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The protective role of glutamine on enteropathy induced by high dose of soybean meal in turbot, *Scophthalmus maximus* L.

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ABSTRACT

The aim of this study is to investigate the protective effect and regulatory mechanism of Gln on the enteropathy of turbot induced by soybean meal. Three isonitrogenous and isolipidic practical diets were formulated with fish meal basis (FM), soy protein replacing 40% fish meal protein (SBM) and dietary 2% Ala-Gln in SBM (Ala-Gln) for sextuple replicates of 30 fish each tank for 12 weeks. The fish in SBM group presented distinct enteropathy including declined absorptive surface and overt infiltration of mixed leukocytes in the lamina propria. However, the turbot fed Ala-Gln diet showed the integrity of the intestinal tissue structure and higher ADC and AKP levels like fish in FM group. Compared to SBM group, the inclusion of 2% Ala-Gln in SBM-basal diet significantly elevated the expression of barrier-forming tight junction proteins claudin-4, occludin and ZO-1 and depressed the expression of pro-inflammatory cytokines TNF-α in the intestine. Fish fed Ala-Gln diet showed remarkable increased MUC-2 and PPAR-γ gene expression as well as reduced expression of NF-κB and MLCK equivalent to the level of FM group. Additionally, Gln significantly elevated the relative abundance of Proteobacteria (*Vibrio* spp.) and declined Bacteroidetes (*Bacteroides* spp.) relative abundance. The similarity of intestinal microbial communities in Ala-Gln group was notably closer to FM group from PCoA, UPGMA and Heatmap analyses based on weighted UniFrac distance. In conclusion, dietary Gln could probably enhance intestinal barrier function to alleviate enteropathy by improving MUC-2 and PPAR-γ expression and inhibiting NF-κB-MLCK signaling pathway, as well as altering microbiota.

1. Introduction

The intestine is one of the major organs in fish involved in digestion, absorption and immunity, as well as interaction with the environment (Ray et al., 2012). In human and terrestrial animal studies, nutritional strategies such as Gln, have been widely used in intestinal disturbances. For instance, De Oliveira et al. (2006) reported that an intestinal obstruction triggered by ^{99m}Tc-*E. coli* translocation in rats was mitigated by 500 mg kg^{-1} d^{-1} Gln treatment. As the case of aquatic animals, little attention was focused on the effects of the broad-spectrum Gln on intestinal health. Owing to the distinctiveness in evolution, fish with inferior intestinal length index to other species has to perform further responsibilities on nourishment and growth, especially the carnivorous one. Thereby, the impact of Gln on the intestinal health in carnivorous fish merits further investigation.

Evidence appears to be mounting that the intestinal mucosal barrier dysfunction is a prerequisite for enteropathy (Bron et al., 2017; Turner, 2009). As the first line of defense, the mechanical barrier mainly comprises epithelial cells, tight junctional complex and the mucus above (Reynolds, 1996). Usually, the intestinal inflammation

Abbreviations: ADC, Apparent digestibility coefficient; AKP, Alkaline phosphatase; Ala-Gln, Alanyl-glutamine; AMPK, Adenosine 5′-monophosphate-activated protein kinase; DAO, Diamine oxidase; FER, Feed efficiency ratio; FI, Feed intake; FM, Fish meal; FMW, Final mean weight; GC, Goblet cell; Gln, Glutamine; IL-1β, Interleukin-1β; ILI, Intestinal length index; ISI, Intestinal somatic index; LP, Lamina propria; MLCK, Myosin light-chain kinase; MUC-2, Mucin-2; NF-κB, Nuclear factor-kappa B; OTU, Operational taxonomic unit; PCoA, Principle coordinate analysis; PPAR-γ, Peroxisome proliferator-activated receptor-γ; PR, Perimeter ratio; SBM, Soybean meal; SGR, Specific growth rate; SR, Survival rate; TGF-β, Transforming growth factor-β; TNF-α, Tumor necrosis factor-α; UPGMA, Unweighted pair group method with arithmetic; ZO-1, Zonula occluden-1

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accompanies with abnormal expression of MUC-2 and tight junction proteins such as claudin-4, occludin and ZO-1 (Jiang et al., 2015; Velcich et al., 2002; Hara et al., 2000). Moreover, marine fish like turbot (Gu et al., 2016) and Atlantic salmon (Marjara et al., 2012; Bakke-McKellep et al., 2007) fed high dose of soybean meal presented gross intestinal inflammation coupled with increased expression of pro-inflammatory cytokines such as IL-1β and TNF-α. In addition, the intestinal microbiota was referred as the key factor involved in sustaining barrier function and homeostasis (Bron et al., 2017), which could provide the host with a biological barrier to the pathogen infection by competitive exclusion and production of antimicrobial substances, vitamins and enzymes (Sekirov et al., 2010). To date, despite much work on growth-promotion and anti-oxidation of Gln in fish (Jiang et al., 2015; Cheng et al., 2012), little is known about the impact of Gln supplementation on intestinal mucosal integrity and function, particularly microbiota.

To develop effective strategies of Gln, the underlying mechanisms of Gln on the intestinal mucosal barrier need to be determined. However, relative mechanisms in cell and mammals have not been in unison, not alone in fish. A growing body of literature demonstrates that NF-κB is a crucial regulator for the expression of genes involved in inflammation (He et al., 2012; Chen et al., 2008; Kisseleva et al., 2006) via translocating to the nucleus inducing inflammatory cytokines and recruiting immune cells (Hanning and Diaz-Sanchez, 2015). Moreover, Ye and Ma (2008) pointed out that NF-κB binding motifs were present on the MLCK promoter region to induce MLCK gene expression, which modulated contraction of actomyosin ring and exerted a key role in regulation of tight junction (Costantini et al., 2009; Shen et al., 2006). Thus, the activation of NF-κB could lead to inflammation and hyperpermeability in intestinal epithelial cells (He et al., 2012). However, PPAR-γ and AMPK were both recorded to have the efficiency of NF-κB inhibition via protein-protein interaction (Scott et al., 2008; Kelly et al., 2004). Nevertheless, knowledge about the signaling pathways of Gln inhibiting inflammation in fish is still unknown.

As the most important alternative protein source, SBM has been widely replacing fish meal in commercial aqua-feeds production. Turbot (*Scophthalmus maximus* L.), a highly valued and extensively cultured marine carnivorous fish, is sensitive to the dietary dose of SBM. The focus of this study is to highlight the protective effect and potential mechanisms of Gln on the intestinal function and health of turbot suffering the SBM induced enteropathy that would allow a better understanding of Gln and the utilization of SBM in carnivorous fish. Meanwhile, due to thermolability of Gln in the process of making feed, the alternative of alanyl-glutamine dipeptide addressed the problem (Fürst et al., 1997; Wu et al., 1996).

2. Materials and methods

2.1. Ethical considerations

The protocols for animal care and handling used in this study were approved by the Animal Care Committee of Ocean University of China. Facilities for turbot husbandry were optimally equipped to ensure refinement of breeding and accommodation to minimize fish suffering. No clinical symptoms were observed within or outside the experimental periods.

