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Chitosan and chitooligosaccharides attenuate soyabean meal-induced intestinal inflammation of turbot (*Scophthalmus maximus*): possible involvement of NF-κB, activator protein-1 and mitogen-activated protein kinases pathways

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Abstract

An 8-week feeding experiment was conducted to investigate and confront the putative functions of chitosan (CTS) and chitooligosaccharide (COS) in the growth and homoeostasis of distal intestine in juvenile turbots fed diets containing soyabean meal (SBM). Three isolipidic and isonitrogenous diets were formulated by supplemented basal diet (based on a 400 g/kg SBM) with 7·5 g/kg CTS or with 2·0 g/kg COS. Our results indicated that both CTS and COS supplementation could significantly improve (i) the growth performance and feed efficiency ratio; (ii) antioxidant activity driven by metabolic enzymes (i.e. catalase, glutathione reductase, glutathione peroxidase and superoxide dismutase); (iii) glutathione levels; (iv) acid phosphatase and lysozyme activity and (v) IgM content. As a result, these two particular prebiotics were able to significantly attenuate the histological alterations due to local inflammation as well as to decrease the transcriptional levels of proinflammatory cytokines (i.e. IL-1β, IL-8 and TNF-α) and major pathway effectors (i.e. activator protein-1 (AP-1), NF-κB, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase and extracellular regulated kinase). High-throughput sequencing data indicated that dietary CTS and COS could significantly decrease the diversity of intestinal bacteria but elevate the relative abundances of *Bacillus, Lactobacillus* and *Pseudomonas* genera. Altogether, these findings suggest that CTS and COS can improve growth of turbot, enhance intestinal immune and anti-oxidant systems and promote the balance of intestinal microbiota. The protective effects, elicited by these two prebiotics, against SBM-induced inflammation could be attributed to their roles in alleviating the overexpression of inflammatory cytokines by possibly down-regulating NF-κB, AP-1 and/or mitogen-activated protein kinases pathways.

Key words: ***Scophthalmus maximus*: Chitosan: Chitosan oligosaccharide: Enteropathy: Enteritis**

Besides being responsible for the digestion and absorption of nutrients, the fish gut is also an important barrier of defence against a number of aquatic pathogens and adverse environmental stimuli. It has been reported that diet-induced enteritis can dramatically affect fish production and, ultimately, threaten the sustainable development of fish cultures[1]. Soyabean meal-induced enteropathy (SBMIE) has been considered a limiting condition that abrogates fish feeding in several freshwater and marine fish species[2]. The SBMIE in turbot is characterised by a series of histopathological changes, including (i) reduction of the mucosal folds, (ii) bulging of the sub-epithelial mucosa and lamina propria, (iii) prominent infiltration of pro-inflammatory cells, (iv) induction of IL and TNF-α, (v) down-regulation of antioxidant enzymes (due to oxidative stress) and (vi) increased apoptosis of intestinal epithelial cells[3,4].

Secreted pro-inflammatory cytokines and reactive oxygen species, produced by recruited immune cells in mammals, can promote apoptosis of intestinal epithelial cells and therefore compromise the integrity of the intestinal epithelial barrier as well as exacerbate the mucosal inflammatory response in inflammatory bowel disease[5]. The process of inflammatory response can activate a subset of transcription factors and related signalling cascades, including activator protein-1 (AP-1), mitogen-activated protein kinases (MAPK) and NF-κB, which together contribute to the expressions of many pro-inflammatory genes[6–8]. Hence, it would be appealing to investigate possible bioactive agents that can attenuate the induction of cytokines, down-regulate pro-inflammatory cascades (i.e. AP-1, MAPK and NF-κB) and promote an oxidative homoeostasis under certain inflammatory conditions[8,9].

Abbreviations: AP-1, activator protein-1; CAT, catalase; COS, chitooligosaccharides; CTS, chitosan; DI, distal intestine; ERK, extracellular regulated kinase; GPX, glutathione peroxidase; GR, glutathione reductase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MDA, malondialdehyde; p38, p38 mitogen-activated protein kinase; SBM, soyabean meal; SBMIE, soyabean meal-induced enteropathy; SOD, superoxide dismutase.

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Chitosan (CTS), the deacetylated derivative of chitin, is a linear polymer of *β*-(1,4)-linked D-glucosamine. Chitooligosaccharides (COS) correspond to a depolymerised form of CTS, generated by either chemical or enzymatic hydrolysis[10]. Over the past decades, both CTS and COS have received considerable attention because of their anti-inflammatory, antioxidant, immunomodulatory and prebiotic properties. These functions have been related to their regulatory impact on several signalling pathways[11,12]. Similarly, CTS and COS have also been utilised as functional additives for aquatic animals[13–15] and proved to improve the morphological structure of intestine of fish and shrimp[16–19]. Specifically, these studies have suggested that CTS and COS may potentially attenuate SBMIE. However, other reports have also shown some differences on the bioactivity of CTS and COS. Hu *et al.*[20] and Seyfarth *et al.*[21] have shown that the antibacterial and antifungal activities of CTS and its derivatives are significantly decreased by a decline on its molecular mass. Chiu *et al.* have also reported that CTS can greatly improve the lipid metabolism of high-fat-diet-fed rats when compared with COS[22]. Upon evaluation of aquatic animals, Niu *et al.* have demonstrated that only CTS (not COS) is capable of enhancing growth and resistance to low oxygen stress in *Penaeus monodon* (tiger shrimp)[23]. Thus, the effects of CTS or COS in fish affected by SBMIE, particularly involving the intestinal homoeostasis, require further investigations.

Due to the high quality of its meat and rapid growth, *Scophthalmus maximus* (turbot) has become the most prominent cultured flatfish in Asia and Europe, reaching a global production of about 57 000 tons in 2017[24]. Hence, turbot could be utilised as a model for causal and mechanistic studies related to SBMIE and for the mitigation of technology development[3]. In this research, we examined and also compared the potential positive effects of CTS and COS on the growth, histological structure, inflammation-related cytokines expression, immune and antioxidative parameters as well as mucosal microbiota, in the distal intestine (DI) of juvenile turbot fed with soyabean meal (SBM) diet. Functionally, we also explored related mechanisms by examining the expression and activity of NF-κB, AP-1 and MAPK-related genes.

