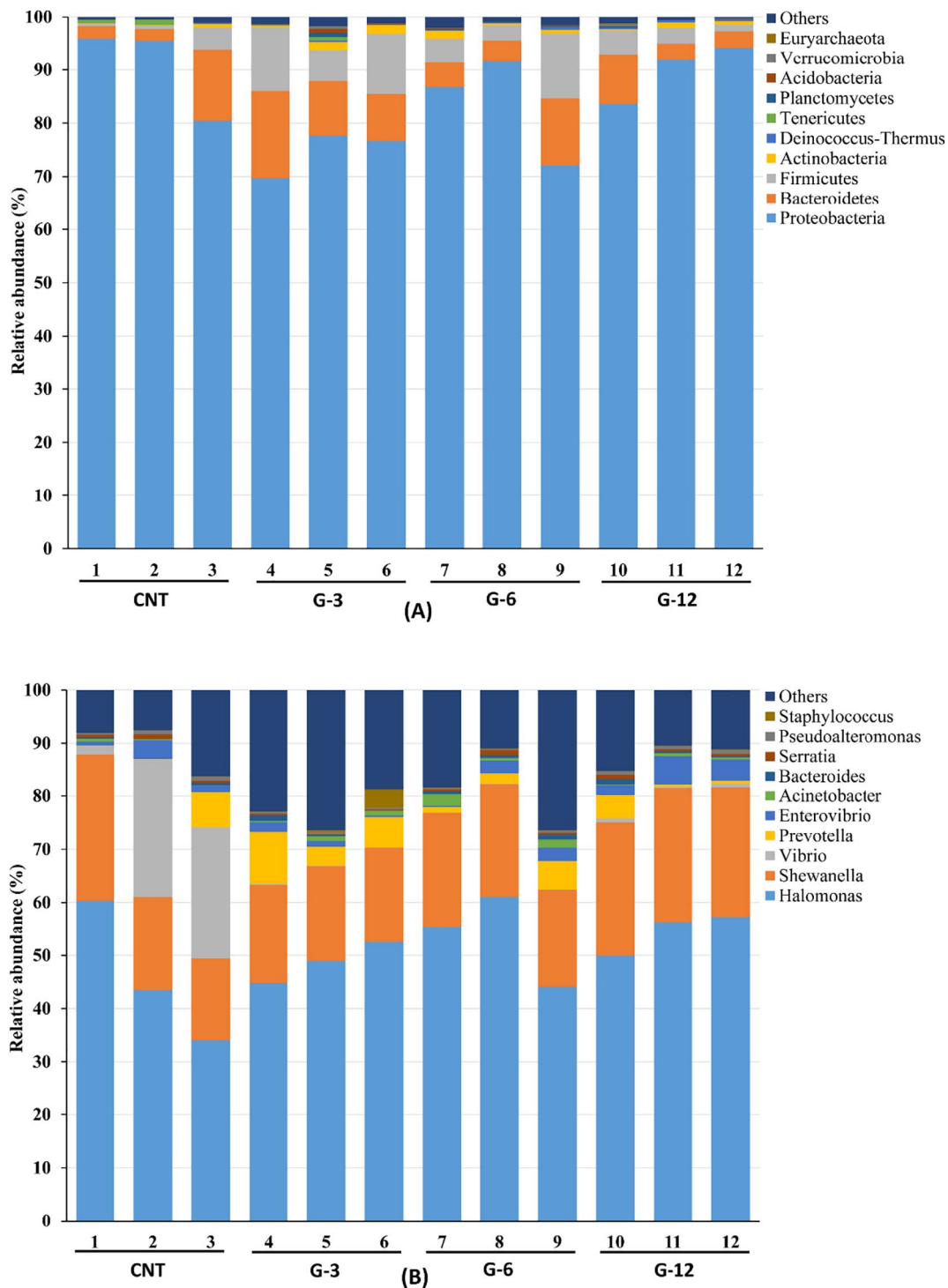


Fig. 4. Composition of gut microbiota in juvenile turbot fed increasing levels of glycinin at phylum and genus level. Each bar represents average relative abundance of top 10 most abundant (based on relative abundance) bacterial taxon within a sample at phylum (A) and genus (B) level.