2.2. Experimental diets

Three isonitrogenous and isolipidic experimental diets were formulated to contain approximately 52% crude protein and 10% crude lipid (Table 1). A fish meal based diet named as FM group, and a diet replacing 40% fish meal protein by soybean protein named as SBM group. Then a glutamine-supplemented diet was formulated with the inclusion of 2% alanine-glutamine (2% Ala-Gln) into the SBM diet. Alanine was added to make the diets isonitrogenous, and Yttrium oxide (Y_2O_3, 1 g kg^{-1}) was used as an inert tracer in each diet for determining apparent digestibility coefficient of dry matter.

Table 1
Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	FM	SBM	2% Ala-Gln
Fish meal[a]	68	40.80	40.80
Soybean meal[b]	0	37.90	37.90
α-Starch	16	9.55	9.55
Fish oil	4.80	6.70	6.70
Soy lecithin	0.50	0.50	0.50
Vitamin premix[c]	1	1	1
Mineral premix[d]	0.50	0.50	0.50
Choline chloride	0.30	0.30	0.30
Monocalcium phosphate	0.50	0.50	0.50
Ethoxyquin	0.05	0.05	0.05
Yttrium oxide	0.10	0.10	0.10
Calcium propionate	0.10	0.10	0.10
Cellulose	6.15	0	0
Ala-Gln[e]	0	0	2
Alanine[f]	2	2	0
Sum	100	100	100
Component analysis			
Dry matter content	95.68	96.45	96.39
Crude protein (%)	52.13	53.27	52.05
Crude lipid (%)	9.82	10.49	10.19

Abbreviations: FM, fish meal diet; SBM, soybean meal diet; Ala-Gln, alanyl-glutamine dipeptide diet.

[a] Purchased from Qingdao Seven Great Bio-tech Company Limited (Qingdao, China), crude protein: 74.04%, crude lipid: 9.97%.

[b] Purchased from Qingdao Seven Great Bio-tech Company Limited (Qingdao, China), crude protein: 53.12%, crude lipid: 2.12%.

[c] Vitamin mixture: providing for per kg diet: VA 32 mg, VB1 25 mg, VB2 45 mg, VB6 20 mg, VB12 10 mg, Niacinaminde 200 mg, Inositol 800 mg, Calcium pantothenate 60 mg, VH 60 mg, Folate 20 mg, VE 240 mg, VK 10 mg, VC phosphate 2000 mg, VD 5 mg, Antioxidant 3 mg, Microcrystalline cellulose 6470 mg.

[d] Mineral mixture: providing for per kg diet: Mg 313 mg, Fe 79.10 mg, Zn 62.60 mg, Mn 46.60 mg, I 2 mg, Se 0.90 mg, Cu 6.40 mg, Zeolite powder 3485 mg.

[e] Purchased from Shanghai Wintide Biotechnology Co.,Ltd. (Shanghai). The purity was > 99.30%.

[f] Purchased from Shanghai Macklin Biochemical Co.,Ltd. (Shanghai). The purity was > 99.26%.

Ingredients were grounded into fine powder through a 320-μm mesh. All the ingredients were thoroughly mixed with fish oil and soy lecithin, and water was added to produce stiff dough. Then the dough was pelleted with an experimental feed mill (F[II]-26, South China University of Technology, Guangzhou, China), dried for about 12 h in a ventilated oven at 50 °C, and stored at −20 °C prior to usage.

2.3. Fish husbandry

Juvenile turbot (*Scophthalmus maximus* L.) in this experiment were yearlings obtained from a commercial farm in Weihai, Shandong Province, China. After arriving, all the fish were fed the control diet and reared in experimental system for 2 weeks to acclimate the experimental conditions. During the acclimation procedure, fish with malformation, surface damage, incomplete gill cover, low vitality and extreme sizes were excluded. At the initiation of the experiment, all the fish were fasted for 24 h and weighted. Fish of similar size (initial mean weight 9.57 g) were randomly distributed into 18 tanks in the free-flowing system. Three experimental diets were randomly assigned to six replicate tanks and 30 fish in each tank were fed by hand to apparent satiation twice daily (8:00 and 18:00) for 12 weeks. The uneaten feed was collected in 30 min after each meal, dried to a constant weight, and weighed to allow calculation of feed intake. During the feeding period, water conditions and fish behavior were observed and noted every day.

Table 2
Primers used in quantitative real-time PCR (qRT-PCR).

Target gene	Primer sequence (5′-3′)	Amplicon size (bp)	AE	Genbank accession number
Claudin-4	F: ATGTGGAGTGTGTCGGCTT R: AGACCTTGCACTGCATCTG	237	1.06	MF370857
Occludin	F: ACTGGCATTCTTCATCGC R: GGTACAGATTCTGGCACATC	583	1.01	KU238181
ZO-1	F: AGAGAACCTGTCACTGATAGATGC R: CTGTCGGAATTGTTGCCTGATG	1697	0.95	KU238184
MLCK	F: TGTGCTGGGAAGTTCTACAAAGG R: CAATCTCAGGCTTGTGGTCGTAG	551	0.90	MF370856
MUC-2	F: GTTGGTGCAGCCGCATAG R: CACTGGACGCTGGGAATG	534	0.95	KU238186
IL-1β	F: ATGGTGCGATTTCTGTTC R: CACTTTGGGTCGTCTTTG	1257	0.97	AJ295836.2
TNF-α	F: GGACAGGGCTGGTACAACAC R: TTCAATTAGTGCCACGACAAAGAG	186	0.91	AJ276709.1
TGF-β	F: CTGCAGGACTGGCTCAAAGG R: CATGGTCAGGATGTATGGTGGT	749	0.97	KU238187
NF-κB	F: TGTCGTAGATGGGGTTGGA R: AGGAGCTGGGGAAGGTGAT	764	1.04	MF380879
PPAR-γ	F: TAATGGAAGGAGAGCAGTTC R: CTGTGGAAGAAGCGTAGC	121	0.94	results not shown
AMPK	F: TGGAGCAGTGGGGTCATTC R: ATGGGGTCCACCTGAAGCA	158	0.92	results not shown
β-actin	F: GCTGTCTTCCCTTCTATCGTCG R: TCCATGTCATCCCAGTTGGTC	543	0.97	AY008305.1
RPSD	F: CCTCATGTCGCGGATGCT R: CCTCGGAAAGTTCCTGCTC	545	1.10	DQ848899

F, forward primer; R, reverse primer; AE, the primer amplification efficiencies.

Table 3
Effects of Gln on growth performance of juvenile turbot fed high dose of soybean meal in diet.

	FM		SBM		2% Ala-Gln	
	Mean	S. E.	Mean	S. E.	Mean	S. E.
SR(%)	100	0.00	96.67	1.93	96.67	1.93
IMW(g)	9.58	0.05	9.55	0.02	9.62	0.04
FMW(g)	57.68^{b}	2.99	46.58^{a}	2.46	55.97^{ab}	0.53
SGR(% d^{-1})	2.13^{b}	0.06	1.88^{a}	0.07	2.10^{ab}	0.01
FI(%)	1.50	0.04	1.65	0.03	1.60	0.04
FER	1.14^{b}	0.05	0.93^{a}	0.01	1.04^{ab}	0.03
ILI(%)	51.91^{b}	1.31	46.99^{a}	0.52	48.66^{ab}	0.27
ISI(%)	1.14	0.02	1.03	0.05	1.19	0.06

Values are means and S. E. values within the same row with different letters are significantly different ($P < .05$).
SR, survival rate; IMW, initial mean weight; FMW, final mean weight; SGR, specific growth rate; FI, feed intake; FER, feed efficiency ratio; ILI, intestinal length index; ISI, intestinal somatic index; S. E., standard error.