Materials and methods

Feed content and formulation

A SBM-based diet was prepared as previously[3]. This diet contained 48 % protein and 12 % lipids, which included soyabean and fishmeal. Wheat flour was used as carbohydrate source, while fish and soyabean oil were added as lipid sources (Table 1). Two isolipidic and isonitrogenous diets were also designed by supplementing the SBM diet (control) with 7·5 g/kg CTS (CTS diet) or 2 g/kg COS (COS diet)[23,25–28]. All formulations were designed to meet the essential amino acid requirements for juvenile turbot, according to the whole-body amino acid profiling[29,30]. Standard methods were used to evaluate the nutritional values of these diets[31]. Ash and moisture contents were gravimetrically determined after heating procedures (550 °C and 105 °C, respectively). Crude lipid content was also gravimetrically analysed following ethyl ether extraction (Extraction System B-811, BUCHI). According to the Kjeldahl method, the content of crude protein was evaluated by a Kjeltec 2300 Autoanalyzer (FOSS), with the use of boric acid to trap the released ammonia. Gross energy was examined using a calorimetric pump (Parr). Amino acid profiles related to both ingredients and whole diet were determined accordingly (S-433D, Sykam). The preparation and storage of respective diet formulations were conducted as previously[32]. All ingredients except fish oil, soyabean oil and soyabean lecithin were first ground into fine powder through 180-μm mesh and were mixed thoroughly. Fish oil, soyabean oil and soyabean lecithin were also mixed. The oil and water were mixed with other ingredients thoroughly to produce stiff dough. Finally, the dough was pelleted by experimental feed mill (SKJ120, Shandong Minglun machinery factory) at the length and diameter of 2 mm and dried for about 12 h at 45 °C. The dried feed was stored in a freezer at −20 °C until used.

Table 1. Ingredients and composition of the experimental diets (DM basis)

	Experimental diet*		
	SBM	CTS	COS
Ingredients (g/kg)			
Fishmeal†	360	360	360
Soyabean meal	400	400	400
Wheat meal	118	118	118
Fish oil	48	48	48
Soyabean oil	9	9	9
Soyabean lecithin	20	20	20
Vitamin and mineral premix‡	25	25	25
Monocalcium phosphate	5	5	5
Choline chloride	5	5	5
Yttrium premix	1	1	1
Calcium propionic acid	1	1	1
Ethoxyquin	0·5	0·5	0·5
Cellulose	7·5	0	5·5
Chitosan§	0	7·5	0
Chitooligosaccharide‖	0	0	2
Proximate composition (%)			
DM	93·6	93·7	93·5
Crude protein	48·6	48·7	48·5
Crude lipid	12·3	12·5	12·6
Ash	11·6	11·2	11·4
Gross energy (kJ/g)	20·6	20·7	20·5

* SBM, a basal diet containing 400 g/kg of soyabean meal; CTS, inclusion of 7·5 g/kg of chitosan in SBM diet; COS, inclusion of 2 g/kg of chitooligosaccharide in SBM diet.
† Steam-dried fish-meal (COPENCA Group).
‡ Vitamin premix consisted of the following compounds (mg kg^{-1} diet): retinyl acetate, 32; vitamin D_3, 5; DL-α-tocopherol acetate, 240; vitamin K_3, 10; thiamin, 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; and cellulose, 11 473. Mineral premix composed of the following ingredients (mg/kg diet): $FeSO_4·H_2O$, 80; $ZnSO_4·H_2O$, 50; $CuSO_4·5H_2O$, 10; $MnSO_4·H_2O$, 45; KI, 60; $CoCl_2·6H_2O$ (1 %), 50; Na_2SeO_3 (1 %), 20; $MgSO_4·7H_2O$, 1200; and zeolite, 8485.
§ Procured from Sigma-Aldrich; No. 417963.
‖ Procured from Sigma-Aldrich; No. 523682.

Fish storage and culture

Procedures of fish manipulation were initially approved by the Ethical Scientific Committee for Animal Experimentation of the Shandong University and performed in compliance with the European directive 2010/63/UE.

Healthy juvenile turbots were supplied by a commercial farm in Haiyang (China). The fish were allocated into an indoor

flow-through water system (Haiyang Yellow Sea Aquatic Product Co. Ltd), acclimated accordingly and fed with a commercial diet for 2 weeks. Subsequently, turbots with an initial body weight of about 11·7 g were randomly placed into nine tanks (thirty-five fish in each tank; 300 litres of seawater per tank). For seawater acquisition, adjacent coastal water was filtered by a sand filter and then transferred to each fish tank at the flow rate of about 2·0 litres/min. Respective fish diet (total of 3) was randomly administered to each three tanks. The fish were fed twice a day, at 07.00 and 06.00 hours. Feed consumption and feed intake were recorded accordingly. Water temperature was adjusted between 12 and 16 °C, pH was in the range of 7·8–8·2 and the salinity was at 28–30 g/l.

Fish sampling

After 56 d of feeding, all fish were anaesthetised with eugenol (1:10 000 dilution; Shanghai Reagent Co.) and then weighted. Afterwards, six fish were selected from each tank and further dissected. For this, both liver and intestine were removed (cleared of any mesenteric and adipose tissues) and then washed with cold PBS to eliminate any remaining gut content. Body length and weight, as well as intestine and liver weight, were measured and recorded for further calculation of condition factor, hepatosomatic index and intestosomatic index. To ensure dietary exposure, only the fish with digested food along the intestinal tract were sampled. Four fish were randomly selected from the six selected fish, followed by the removal of their respective DI. Tissues were then divided into two sections to allow both histological and gene expression analyses. A section of DI was added into 4 % formaldehyde in PBS for 24 hours and, thereafter, kept in 70 % ethanol until microscopy analyses. The other tissue section was placed in RNAlater (Ambion) and stored at −80 °C before expression analysis. For both analyses, tissues were treated, stored and coded individually. To assess their enzymatic activity, sections of dissected DI from another fish (four per tank) were frozen in liquid N_2 and stored at −80 °C until further use.