inducing both local and systematic immune responses, i.e., an increased infiltration of leukocytes in the lamina propria and the production of specific antibodies. The presence of glycinin specific IgM in serum is in agreement with previous findings in humans and farmed animals that serum specific antibody against glycinin was detected after the ingestion of insufficiently processed soy products that contained immunologically active glycinin (Lallès et al., 1995; Johnston et al., 1996; Holzhauser et al., 2009; Song et al., 2010). However, Burrells et al. (1999) failed to detect antibodies to soya proteins in serum samples from rainbow trout (initial body weight, 5 g) fed diets containing SBM

at levels from 0 to 89% for 12 weeks. They suggested that fish may be immunosuppressed, or, soya proteins are not exposed to the circulatory immune system.

Intestinal microbiota profiling was included in this study as an integral part of the gut health evaluation. Our results showed that intestinal bacterial community of turbot fed the control diet was overly dominated by Proteobacteria (90.64%) at phylum level, followed by Bacteroidetes (6.01%) and Firmicutes (1.73%). This is in accordance with previous results from farmed adult turbot, whose gastrointestinal microbiome was unveiled by metagenomic sequencing using the whole

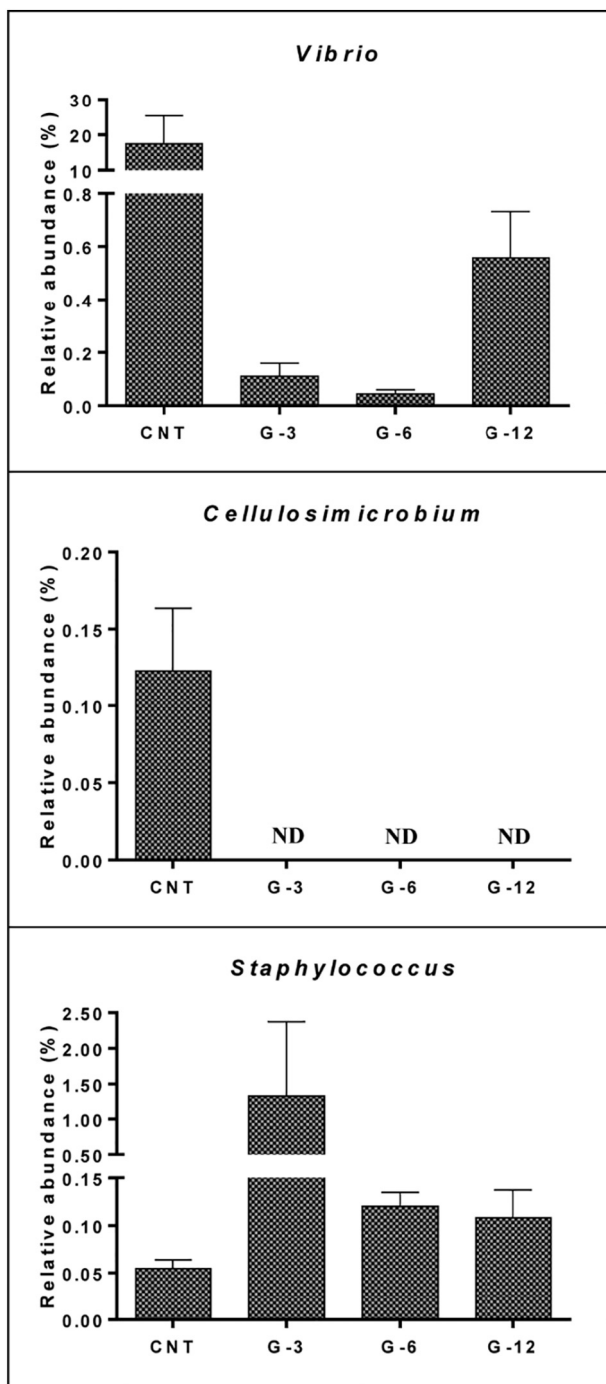


Fig. 5. The relative abundance of *Vibrio*, *Cellulosimicrobium* (*C. funkei*) and *Staphylococcus* (*S. capitis*) in turbot's gut exposed to increasing doses of glycinin. ND, not detected.