The water temperature was 19–25 °C, DO > 7.0 mg L^{-1}, salinity 23–26, NH_4^+-N < 0.3 mg L^{-1}, and pH 7–8.

2.4. Sample collection

Three out of six tanks were for periodic sampling at the 2nd, 4th and 8th weeks and the other three were sampling at the 12th week. Fish were fully anesthetized with eugenol (1:10000) (purity 99%, Shanghai Reagent Corp, Shanghai, China) before handling to ameliorate suffering. Samples were taken only from fish with digesta throughout the intestinal tract, to ensure intestinal exposure to the diets until sampling. To evaluate the intestinal morphology, distal intestine samples of three fish were selected randomly per tank and fixed at Bouin's fixative solution. Distal intestine samples from another 4 fish per tank were taken and frozen in liquid nitrogen immediately and stored at −80 °C for gene expression analysis.

At the end of the trial, the last three tanks per diet were used for terminal sampling, and total number and body weight of fish in each tank were counted and measured. Following the above method, three distal intestine samples were for morphological measurements per tank after weighing and testing length and five distal intestine samples per tank were collected for gene expression analysis. Another five distal intestine samples were collected for enzyme activity analysis. Blood samples from ten fish in each tank were collected from the caudal veins and centrifuged (4000 rpm, 10 min) at 4 °C to obtain serum samples. Then the serum samples were immediately stored at −80 °C prior to analysis. Feces was collected quantitatively after sampling, and then frozen in liquid nitrogen and stored at −80 °C. To analyse intestinal microbiota, the three fish in each tank was sterilized with 70% alcohol, and then the whole distal intestinal mucosa layer of distal intestines were removed with sterilized scissors and bistouries around an alcohol lamp and then transferred to 2 ml sterile tubes (Axygen, America) stored at −80 °C.

2.5. Enzyme activities

The activities of intestinal AKP and serum DAO at the 12th week were determined spectrophotometrically according to the demands of the usage manual of commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China).

2.6. Intestinal morphology

The distal intestine samples were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin wax according to the standard histological procedure (Li et al., 2016; Hu et al., 2015; Ross and Pawlina, 2006). Then the samples were cut in 7 μm longitudinal sections with a rotary microtome (Lecia Jung RM 2016, Germany) and stained with hematoxylin-eosin method (HE). Observation of sections was performed by Nikon eclipse Ti−S microscope (Japan). The thickness of the LP, the PR and the number of goblet cells were determined by analyzing the micrographs from light microscopy with the image analysis software, Image Pro Plus® 6.0 (Media Cybernetics, Silver Spring, MD, USA) as described (Li et al., 2017; Hu et al., 2015; Krogdahl et al., 2015; Dimitroglou et al., 2009).

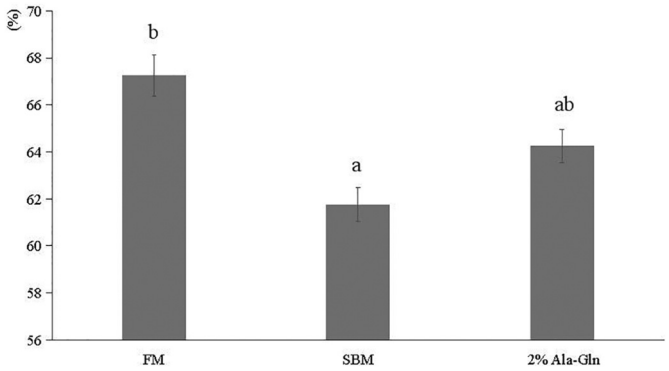

Fig. 1. Effects of Gln on apparent digestibility coefficient of juvenile turbot fed high dose of soybean meal in diet.
[ab]Different superscript letters indicate significant difference ($P < .05$).

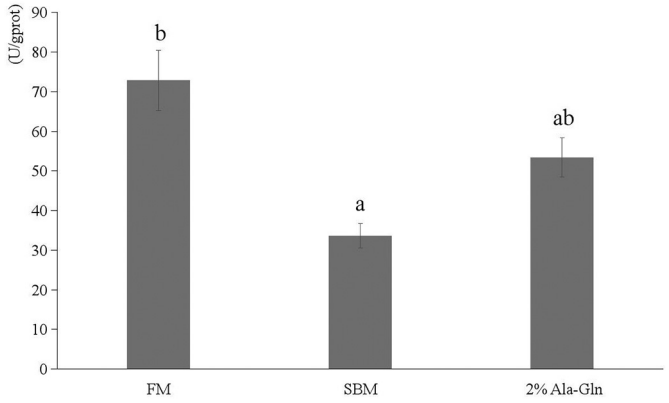

Fig. 2. Effects of Gln on intestinal alkaline phosphatase activity of juvenile turbot fed high dose of soybean meal in diet.
[ab]Different superscript letters indicate significant difference ($P < .05$).

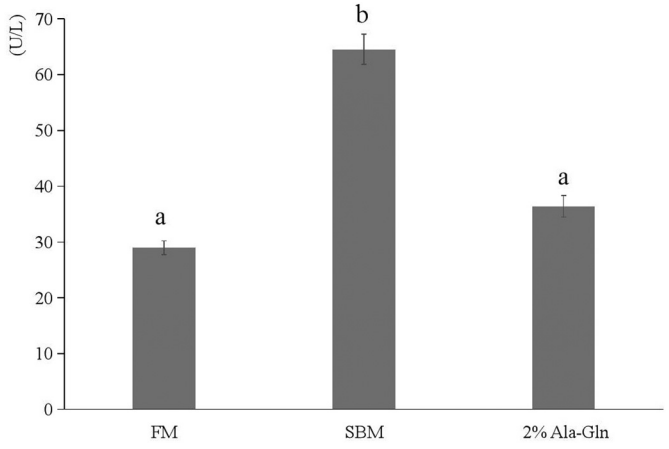

Fig. 3. Effects of Gln on serum diamine oxidase of juvenile turbot fed high dose of soybean meal in diet.
[ab]Different superscript letters indicate significant difference ($P < .05$).

2.7. Real-time quantitative PCR

To extract and purify the total RNA, distal intestines of 9 samples each group were ground to powder in liquid nitrogen and added to RNAiso Plus (9109; Takara Biotech, Dalian, China). The quality and quantity of RNA were detected by electrophoresis on 1.2% denatured agarose gels and then assessed concentration by a Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, USA). The cDNA was synthesized by reversely transcribing 1 μg total RNA using PrimeScript RT reagent Kit with gDNA Eraser (RR047A; Takara Biotech, Dalian) according to the manufacturer's instructions.