For autochthonous microbiota analysis, the remaining fish were starved for 24 h and then sampled as previously reported[33]. For this, fish were anaesthetised and then cleaned with 70 % ethanol before assessing their abdomen at the ventral midline. Whole intestines were then removed, under aseptic conditions, from the abdominal cavity. Fish intestine was opened longitudinally, and the mucosa-associated microbiota was further extracted by scraping the mucosal layer using a sterile scalpel. Mucosal layers (from at least twenty fish per tank) were pooled as single samples, frozen in liquid N_2 and stored at −80 °C until further analysis. During the feeding, sampling and the following analysis and data statistics, the researchers excluding corresponding author were blinded for the sampling sources.

Histological analysis

Fixed tissue samples were prepared accordingly before staining with haematoxylin–eosin. Tissue examination was performed blindly using a light microscope. According to Penn *et al.*[34], a progressing scoring scale (0–10) was defined. For this, the following histological characteristics were considered: (i) fusion and extension of the mucosal folds, (ii) cell infiltration and width along the submucosa and lamina propria (iii), enterocyte vacuolisation and (iv) position of the nucleus within enterocytes.

Quantitative real-time PCR

Total RNA was isolated from DI tissues (about 50 mg per sample) by following the manufacturer's instructions (RNeasy Protect Mini Kit, catalogue no. 74126, Qiagen, GmbH, Hilden). Purified RNA was further quantified using a NanoDrop® ND-1000 spectrophotometer (Nano-Drop Technologies). RNA quality was examined using an Agilent Bio-Analyzer (Agilent Technologies). First-strand cDNA synthesis was conducted using one microgram of RNA/sample, as indicated by the manufacturer's protocol (QuantiTect Reverse Transcription Kit, catalogue no. 205311, Qiagen, GmbH).

The expression profiles of selected genes, including AP-1, IL-1β, IL-8, NF-κB, TNF-α, p38 mitogen-activated protein kinase (p38), c-Jun N-terminal kinase (JNK) and extracellular regulated kinase (ERK) were determined by quantitative PCR. The expression ribosomal protein S4 was utilised for data normalisation. Validated gene-specific primers were provided by Gu *et al.*[3] and Zhao *et al.*[35]. QPCR reactions were conducted as described previously[32] in 25 μl reaction volume including 12·5 μl of SYBR Green PCR Master Mix (QuantiTect SYBR Green RT-PCR Kit, Qiagen, 204243), 10·5 μl of ultrapure water (Sigma-Aldrich), 1·0 μl of each specific primer (10 μM) and 1·0 μl of cDNA template. The thermal profile was 95 °C for 20 s, followed by 40 cycles of 95 °C for 5 s, 56 °C for 30 s and 72 °C for 30 s. At the end of each cycle, fluorescence readings were recorded to estimate quantification cycle values (Cq). Melting curve analysis was performed to verify that only one PCR product was present in each reaction. Raw Cq values were normalised to ribosomal protein S4 using a relative quantitative method ($2^{-\Delta\Delta CT}$) and expressed as fold change.

Intestinal biochemical analysis

Gut samples were homogenised in cold saline solution (10 volumes, w/v) and then centrifuged at 5000 × ***g*** for 20 mins at 4 °C. Thereafter, supernatants were separated and split into twenty pieces. Respective solutions were kept at –80 °C until further analysis.

The concentrations of malondialdehyde (MDA) in the gut were assessed as an indicator of lipid peroxidation. The activities of other enzymes, such as acid phosphatase, catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX), lysozyme and superoxide dismutase (SOD), were also assayed. Additionally, the levels of intestinal glutathione (GSH), complement 3, complement 4 and IgM were also measured accordingly[36]. All the analysis was conducted by the kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China; Product code: MDA, A003-1-1; acid phosphatase, A060-1-1; CAT, A007-1-1; GR, A062-1-1; GPX, A005-1-1; lysozyme, A050-1-1; SOD, A001–1-1; GSH, A006-1-1; complement 3, H186-1; complement 4, H186-2; IgM, H109).

Analysis of autochthonous microbial community in the fish gut

DNA sequencing. Based on our previous work[37], bacterial DNA was extracted using the CTAB/SDS method. The DNA

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quality was monitored on a 1 % agarose gel electrophoresis, and the concentration was determined using a NanoDrop® ND-1000 spectrophotometer (Nano-Drop Technologies). After diluting to 1 ng/μl with sterile water, DNA samples were subjected to amplification using 341F and 806R primers with barcode specific for 16SV3-V4 regions. All PCR were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Thermal cycling consisted of initial denaturation 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 elongation at 72 °C for 30 s. The PCR products were mixed with same volume of 1 × loading buffer (contained SYBR Green), and the quantification and qualification were assayed in electrophoresis on 2 % agarose gel for detection of bands between 400 and 450 bp. After mixing in equidensity ratios, PCR products from all samples were purified with a Qiagen Gel Extraction Kit (Qiagen). Sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina), and index codes were added. The library quality was assessed on the Qubit@ 2·0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. DNA sequencing library was constructed using an Illumina HiSeq 2500 platform, upon generation of 250-bp paired-end reads.

Data analysis. The assembly and quality control of respective paired-end reads as well as species annotation and OTU-based clustering were carried out as previously[37]. Paired-end reads were merged using FLASH (http://ccb.jhu.edu/software/FLASH/; version 1.2.7). High-quality clean tags were obtained by QIIME (http://qiime.org/index.html; version 1.7.0). Effective tags were obtained by removing chimera sequences detected UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html). Sequences were analysed by Uparse (http://drive5.com/uparse/; version 7.0.1001), and sequences with ≥97 % similarity were assigned to the same OTU. Representative sequence for each OUT was annotation using the Green Gene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) based on RDP 3 classifier (http://sourceforge.net/projects/rdpclassifier/; version 2.2). Multiple sequence alignment was conducted using the MUSCLE software (http://www.drive5.com/muscle/; version 3.8.31). OTU abundance information was normalised using a standard of sequence number corresponding to the sample with the least sequences. Alpha- and beta-diversities were further analysed according to the normalised output data. Alpha diversity metrics, such as Chao 1 richness and Shannon diversity index, were analysed by QIIME (version 1.7.0) and then interpreted using R software (version 2.15.3). In the context of the beta-diversity, a cluster analysis was performed by nonmetric multidimensional scaling using the vegan package in R language (https://www.r-project.org/; version R-2.15.3).