digestive tract containing digesta (Xing et al., 2013), and general findings on fish autochthonic (mucosal) intestinal microbiota (Llewellyn et al., 2014; Wang et al., 2017). At genus level, our results that *Halomonas* (45.97%), *Shewanella* (20.14%) and *Vibrio* (17.46%) are the most prominent indigenous genera in the intestine of turbot show some disagreements with Xing et al.'s (2013) study, whence they found *Vibrio* (72%) and *Photobacterium* (7.4%) to be the core microbes. The disagreements may be caused by the difference in sample type, life stage, sequencing method, diet composition, growth environment and genetic background. *Halomonas*, *Shewanella* and *Vibrio* are protease-producing bacteria commonly found in the fish gut (Zhou et al., 2009; Givens et al., 2015). In particular, *Halomonas* was found to be specially enriched in the gut of carnivorous fish species compared to that of the

herbivorous species captured in the same freshwater lake (Liu et al., 2016). The over-dominance of *Halomonas* in turbot's intestine is indicative of associations between the host trophic level and its intestinal microflora. Though the addition of glycinin showed no significant effects on intestinal microbiota at community level, some bacteria seemed to be sensitive to this dietary intervention. Notably, the relative abundance of *Vibrio*, one of the core microbes, diminished from 17.46% to < 1% in all the treatment groups, implying that glycinin might be suppressive to the growth of *Vibrio*. Indeed, glycinin was reported to possess antimicrobial effects on several food-borne pathogens, including *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus aureus* (Sitohy et al., 2012; Vasconcellos et al., 2014; Xiang et al., 2016). Furthermore, Merrifield et al. (2009) also found decreased richness of autochthonous *Vibrio* genus in the gut of rainbow trout fed SMB based diet for 16 weeks, relative to those fed the FM based diet. As some members of the *Vibrio* genus, such as *Vibrio anguillarum* and *Vibrio ordalii*, can cause serious infections to fish, it's tempting to test in future studies whether glycinin possess antimicrobial effects on these pathogens. Meanwhile, *S. capitis* was found to thrive in turbot's gut exposed to glycinin, showing an opposite response than that of *S. aureus*, a pathogen causing food poisoning and many forms of infection in human. Our results indicate that different species of *Staphylococcus* might have divergent responses to the glycinin exposure.

Beta-conglycinin is another major soy allergenic protein and it also manifested a strong cholesterol-lowering effect (Moriyama et al., 2004; Martinez-Villaluenga et al., 2009; Mochizuki et al., 2009; Inoue et al., 2015). Our another parallel study using the same experimental setups showed that dietary supplementation of 1.55% immune-reactive β -conglycinin did not induce a local immune response or a reduction in carcass lipid content in turbot (Li et al., 2016). Accordingly, it's unlikely that the presence of 0.31% β -conglycinin in diet G-12 may contribute to the lower whole-body lipid content or the increased infiltration of leukocytes in the lamina propria.

In conclusion, results from the present study suggest that turbot can tolerate a relatively high level of immunologically active glycinin (8.31%) in the diet without showing significant impairments of growth performance and gut function. Glycinin could be an inhibitory agent to species of the *Vibrio* genus in the intestine, which merits further investigation. Among soybean-derive anti-nutrients, glycinin is probably not a major contributing factor that limits the use of less refinery soya products in turbot's feeds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.aquaculture.2017.05.008>.

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