Specific primers for the genes were synthesized by Sangon (Shanghai, China) and assessed to determine the application efficiency (Table 2). RPSD and β-actin were used as the reference gene to normalize cDNA loading. The quantitative Real-time PCR assays were conducted in a quantitative thermal cycler (Mastercyclerep realplex, Eppendorf, Germany) in a total volume of 20 μl containing 10 μl of EvaGreen Express 2 × qPCR MasteMix (MasterMix-ES, Applied Biological Materials, Canada), 1 μl of the cDNA product, 0.6 μl of each primer (10 mM) and 7.8 μl of diethylpyrocarbonate-treated water. The PCR conditions began with 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C and 40 s at 72 °C. When PCR amplification was finished, melting curve analysis was performed to verify that only one PCR product was present in each of these reactions.

2.8. Bacterial DNA extraction, amplification and sequencing

Bacterial DNA was extracted according to the manufacturer's instruction of the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with slight modifications as follows: (i) the distal intestinal samples were transferred from −80 °C to ice for a short while; (ii) the samples were opened longitudinally on sterile petri dishes on ice before thawing; (iii) the mucosal layers and contents were scraped and transferred immediately to a 5 ml sterile tubes containing sufficient InhibitEX buffer (proportional to the tissue weight); (iv) the tubes were subjected to vortex at maximum speed for 1 min and 1 ml of the homogenate was taken for the downstream DNA extraction (Li et al., 2017). The quality and integrity of each DNA sample were monitored on a 1% agarose gel electrophoresis. DNA purity and concentration were determined using a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). DNA was diluted to 1 ng μl^{-1} using sterile water according to the concentrations.

The bacteria genomic DNA was amplified with the 515F and 806R primers specific for the V4 hypervariable regions of the 16S rDNA gene with the barcode. All the PCR reactions were carried out in 30 μl volume with 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, 10 ng template DNA, and nuclease-free water. Thermal cycling was conducted at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s, finally at 72 °C for 5 min. The PCR products were sequenced on an Illumina MiSeq platform according to the manufacturer's recommendations at Novogene Bioinformatics Technology Co., Ltd., Beijing, China (Liu et al., 2016; Wang et al., 2015; Yu et al., 2015).

2.9. Bioinformatic analysis

Paired-end reads from the original DNA fragments were merged using FLASH, which was designed to merge paired-end reads when original DNA fragments were shorter than twice the length of reads. Sequencing reads were filtered using the open-source software system QIIME (Quantitative Insights into Microbial Ecology) (Caporaso et al., 2010) and the tags were then clustered to OTUs using UPARSE pipeline at an identity threshold of 97% (Wang et al., 2015; Liu et al., 2016). RDP Classifier was used to annotate taxonomic assignment for each representative sequence. To estimate individual hosts' microbial alpha diversity, an OTU table including Good's coverage was produced and the rarefaction curve was generated based on the metric of observed species. A PCoA, UPGMA clustering and Heatmap based on weighted UniFrac phylogenetic distance helped to visualize the distance matrix from complex and multidimensional data. In order to assess the changes in microbial community structure among groups, differentially abundant taxa between the FM group and the other groups were identified by Metastats analysis (White et al., 2009). Tukey's test and wilcox's test

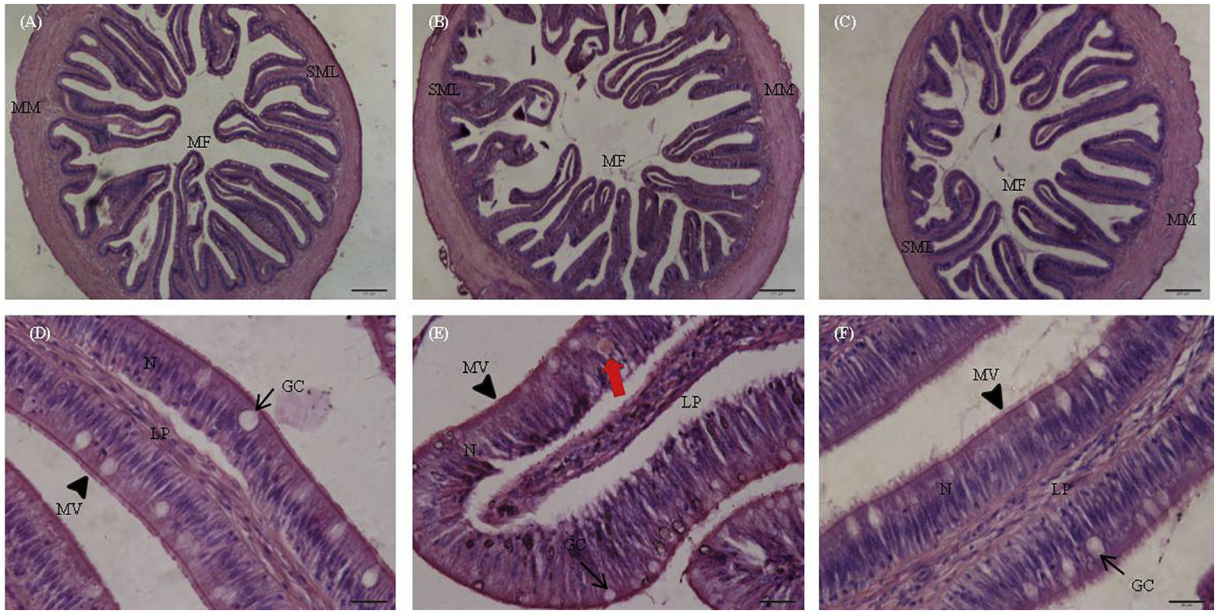

Fig. 4. Effects of Gln on the distal intestine morphology of juvenile turbot fed high dose of soybean meal in diet for twelve weeks.
Details of distal intestine section from fish in FM (A and D), SBM (B and E) and 2% Ala-Gln (C and F) groups. MF, mucosal fold; MM, muscularis mucosa; SML, submucous layer; LP, lamina propria; GC, goblet cell (arrows); MV, microvilli (arrowheads); Infiltration of mixed leukocytes (red arrows); N, nucleus. Scale bar of A, B and C, 200 μm; scale bar of D, E and F, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Effects of Gln on the distal intestine morphometry of juvenile turbot fed high dose of soybean meal in diet (n = 3).

	FM		SBM		2% Ala-Gln	
	Mean	S. E.	Mean	S. E.	Mean	S. E.
PR	4.83^{b}	0.13	3.97^{a}	0.23	4.46^{ab}	0.09
LP (μm)	25.11^{a}	1.13	34.70^{b}	0.70	30.82^{ab}	1.73
GC	46.55^{b}	2.18	33.58^{a}	4.68	48.23^{b}	3.65

Values are means and S. E. values within the same row with different letters are significantly different ($P < .05$).
PR, perimeter ratio; LP, lamina propria; GC, goblet cell.

were used to test statistical difference of α-diversity and β-diversity between groups.

2.10. Calculations and statistical analysis

Survival rate, SR (%) = 100 × (final amount / initial amount).

Specific growth rate, SGR (% d^{-1}) = ($ln_{final\ body\ weight}$ - $ln_{initial\ body\ weight}$) / days × 100.

Feed intake, FI (% d^{-1}) = 100 × total amount of feed consumption (g) / [(initial body weight + final body weight) / 2] / days.

Feed efficiency ratio, FER = weight gain (g) / total amount of feed consumption (g).

Intestinal length index, ILI (%) = intestinal length (cm) / body length (cm).

Intestinal somatic index, ISI (%) = intestinal weight (g) / body weight (g).