Calculation and statistics

Growth performance and food acquisition were quantified by calculating the respective feed efficiency ratio and specific growth rate as follows:

$$\text{Feed efficiency ratio} = (\text{final body weight} - \text{initial body weight})/\text{total amount of the food consumed.}$$

Table 2. Growth performance and biological parameters of the turbots fed the experimental diets for 8 weeks*

	SBM	CTS	COS	Pooled SE	*P*
IBW (g)	11·6	11·7	11·7	0·11	0·806
FBW (g)	40·1a	43·0^{b}	43·6^{b}	0·31	<0·001
SGR	2·21^{a}	2·33^{b}	2·35^{b}	0·02	<0·001
FI (%/d)	1·42	1·41	1·40	0·02	0·752
FER	1·39^{a}	1·43^{b}	1·46^{b}	0·02	0·003
CF	2·77^{a}	3·08^{b}	3·13^{b}	0·09	0·012
ISI (%)	4·72	4·74	4·80	0·18	0·889
HSI (%)	1·41	1·36	1·37	0·05	0·644

CF, condition factor; FBW, final body weight; FER, feed efficiency ratio; FI, feed intake; HSI, hepatosomatic index; IBW, initial body weight; ISI, intestosomatic index; SGR, specific growth rate; SBM, a basal diet containing 400 g/kg of soyabean meal; CTS, inclusion of 7·5 g/kg of chitosan in SBM diet; and COS, inclusion of 2 g/kg of chitooligosaccharide in SBM diet.

* All values are expressed as means of three replicate measurements. For IBW, FMW, SGR, FI and FER, the result of a single replicate measurement was calculated by the body weight data and feed consumption data of all thirty-five fish in one tank. For CF, ISI and HIS, the result of a single replicate measurement was calculated by the average of six randomly selected fish in one tank.

[a,b]Mean values in the same row with different superscript letters denote a statistically significant difference ($P < 0·05$).

$$\text{Specific growth rate} = ((\ln \text{mean final body weight} - \ln \text{mean initial body weight})/\text{number of days}) \times 100$$

$$\text{Condition factor} = \text{fish weight}/(\text{body length})^3 \times 100$$

$$\text{Hepatosomatic index (\%)} = (\text{hepatopancreas weight}/\text{body weight}) \times 100$$

$$\text{Intestosomatic index (\%)} = (\text{intestine weight}/\text{body weight}) \times 100$$

Statistical analysis was performed using SPSS 11.0. All data (except the light microscopy and quantitative PCR assay) were analysed by the one-way ANOVA test. The difference among the means was examined using Duncan's multiple range test. *P*-value of <0·05 was deemed statistically significant. Light microscopy analysis and quantitative PCR data were analysed by Kruskal–Wallis/Wilcoxon test, followed by the tech *post hoc* Wilcoxon method for comparison of the means.

Results

Dietary impact on turbot growth and biometric parameters

As indicated in Table 2, the final body weight, specific growth rate, feed efficiency ratio and condition factor of fish fed with CTS or COS diets were remarkably increased compared with those of fish fed with SBM diet only ($P < 0·05$), but no obvious difference was found between these two groups ($P > 0·05$). In addition, no significant differences were found among the three groups in regard to FI, intestosomatic index and hepatosomatic index ($P > 0·05$).

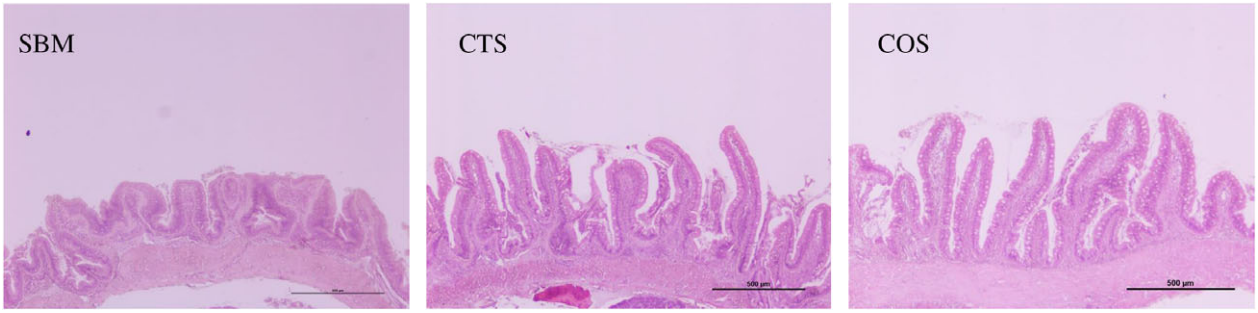

Fig. 1. Representative images of H&E-stained distal intestinal sections from turbots fed with the three diets. Abbreviations: SBM, a basal diet containing 400 g kg^{-1} of soybean meal; CTS, SBM diet containing 7.5 g kg^{-1} chitosan inclusion; COS, SBM diet containing 2 g kg^{-1} of chitooligosaccharides. Bar represents 500μm.

Table 3. Distal intestine tissue variable scores of the turbots fed the experimental diets for 8 weeks

	SBM	CTS	COS	Pooled SE	*P*
MF height	4·7[a]	5·9[b]	6·0[b]	0·19	<0·001
MF fusion	4·6[b]	3·6[a]	3·4[a]	0·21	<0·001
LP width	4·4[b]	3·8[a]	3·5[a]	0·23	0·003
LP cellular infiltration	4·6[b]	3·5[a]	3·3[a]	0·24	<0·001
SM width	3·9[b]	3·2[a]	3·3[a]	0·19	0·008
SM cellular infiltration	3·8[b]	3·3[a]	3·2[a]	0·22	0·031
ent vacuolisation	4·7[a]	5·8[b]	6·0[b]	0·21	<0·001
ent nucleus position	3·5[b]	3·2[a]	2·8[a]	0·17	<0·001

LP, lamina propria; MF, mucosal fold; SM, submucosa; ent, enterocyte; SBM, a basal diet containing 400 g/kg of soyabean meal; CTS, inclusion of 7·5 g/kg of chitosan in SBM diet; and COS, inclusion of 2 g/kg of chitooligosaccharide in SBM diet.
* All values are expressed as means of twelve fish randomly selected from three replicate groups.
[a,b]Mean values in the same row with different superscript letters indicate a statistically significant difference ($P < 0·05$).