Absorptive surface (Perimeter ratio, PR) = IP / EP, arbitrary units. IP, the internal perimeter of the gut lumen (villi and mucosal folding length). EP, the external of the gut lumen (perimeter of internal muscularis mucosa) (Dimitroglou et al., 2009).

SPSS 17.0 microcomputer software was used for all statistical analyses. Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test. Homogeneity of variance test was conducted to ensure that variance was homogeneous. The results were presented as means ± standard error. In all statistical testing, differences between means at $P < .05$ were considered as significant.

3. Results

3.1. Growth performance

The SR was high for fish fed all diets (ranging from 96.67% to 100%) and no significant differences were observed among treatments after 12-week feeding trial ($P > .05$). Compared with FM group, the fish from SBM group showed the significantly decreased FMW, SGR, FER and ILI ($P < .05$); however, the inclusion of 2% Ala-Gln in the diet significantly increased the FMW, SGR, FER and ILI to the similar level with FM group ($P > .05$) (Table 3).

3.2. Intestinal digestive, absorptive ability and cell integrity

The fish from SBM group recorded the lowest ADC of dry matter and intestinal AKP activity among treatments ($P < .05$), and there was no significant differences of ADC of dry matter and intestinal AKP activity observed between 2% Ala-Gln and FM groups ($P > .05$) (Figs. 1 and 2). In addition, the content of serum DAO of juvenile turbot fed SBM diet was significantly higher than that of fish in FM and 2% Ala-Gln groups ($P < .05$) (Fig. 3).

3.3. Intestinal morphology and cell membrane integrity

With the supplementation of high dose of soybean meal alone, the intestine of fish exhibited typical inflammatory symptoms including declined absorptive surface ($P < .05$), wider LP ($P < .05$), decreased GC ($P < .05$), wizened enterocytes, profound infiltration of mixed leukocytes in the lamina propria and disarranged enterocyte nucleus position; however, the fish in 2% Ala-Gln group presented regular intestinal morphology without inflammation equivalent to that of FM group (Fig.4 and Table 4).

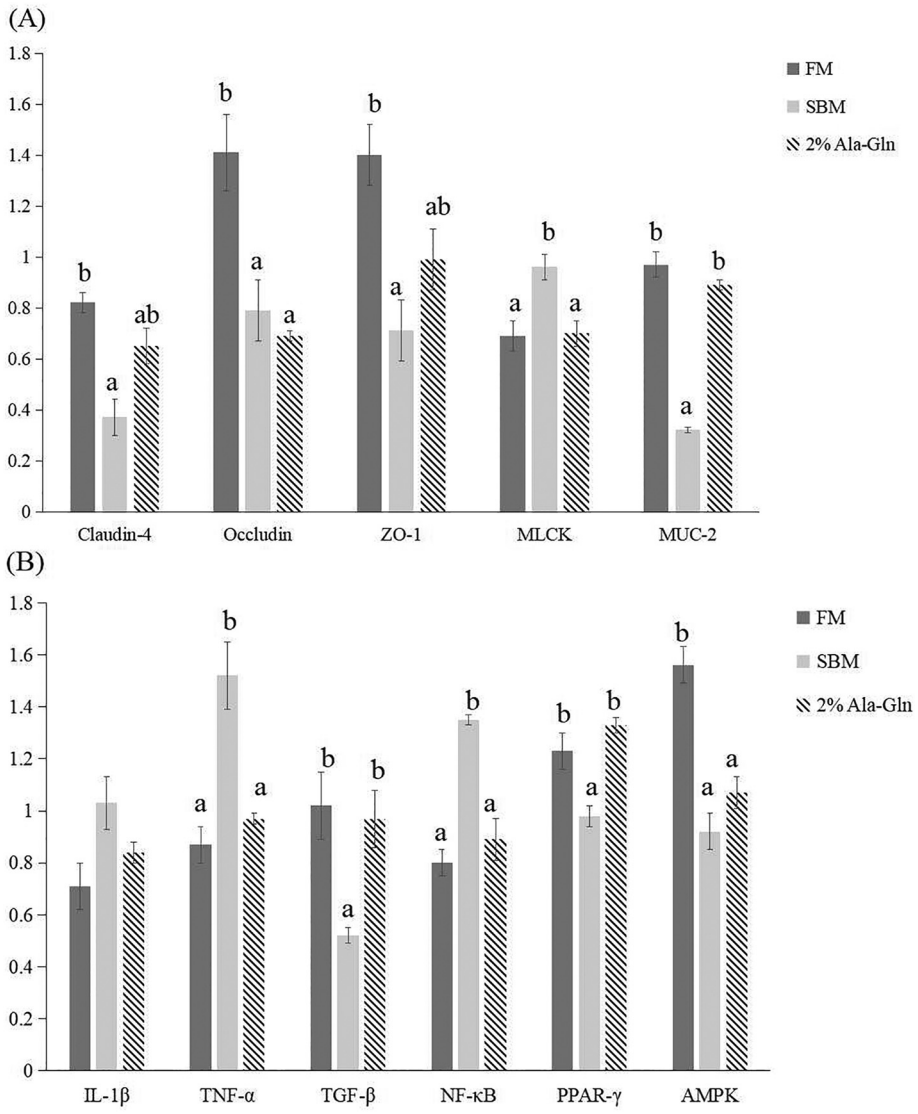

Fig. 5. Effects of Gln on the expression of key genes in intestinal mucosal barrier of juvenile turbot fed high dose of soybean meal in diet for 12 weeks. (A) the expression of distal intestine tight junction proteins and key genes in mechanical barrier; (B) the expression of distal intestine cytokines and key genes in immune barrier.

[ab]Different superscript letters indicate significant difference ($P < .05$).

3.4. Intestinal mucosal barrier

After 12-week feeding trial, the fish subjected to SBM treatment alone presented the significantly decreased intestinal gene expression levels of claudin-4, occludin, ZO-1 and MUC-2 ($P < .05$) and significantly increased MLCK gene expression ($P < .05$) compared to FM group, whereas Ala-Gln supplementation increased notably claudin-4, ZO-1 and MUC-2 gene expression ($P < .05$) and decreased significantly intestinal gene expression of MLCK ($P < .05$) (Fig. 5A). From different time points (the 2nd, 4th and 8th weeks), the inclusion of Gln significantly increased gene expression of claudin-4, ZO-1 and MUC-2 (except for no change of ZO-1 at the fourth week) compared to SBM group ($P < .05$). In addition, MLCK gene expression of fish distal intestine in GLN group was notably down-regulated compared to SBM group ($P < .05$) at the fourth week (Fig. 6).

At the 12th week, supplementing high dose of SBM alone significantly increased the intestinal gene expression of TNF-α and NF-κB ($P < .05$) and significantly decreased gene expression levels of TGF-β, PPAR-γ and AMPK ($P < .05$), while dietary 2% Ala-Gln notably reduced TNF-α and NF-κB gene expression ($P < .05$) as well as significantly increased the gene expression of TGF-β and PPAR-γ ($P < .05$) (Fig. 5B). From different time points, no significant effects on the gene expression of immune barrier function detected were observed among treatments ($P > .05$) at the second and eighth weeks (Fig. 7A and C). However, at the fourth week, supplementing 2% Ala-Gln in high dose of soybean meal diet significantly down-regulated the intestinal gene expression of IL-1β, TNF-α and NF-κB ($P < .05$) as well as notably up-regulated TGF-β and PPAR-γ gene expression ($P < .05$) compared to SBM group (Fig. 7B).

3.5. Intestinal microbiota

Illumina HiSeq sequencing platform was adopted to sequence bacterial hyper-variable V4 region of the 16S rRNA in samples of distal intestinal mucosa. A total of 583,502 effective tags were obtained from nine samples (three samples each treatment), clustering 4446 OTUs with over 97% sequence similarity in total (Table 5). As was shown in Fig. S1 and Table 5, the rarefaction curves approached the saturation plateau and the Good's coverage of all samples reached 0.998, indicating adequate sequencing depth.

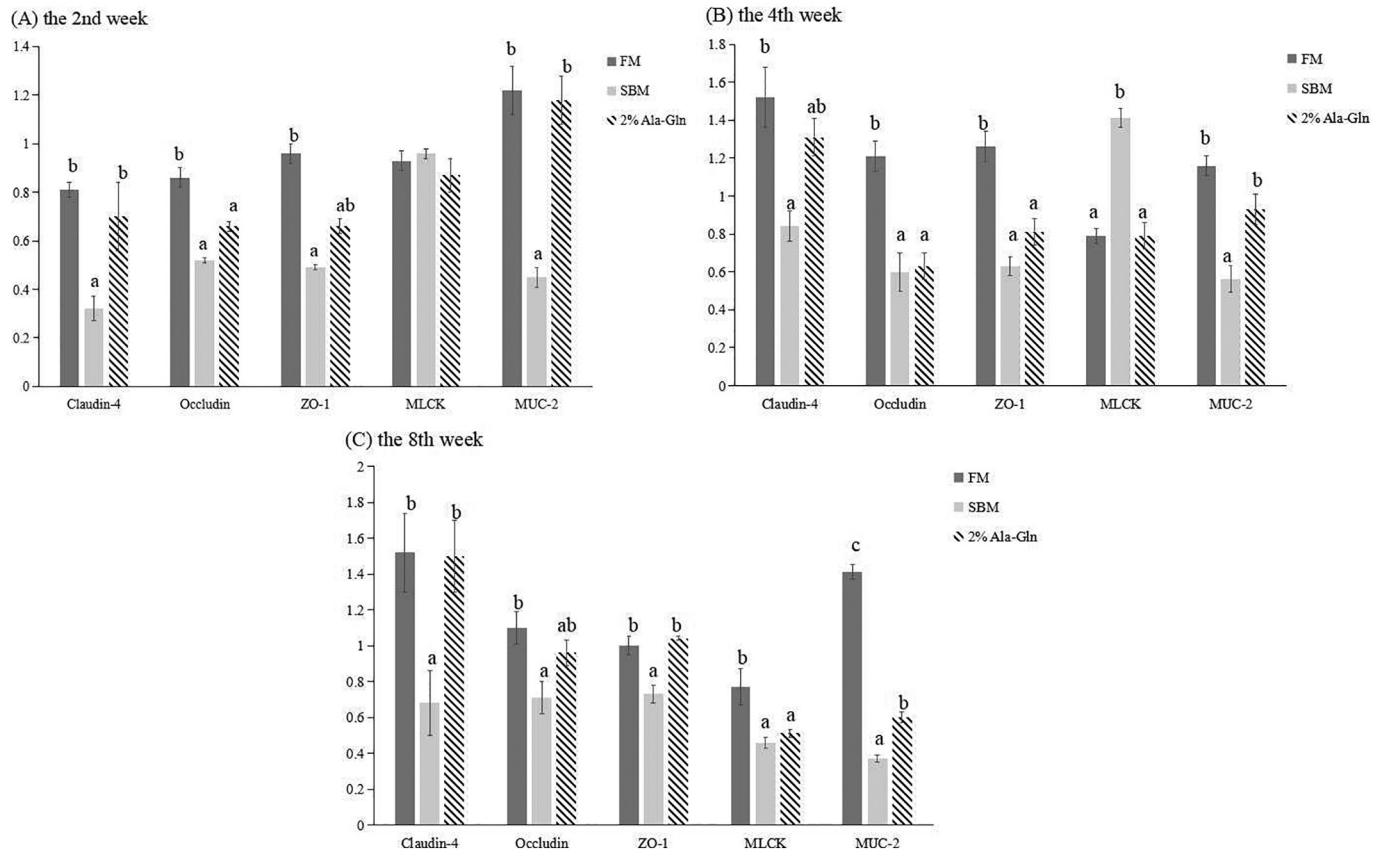

Fig. 6. Effects of Gln on the expression of distal intestine tight junction proteins and key genes in mechanical barrier of juvenile turbot fed high dose of soybean meal in diet.
[abc]Different superscript letters indicate significant difference ($P < .05$).

A PCoA (Fig. 8), UPGMA (Fig. 8) and Heatmap analyses (Fig. 8) based on weighted unifrac distance were used to compare the similarity in the microbial community composition of samples among treatments. The scatter plot manifested a clear separation based on diet treatments, and the Gln cluster was relatively closer to FM cluster. Moreover, the similar result as UPGMA analysis was performed. Several representative genera yielded statistical differentiation from the MetaStat analysis (Table 6). Compared to SBM group, supplementing Ala-Gln in high dose of soybean meal diet significantly increased the relative abundance of *Vibrio* spp. ($P < .05$) but reduced markedly the relative abundance of *Bacteroides* spp. ($P < .05$).

4. Discussion

Over the course of 12-week feeding trial, dietary 2% Ala-Gln in SBM-basal diet remarkably alleviated the enteropathy and elevated MUC-2 gene expression, as well as improved intestinal digestive and absorptive ability and growth performance of fish, like mammals (Jiang et al., 2015; Wise and Thompson, 2010; Rhoads and Wu, 2009; Chen et al., 2008). Jiang et al. (2015) have reported that a dosage of 12.0 g kg^{-1} Gln had an efficacy on maintaining intestinal structure and reducing the expression of pro-inflammatory cytokines in juvenile Jian carp induced by glycinin. Moreover, the inclusion of Gln mitigated intestinal atrophy, declined the expression of the inflammatory cytokines and recovered the intestinal function in rats and weaned pigs (Jiang et al., 2009; Chen et al., 2008; Wang et al., 2008). As the preferable 'fuel' substrate for enterocyte, Gln could promote protein synthesis and intestinal development (Wu et al., 2011; Jiang et al., 2009; Wang et al., 2008). However, the underlying mechanisms behind the positive effect of Gln remain unknown in fish and need to be addressed. The integrate function of the barrier depends on tight junctions anchored in the epithelial cells (He et al., 2012; Al-Sadi et al., 2009). As the pivotal defensive line in the epithelium, the mechanical barrier disorders might trigger microbial challenge and immune response (Ivanov et al., 2010; Hartsock and Nelson, 2008). In the present study, dietary 2% Ala-Gln increased intestinal expression of barrier-forming tight junction proteins (claudin-4 and ZO-1) as well as declined pro-inflammatory cytokine (TNF-α) expression. That was probably due to the fact that as a major metabolic fuel and a key regulator of gene expression, Gln could be involved in related immune response signaling pathways (Dai et al., 2012; Kim et al., 2011; Wu et al., 2011).