Distal intestinal inflammation

Morphological characteristics of the distal intestine. According to our previous studies, here we confirmed that dietary SBM was capable of inducing SBMIE[3]. As demonstrated in Fig. 1 and Table 3, the fish fed with CTS or COS diets presented a significant increase on the height of mucosal folds and enterocyte nucleus, as well as a decrease on the width and cellular (leucocyte-based) infiltration in both submucosa and lamina propria when compared with those fed with SBM diet only ($P < 0·05$). The fusion of the mucosal folds was also markedly reduced in CTS or COS diet groups *v.* SBM diet only group ($P < 0·05$). In regard to all evaluated parameters, no noticeable differences were observed when comparing both CTS and COS groups ($P > 0·05$).

Gene expression analysis. Compared with SBM-fed fish (control), fish receiving either CTS or COS diets showed significantly lower expression levels of IL-1β, IL-8, AP-1, NF-κB, TNF-α, p38, JNK and ERK ($P < 0·05$; Fig. 2). Although the expression levels of all genes presently tested in COS group were slightly up-regulated compared with CTS group, such difference was not statistically significant ($P > 0·05$).

Intestinal oxidant and antioxidant parameters

As presented in Table 4, MDA concentrations in the fish fed with CTS and COS diets were remarkably lower than those fed with SBM diet only ($P < 0·05$). In contrast, the levels of CAT, GPX, GR, GSH and SOD in fish fed with CTS and COS diets were markedly higher than those receiving SBM diet only ($P < 0·05$). However, no remarkable differences in the levels of MDA, SOD, CAT, GSH and GR was detected upon comparing the CTS and COS groups ($P > 0·05$). Nevertheless, the activity of GPX in COS group was obviously increased when compared with CTS group ($P < 0·05$).

Immune-related parameters

Fish fed with either CTS or COS diets presented a significant increase on lysozyme and acid phosphatase activities as well as IgM concentration when compared with those fed with SBM diet only ($P < 0·05$; Table 5). In particular, the acid phosphatase activity of fish was markedly higher in COS diet group than in CTS diet group ($P < 0·05$). There were no obvious differences noted among the three groups at complement 3 and complement 4 concentrations ($P > 0·05$).

Mucosal microbiota in the intestine

Phylotype coverage. After quality control analysis, 467 436 reads were obtained, covering 2034 OTU (97 % of similarity) in a total of nine samples. The average number of reads per treatment was 52 011, 51 435 and 52 366 reads. The Good's coverage of all samples was 0·999, implying that the sequence depth was sufficient.

Bacterial diversity in the intestine mucosa. Changes in the bacterial richness (estimated with the Chao1 index) and diversity (estimated with the Shannon index) of fish fed with CTS or COS diet are presented in Fig. 3(a) and (b). Fish fed with either CTS or COS diets had a significantly lower Chao1 and Shannon indices than those receiving SBM diet only ($P < 0·05$). The beta diversity among fish groups was evaluated by nonmetric multidimensional scaling. Three diet-related nonmetric multidimensional scaling clusters were distinctively separated, suggesting a differential response model of the intestinal microbiota towards each respective diet (Fig. 3(c)). In addition, the relative abundances of top fifty most abundant bacterial genera were analysed (online Supplementary Table S1) and the relative abundance of *Bacillus*, *Lactobacillus* and *Pseudomonas* was compared among groups. Consistently, CTS or COS treatment markedly elevated the relative abundances of all three bacteria genera ($P < 0·05$; Fig. 3(d), (e) and (f)). However, no noticeable differences in Chao1 and