The up-regulation of NF-κB is a hallmark of common intestinal bowel diseases such as inflammatory bowel disease (O'Dea and Hoffmann, 2009; Courtois and Gilmore, 2006). Moreover, the activation of AMPK and PPAR-γ could diminish the nuclear translocation of NF-κB and inhibit inflammation in human and murine studies (Katerelos et al., 2010; Adachi et al., 2006; Dubuquoy et al., 2006). At the 4th and 12th weeks, the overexpression of inflammatory cytokines of the fish in SBM group was accompanied with the up-regulation of NF-κB in this trial in line with the previous studies (Gu et al., 2016; Jiang et al., 2015). Notably, the inclusion of Gln in diet decreased NF-κB gene expression and elevated the intestinal PPAR-γ gene expression on the 4th and 12th weeks, similar to the study of Sato et al. (2006) that filled with 60 mM Gln significantly increased PPAR-γ expression to attenuate injurious ischemia-reperfusion mediated intestinal inflammation and injury of rats. It was probably because the increased PPAR-γ expression by Gln could suppress inflammation by directing protein-protein interactions with NF-κB in a DNA-binding independent manner (Wahli, 2008; Moraes et al., 2006; Ricote et al., 1998). However, of particular interest, it had no effect of Gln on AMPK gene expression in the present

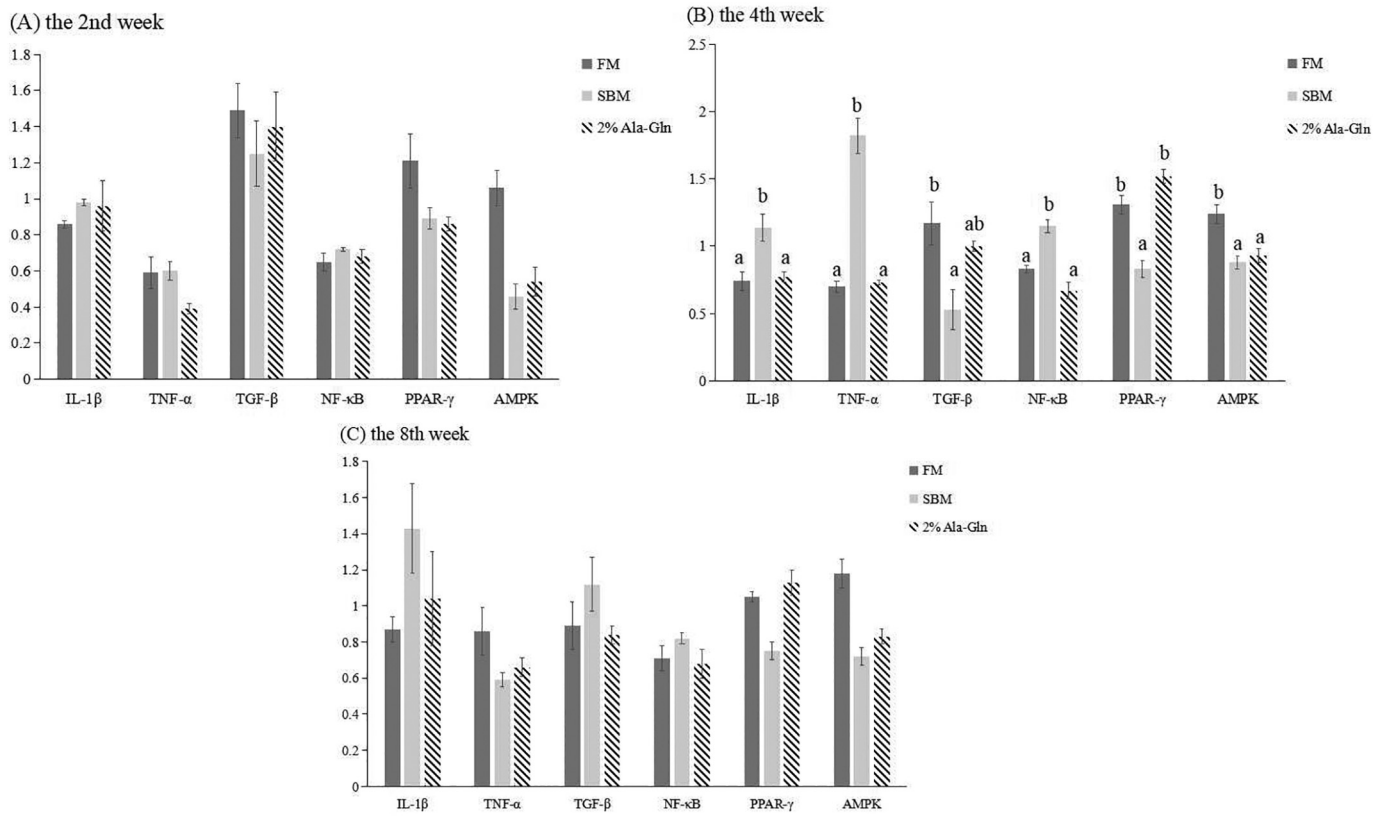

Fig. 7. Effects of Gln on the expression of distal intestine cytokines and key genes in immune barrier of juvenile turbot fed high dose of soybean meal in diet. [ab]Different superscript letters indicate significant difference ($P < .05$).

Table 5
The number of obtained effective tags, OTUs and good's coverage per replicate of each group.

Sample name	Effective tag	OTU	Good's coverage
FM1	63,226	580	0.998
FM2	51,086	723	0.998
FM3	65,642	447	0.998
SBM1	78,573	411	0.998
SBM2	69,949	700	0.999
SBM3	57,613	497	0.998
GLN1	63,340	345	0.998
GLN2	71,363	159	0.999
GLN3	62,710	584	0.998

study, which was line in with the study of Wu (2014) that neither the content nor phosphorylation level of AMPK were changed with Gln supplementation in IEC-6 under hypoxic conditions. Additionally, the increased expression of MLCK appeared to be in keeping with the up-regulation of NF-κB in this study, which was supported by study of Barreau and Hugot (2014) that the activation of NF-κB positively modulated MLCK to promote cytoskeleton contraction further increased epithelial permeability. As such, Gln probably potentiated intestinal immune barrier by elevating the expression of PPAR-γ not AMPK to inhibit NF-κB. Also, Gln could probably enhance the mechanical barrier via suppressing NF-κB and MLCK expression and elevating the expression of tight junction proteins. However, the techniques on in vitro culture of fish intestinal epithelial cells are expected to apply to studies of intestinal health mechanisms.

The microbial homeostasis is usually evaluated as a critical indicator of intestinal health. According to current knowledge, diets exert a key role in shaping intestinal microbiota and altering the metabolism (Xia et al., 2014; Brown et al., 2012; Pop, 2012), which was supported in the present study that the intestinal microbiota was sensitive to dietary components and clustered distinctly. The relative abundances of Proteobacteria and Bacteroidetes were significantly altered in SBM group. The similar results have been showed in turbot (Xing et al., 2013) and Atlantic salmon (Grammes et al., 2013) that soybean meal diets could remarkably reduce the relative abundance of Proteobacteria and notably elevate the relative abundance of Bacteroidetes. These two predominant phyla in turbot intestine were reported to contribute to the digestive process by providing a variety of enzymes (Romero et al., 2014). Furthermore, a large number of the proteins made by bacteria belonged to Bacteroidetes serve to break down polysaccharides and metabolize the sugars (Xia et al., 2014; Xu et al., 2003), which helps to explain the relative high prevalence of Bacteroidetes in the group SBM. The results of the present study showed that the cluster of the bacterial community in Gln group was relatively closer with that in FM group than SBM group in terms of the microbial composition and phylogenetic analysis. The possible reason is that Gln supplementation in diet could change the microenvironment of intestine such as (amino acid utilization and metabolism), thereby altering the relative abundance and composition of intestinal microbiota (Ren et al., 2014; Dai et al., 2013).

As the most predominant genus in the intestine of marine fish (Verner-Jeffreys et al., 2003), *Vibrio* spp. was increased greatly by the inclusion of 2% Ala-Gln in this study. There is evidence that *Vibrio* spp. have the capacities of enzyme-production including amylase, protease and lipase (Ray et al., 2012), as well as some species in this genus exhibited a positive effect against the pathogens *Vibrio harveyi* and white spot syndrome virus in white shrimp due to a stimulation of the immune system (Balcázar et al., 2006). In addition, the relative abundance of *Bacteroides* spp. which might induce inflammation was significantly declined by dietary Gln. Thus, it might be deduced that dietary Gln could alter the intestinal microbiota by facilitating digestion and inhibiting inflammation to maintain intestinal dynamic equilibrium. However, of particular note, any decline of microbial levels or diversity may contribute to a reduction of the effective barrier provided by the

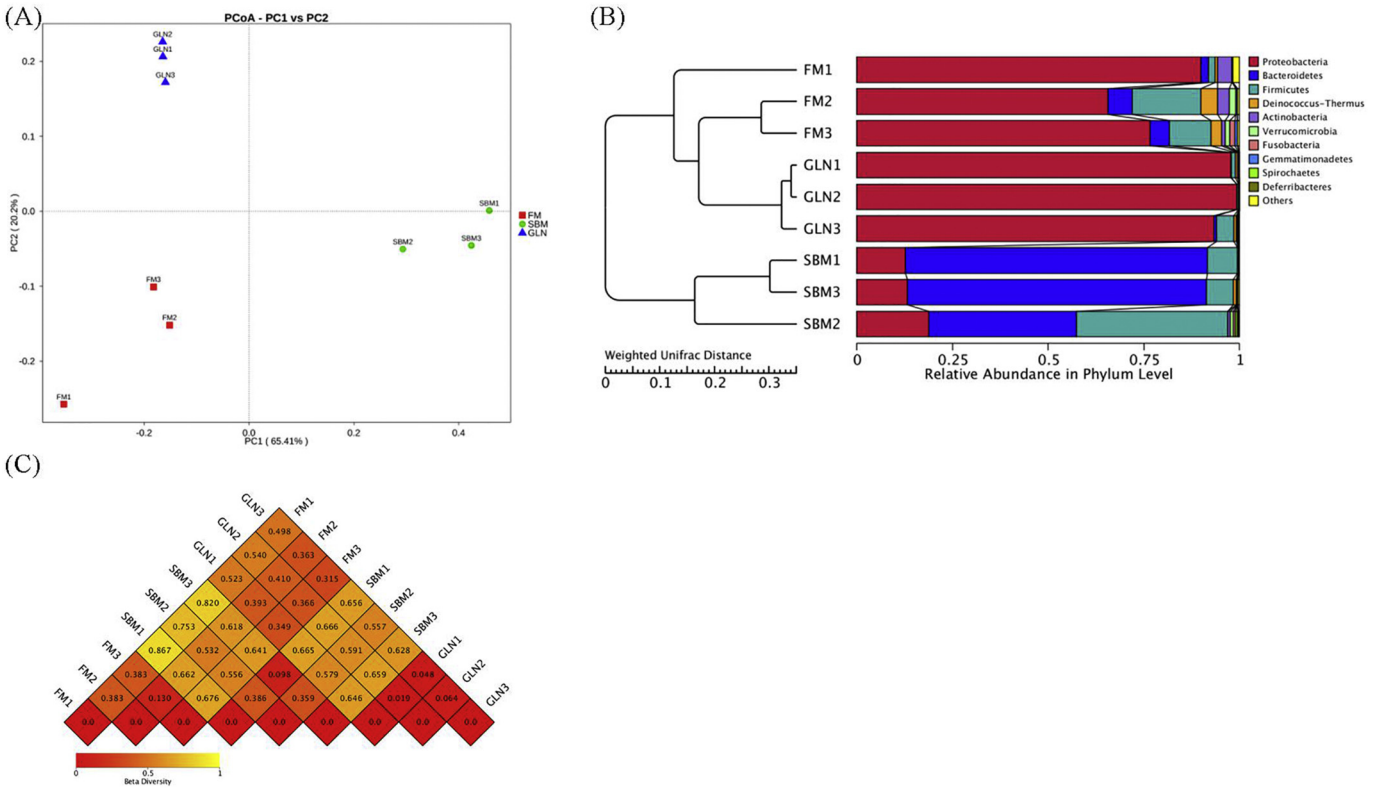

Fig. 8. Beta diversity of intestinal microbiota of juvenile turbot.
Principal Coordinate Analysis (PCoA) against PC1 versus PC2 axes (A), UPGMA-clustering trees (B) and Heatmap (C) of samples were all based on weighted unifrac distance.

Table 6
The MetaStat analysis of intestinal microbiota of fish among groups.

	FM		SBM		GLN	
Vibrio spp.	1057.70[a]	477.81	862.97[a]	148.07	9223.89[b]	390.62
Bacteroides spp.	1.33[a]	1.14	689.65[b]	203.11	3.06[a]	2.56

Values are means and S. E. values within the same row with different letters are significantly different ($P < .05$).
Only the relative abundances of genera with significant difference were shown.

commensal microbiota (Romero et al., 2014; Navarrete et al., 2008). And a manifest reduction of microbial diversity in 2% Ala-Gln group compared to FM and SBM groups was observed (shown in Fig. S1), making opportunists to occupy unavailable ecological niches. Thus, that might be inclined to a dangerous status for fish whose rearing situation spreads bacterial pathogens (Romero et al., 2014). Hence, whether the effect of Gln supplementation on the intestinal microbiota in fish has a certain limitation need further exploration.

In conclusion, our present study revealed that supplementing 2% dietary Ala-Gln could alleviate soybean-induced enteropathy of turbot throughout the whole feeding trial. Consequently, these changes enhanced intestinal morphology, digestive and absorptive ability and increased gene expression of tight junction proteins (claudin-4, occludin and ZO-1), as well as decreased pro-inflammatory cytokine (TNF-α) gene expression. This might be due to that Ala-Gln can improve MUC-2 and PPAR-γ expression and inhibit NF-κB-MLCK signaling pathway to regulate the secretions of tight junction proteins and inflammatory cytokines, as well as alter the intestinal microbiota.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.08.021.

Declaration of interest

None of the authors has any conflicts of interest to declare.

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