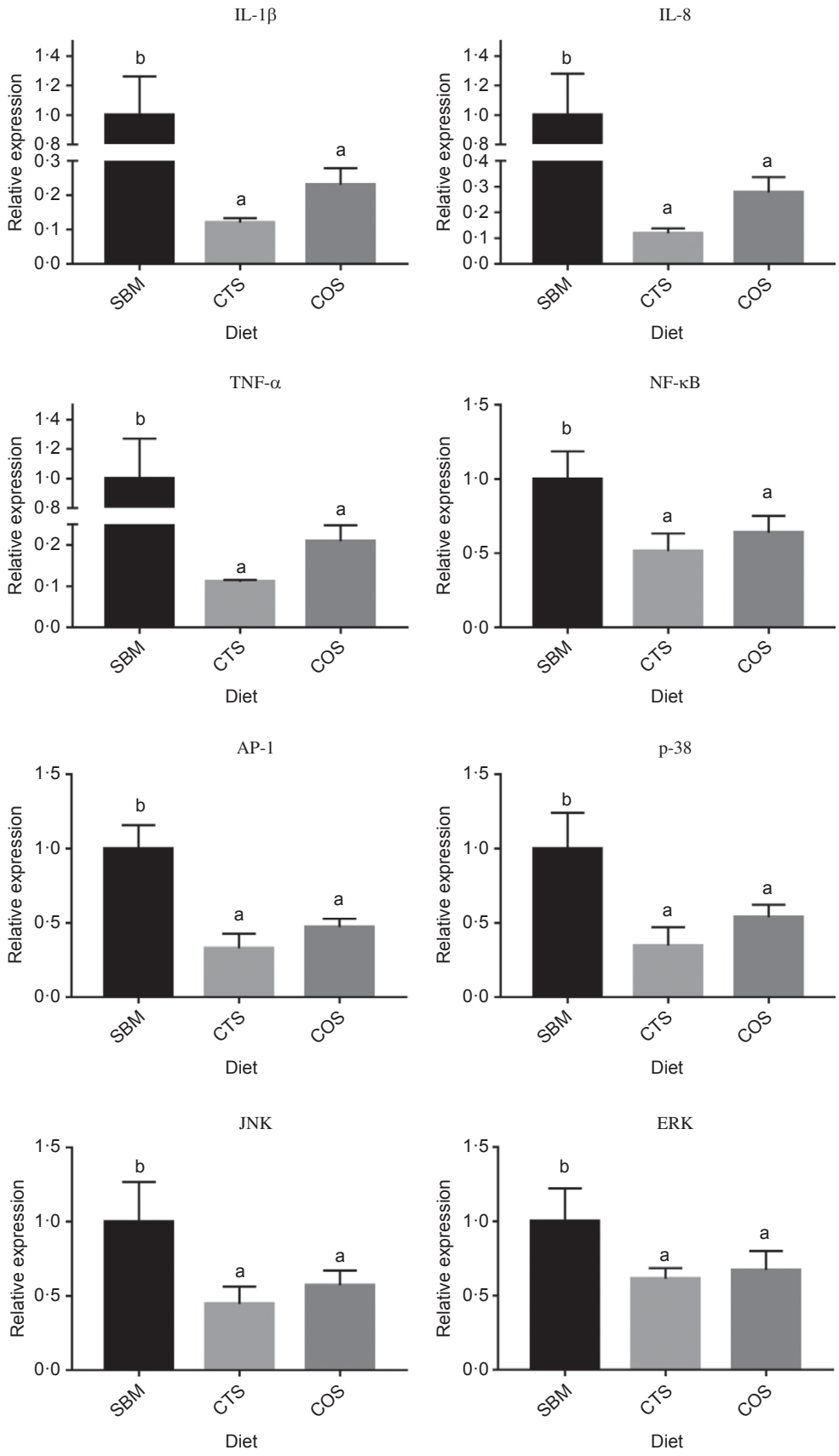

Fig. 2. Relative expression of three inflammation-related genes and five pathway regulatory molecule genes in the intestine of turbots fed with the three diets. Data are presented as means and standard error from three replicates. Data of one replicate was measured from the intestines of four random selected fish in one tank. Mean values for the same gene (with different letters) were significantly different ($P<0.05$). Abbreviations: SBM, a basal diet containing 400 g kg^{-1} of soybean meal; CTS, SBM diet containing 7.5 g kg^{-1} chitosan inclusion; COS, SBM diet containing 2 g kg^{-1} of chitooligosaccharides; AP-1, activator protein^{-1}; IL-1β, interleukin-1 beta; IL-8, interleukin 8; NF-κB, nuclear transcription factor-kappa B; TNF-α, tumor necrosis factor alpha; p38, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular regulated kinase.

Table 4. Intestinal oxidant and antioxidant parameters of the turbots fed the experimental diets for 8 weeks

	SBM	CTS	COS	Pooled SE	*P*
MDA level (nmol/mgpro)	3·9[b]	2·6[a]	2·5[a]	0·20	0·001
SOD activity (U/mg protein)	22·6[a]	28·3[b]	29·5[b]	1·50	0·006
CAT activity (U/mg protein)	2·6[a]	3·3[b]	3·7[b]	0·20	0·009
GSH level (mg/g protein)	7·0[a]	8·2[b]	8·1[b]	0·40	0·048
GPX activity (U/mg protein)	11·1[a]	13·4[b]	18·5[c]	0·70	<0·001
GR activity (U/g protein)	2·8[a]	3·3[b]	3·4[b]	0·11	0·004

CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; SBM, a basal diet containing 400 g/kg of soyabean meal; CTS, inclusion of 7·5 g/kg of chitosan in SBM diet; COS, inclusion of 2 g/kg of chitooligosaccharide in SBM diet.
* All values are expressed as means of three replicates. The data of one replicate were measured from the distal intestines of four randomly selected fish in one tank.
[a,b]Mean values in the same row with different superscript letters denote a statistically significant difference ($P < 0·05$).

Table 5. Intestinal immune parameters of the turbots fed the experimental diets for 8 weeks*

	SBM	CTS	COS	Pooled SE	*P*
LZM activity (U/mg protein)	1·42 [a]	3·68[b]	3·47[b]	0·34	0·001
ACP activity (U/g protein)	81[a]	96[b]	106[c]	4	0·002
C3 level (μg/mg protein)	19·7	27·6	27·9	4·0	0·149
C4 level (μg/mg protein)	11·3	14·3	14·9	2·4	0·334
IgM content (μg/mg protein)	37·4[a]	47·3[b]	50·2[b]	3·5	0·024

ACP, acid phosphatase; C3, complement 3; C4, complement 4; LZM, lysozyme; SBM, a basal diet containing 400 g/kg of soyabean meal; CTS, inclusion of 7·5 g/kg of chitosan in SBM diet; COS, inclusion of 2 g/kg of chitooligosaccharide in SBM diet.
* All values are expressed as means of three replicates. The data of one replicate were measured from the distal intestines of four randomly selected fish in one tank.
[a,b]Mean values in the same row with different superscript letters indicate a statistically significant difference ($P < 0·05$).

Shannon indices as well as *Lactobacillus*, *Bacillus* and *Pseudomonas* abundances were observed between CTS and COS groups ($P > 0·05$).

Discussion

Although some reports have shown differences on the bioactivity of CTS and COS, our present work did not identify any difference between these additives in regard to growth promotion or anti-inflammatory effects in turbots affected by SBMIE. CTS-mediated growth has been demonstrated in other fish models, like *Carassius auratus gibelio* (gibel carp)[25], *Cyprinus carpio* (common carp)[38], *Dicentrarchus labrax* (sea bass)[17], *Misgurnus anguillicaudatus* (loach)[39], *Mugil cephalus* (grey mullet)[40], *Oreochromis niloticus* (Nile tilapia)[41,42], *Rachycentron canadum* (cobia)[43] and shrimps including *Litopenaeus vannamei* (white shrimp)[44] and *P. monodon* (tiger shrimp)[23,45]. Similarly, COS can also enhance the growth performance of numerous fish species, such as *Cyprinus carpio koi* (koi)[26], *Micropterus salmoides* (largemouth bass)[27], *O. niloticus* (Nile tilapia)[46,47], *Pangasianodon hypophthalmus* (striped catfish)[48], *Paramis gurnus dabryanus* (loach)[49], *Takifugu rubripes* (tiger puffer)[28] and *Trachinotus ovatus* (pompano)[50]. Here, we were able to confirm that both CTS and COS can actively promote fish growth. This enhanced growth performance appears to translate into better intestinal health status, in response to CTS and COS supplementation.

SBM-mediated inflammation of fish DI is characterised by swelling of the subepithelial mucosa and lamina propria, as well as by strong infiltration of various pro-inflammatory cells and overexpression of inflammation-related cytokines[51,52]. In turbot, the infiltration of inflammatory cells and expression of a subset of inflammation-related cytokines were promoted by SBM in a dose-dependent fashion, as indicated previously[3]. In this study, fish fed with CTS- or COS-supplemented diet showed a lower content of pro-inflammatory cell infiltration as well as down-regulated gene expression of prototypical cytokines, such as IL-1β, IL-8 and TNF-α, which clearly indicate the protective effects of these additives against SBM-induced inflammation in turbot DI. To our knowledge, the present study inaugurally demonstrates the protective role of CTS and COS against intestinal inflammatory in fish. Based on the present work, other fish species suffering from SBMIE or other kind of inflammation could utilise CTS and COS as treatment strategy.

Compelling investigations in humans and other higher organisms have shown that the secretion of pro-inflammatory cytokines can play an essential role in the progression of inflammatory bowel disease[53]. It has been indicated that both CTS and COS supplementation can attenuate intestinal inflammation by suppressing the expression of certain cytokines as well as the production of inflammatory regulators both *in vivo*[54–56] and *in vitro*[57,58]. Hence, according to our present study, it is reasonable that CTS or COS supplementation in fish diet can attenuate SBM-induced inflammatory response in turbot, partially by suppressing the production of pro-inflammatory cytokines.

AP-1 and NF-κB are two well-studied transcription factors which are capable of inducing the production of inflammatory cytokines such as IL-1β, IL-8 and TNF-α[59,60]. It has been shown that both CTS and COS may reduce an inflammatory response by abrogating AP-1 and NF-κB activation both *in vivo* and *in vitro*[55–58,61–66]. In the present study, dietary CTS or COS could down-regulate the mRNA expression of inflammation-related genes, from which we speculate that the inhibitory effect of these additives towards cytokine production in SBM-fed fish may be due to the suppression of NF-κB and AP-1 activation. The activation of related pathways is modulated by MAPK[67]. LPS-mediated activation of three MAPK, such as p38 MAPK, extracellular (ERK) and c-Jun N-terminal kinase/stress-activated protein (JNK), has been shown to promote gene expression related to an inflammatory response[68]. Activated p38 MAPK can affect cytokine levels by regulating NF-κB-dependent gene expression[68]. Activated JNK initiates the activation of c-Jun that constitutes AP-1, while ERK can act by inducing AP-1 expression[69,70]. In this context, it has been reported that CTS and COS can suppress LPS-induced phosphorylation of p38 MAPK, ERK and JNK[61,66,71]. Taken altogether, our findings suggest that CTS and COS may block SBM-induced signal transduction of inflammatory cytokines by suppressing the mRNA levels of genes involved in NF-κB, AP-1 and MAPK pathways. Nevertheless, further investigations are still necessary to clarify the precise mechanism involved in these events.

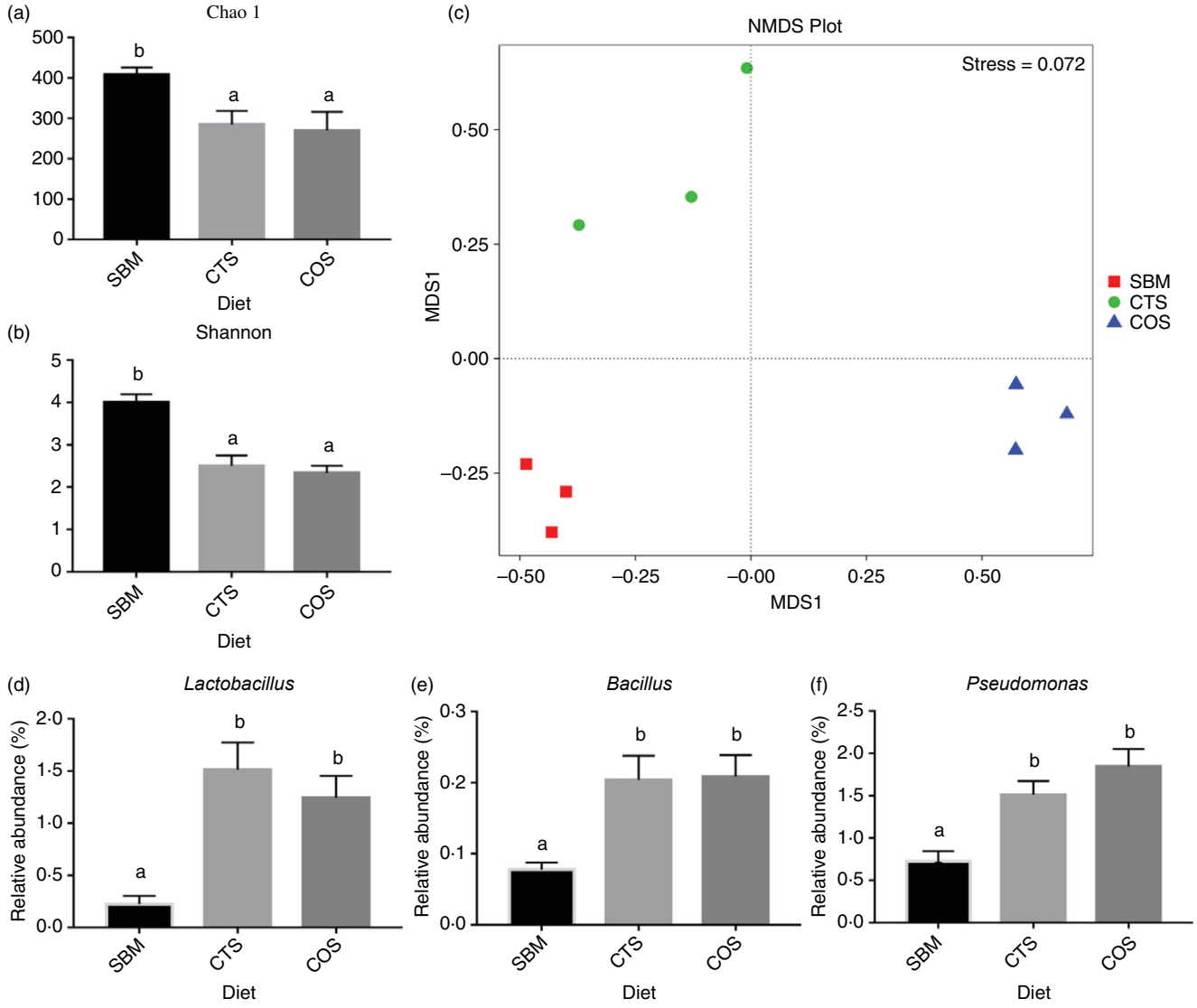

Fig. 3. General structural changes of gut microbiota and relative abundance of selected bacterial genus in turbots fed with the three diets. Chao1 (A) and Shannon (B) indices were used to estimate the richness and community diversity of the gut microbiota. Non-metric multi-dimensional scaling (NMDS) was calculated according to the relative abundances of OUT of turbots in each diet group. Each point represents the microbiota composition in, at least, 20 fish from a single tank (C). Determination of the relative abundance of genus of Lactobacillus (D), Bacillus (E) and Pseudomonas (F). Mean values for the same genus (with different letters) are significantly different ($P<0.05$). Abbreviations: SBM, a basal diet containing 400 g kg^{-1} of soybean meal; CTS, SBM diet containing 7.5 g kg^{-1} chitosan inclusion; COS, SBM diet containing 2 g kg^{-1} of chitooligosaccharides.

Many studies have shown that oxidative stress is largely accompanied by inflammation, thus leading to tissue damage[72]. In regard to SBMIE-affected turbots, Chen *et al.*[73] and Tan *et al.*[4] have reported that SBM may induce oxidative stress in the turbot intestine by elevating the intestinal MDA content and also decreasing the intestinal total antioxidant capacity and the transcript levels of antioxidant enzymes, including SOD, GPx, heme oxygenase 1 and peroxiredoxin 6. It has been considered that these antioxidant enzymes are consumed upon defence against the oxidation caused by SBM[4]. Due to their free radical scavenging activities and proton donation ability, CTS and COS act as efficient additives capable of maintaining expected antioxidant levels[74]. In the present work, the administration of CTS or COS appears to prevent SBM-induced lipid peroxidation and, at the same time, to preserve anti-oxidant substrates (GSH) and anti-oxidative enzyme activity (SOD, CAT, GPX and GR). Here, we validated that either CTS or COS behaves as efficient antioxidants in turbot, similarly to *M. anguillicaudatus* (dojo loach)[39], *O. niloticus* (Nile tilapia)[75], *P. dabryanus* (loach)[49] and *P. monodon* (tiger shrimp)[23].

Wang and co-workers have demonstrated that a substitution of fishmeal for SBM diminishes the immunity of turbot[76]. This effect might be attributed to nutritional imbalances as well as anti-nutritional substances present in SBM[76–79]. The immunostimulating effects of CTS have been widely studied in several aquatic animals (as reviewed by Abdel-Ghany & Salem[15]). Similarly, COS can also increase the immunity of *C. carpio koi* (koi)[26], *L. vannamei* (white shrimp)[80], *M. salmoides* (largemouth bass)[27], *P. hypophthalmus* (striped catfish)[48] and *T. ovatus* (pompano)[50]. Our work validates the fact that CTS and COS can improve the intestinal immunity of turbot, thus playing pivotal roles in the intestinal homoeostasis. Still,

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contrarily to results obtained in *C. auratus gibelio* (gibel carp)[25] and *C. mrigala* (mrigal carp)[81], no positive effects of CTS towards complement activity were detected in the present study. The exact underlying mechanism(s) on how CTS or COS may impact the immune function in SBM-fed fish remains to be further elucidated.

The intestinal microbiota plays important roles in fish health, affecting the gut morphology, nutritional status, disease resistance and immune response[82]. Previous studies have shown that SBM supplementation increases the bacteria diversity and alters the composition of the autochthonous bacterial communities in turbot intestine[37,83]. Similar results were also obtained in *Salmo salar* (Atlantic salmon)[33,84] and *Oncorhynchus mykiss* (rainbow trout)[85] upon supplementation with plant protein sources. According to our high-throughput sequencing data, CTS and COS appear to decrease the diversity and the composition of the intestinal microbiota in SBM-fed fish. This result parallels the findings in *T. rubripes* (tiger puffer), where the microbial abundance and species diversity in fish fed with COS-supplemented diet were lower than those fed with non-supplemented diet[28]. One possible explanation for this effect is that either CTS or COS can foster an ecological niche through secreted materials, thus leading to modifications in the microbiota composition[86]. However, some direct evidence that may support this hypothesis is still missing. We presently observed that both CTS and COS can significantly increase the relative abundance of these three distinct genera (i.e. *Lactobacillus*, *Bacillus* and *Pseudomonas*) with probiotic roles in fish[82,87,88]. This observation reinforces the beneficial effects of these two additives towards fish intestinal microbiota. Similar findings have also been reported in *C. auratus gibelio* (gibel carp) fed with CTS-based diet[25] and *T. rubripes* (tiger puffer) fed with COS-supplemented sources[28].

In conclusion, this study indicates that dietary CTS and COS can improve the performance of fish growth and intestinal immunity, eliciting anti-oxidant effects and ameliorating intestinal microbiota in SBMIE-affected turbot. Furthermore, these two prebiotics are beneficial against SBM-induced inflammation, since they enable a decrease on the expression of inflammatory cytokines by possibly modulating NF-κB, AP-1 and MAPK pathways.

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Supplementary material

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