Received: 12 November 2018 | Revised: 1 January 2019 | Accepted: 26 January 2019

DOI: 10.1111/are.14016

ORIGINAL ARTICLE

WILEY Aquaculture Research

Effect of dietary enzyme-treated soy protein on the immunity and antioxidant status in the intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian)

Weiwei Xiao[1,2] | **Weidan Jiang**[1,3] | **Lin Feng**[1,3] | **Yang Liu**[1,3] | **Pei Wu**[1,3] | **Jun Jiang**[1,3] | **Yongan Zhang**[4] | **Xiaoqiu Zhou**[1,3]

[1]Animal Nutrition Institute, Sichuan Agricultural University, Sichuan, China

[2]Chengdu Mytech Biotech Co., Ltd, Sichuan, China

[3]Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Sichuan Agricultural University, Sichuan, China

[4]Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China

Correspondence
Xiaoqiu Zhou, Animal Nutrition Institute, Sichuan Agricultural University, Sichuan, China.
Emails: zhouxq@sicau.edu.cn, xqzhouqq@tom.com, fishnutrition@126.com

Funding information
Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China, Grant/Award Number: 2012NC0007 and 2013NC0045; Outstanding Talents and Innovative Team of Agricultural Scientific Research (Ministry of Agriculture); Science and Technology Support Program of Sichuan Province of China, Grant/Award Number: 2014NZ0003; National Natural Science Foundation of China, Grant/Award Number: 31672662; National Department Public Benefit Research Foundation (Agriculture) of China, Grant/Award Number: 201003020; Foundation of Sichuan Youth Science and Technology Innovation Research Team, Grant/Award Number: 2017TD0002; Earmarked Found for China Agriculture Research System, Grant/Award Number: CARS-45; Key Fund Project of Sichuan Provincial Department of Education, Grant/Award Number: 16ZA0022; National Basic Research Program of China (973 Program), Grant/Award Number: 2014CB138600; Demonstration of Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China, Grant/Award Number: 2015CC0011

Abstract

This study was conducted to evaluate the hypothesis that enzyme-treated soy protein (ETSP) can save the fish dietary protein, and further investigated the mechanism for saving effect by studying the effects of ETSP on intestinal immune response and antioxidant status in juvenile Jian carp (*Cyprinus carpio* var. Jian). Compared with the high-protein diet, results showed that decreasing 2% of dietary protein increased the protein carbonyl (PC) content, decreased the anti-hydroxyl radical capacity (AHR) and superoxide dismutase (SOD) activity and increased the relative expressions of pro-inflammatory cytokine ($p < 0.05$). The low-protein diet might impair the intestinal health, so as to reduce fish growth performance. After adding 1.5% or 2% of ETSP to the low-protein diet, results indicated that (1) ETSP decreased the relative expressions of pro-inflammatory cytokine and increased the relative expressions of anti-inflammatory cytokine ($p < 0.05$). (2) ETSP decreased malondialdehyde (MDA) and PC content ($p < 0.05$), improved activities of antioxidant enzymes, glutathione (GSH) content, relative expressions of antioxidant enzymes and Nrf2, which were even much higher than the high-protein diet ($p < 0.05$). All the above data suggested that optimal level of ETSP can save 2% of fish dietary protein which might be partly attributed to improve intestinal health through regulating intestinal immunity response and antioxidant status.

KEYWORDS
antioxidant status, enzyme-treated soy protein, inflammatory cytokine, intestine immunity

1 | INTRODUCTION

Recently, plant proteins have become the main protein sources in aquatic feeds due to the cost and shortage of animal protein resources (Hardy, 2010). However, high amount of plant proteins in aquatic feeds usually reduce the growth performance, the digestion and absorption capacity of various fish (Kokou & Fountoulaki, 2018), and also decrease the intestine immune ability of hybrid grouper

(♀ *Epinephelus fuscoguttatus* × ♂ *Epinephelus lanceolatu*) (Yin et al., 2018). Therefore, it is very important to find a path to decrease the dietary protein level which can guarantee the normal growth performance of aquatic animals and reduce the usage of protein raw materials. Enzyme-treated soy protein (ETSP) was produced from dehulled solvent extracted soybean meal (SBM) and soy protein concentrate (SPC) by enzymolysis (Xiao et al., 2017). Our previous study has indicated that adding ETSP to the low-protein diet improved the growth performance and promoted the digestive and absorptive ability referring to TOR signalling in juvenile Jian carp (*Cyprinus carpio* var. Jian), showing the effect of saving dietary protein (Xiao et al., 2017). Study found that the growth performance and the digestion and absorption ability mainly rely on the intestinal health in aquatic animals (Zhao et al., 2014). The fish intestinal health is correlated with its immunological and physical barrier (Niklasson, Sundh, Fridell, Taranger, & Sundella, 2011). However, no report has investigated the effect of ETSP on intestinal immunological and physical barrier of terrestrial and aquatic animals. Research indicated that using enzymes to treat soy protein can release many different kinds of biologically active peptides which have antioxidant and immunomodulatory functions (Singh, Vij, & Hati, 2014). In mice, antioxidant peptides can protect intestinal physical barrier (He et al., 2017), while immunomodulatory peptides can regulate immune response in macrophages (Tuckova et al., 2002). These data suggest that ETSP may affect intestinal immunity and physical barrier to regulate the intestinal health of aquatic animals, which is worthy of investigation.

Fish intestinal immune barrier function mainly depends on immune factors and cytokines (Zhang et al., 2013). As we know, immune factors such as lysozyme activities, C3, C4, IgM content and cytokines such as tumour necrosis factor (TNF), interleukin (IL), transforming growth factor (TGF) play an important role in intestinal immunity (Delcenserie et al., 2008). To date, no information has regarded the effects of ETSP on intestinal immune response in terrestrial and aquatic animals. Weichhart et al. (2008) reported that promotion of mTOR decreased the TNF-α production and increased the release of IL-10 in human peripheral blood mononuclear cells (PBMCs). Our previous study found that ETSP improved TOR gene expression in the intestine of juvenile carp (Xiao et al., 2017). These data indicate that ETSP may be related to the intestinal immune response of fish, further studies are warranted to address these important questions.

The normal physical barrier function is another important factor to fish intestinal health which is associated with the intestinal epithelial cells integrity and can be disrupted by cell apoptosis and oxidative damage (Hoyle, Shaw, & Handy, 2007). Studies revealed that the oxidative damage can be reduced by by enhancing the function of the antioxidant system which could be regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2) in myocardium of mice (Muthusamy et al., 2012). However, no study has investigated the effect of ETSP on the antioxidant gene expression through Nrf2 signalling pathway in terrestrial and aquatic animals. In vitro biochemical studies have shown that ETSP can strongly remove radicals and inhibit lipid peroxidation (Moure, Domınguez, & Parajo, 2006; Sarmadi & Ismail, 2010). Recent research also indicated that ETSP improved the insulin-like growth factor 1 (IGF-1) of plasma in mice (Jayachandran & Xu, 2019), while the IGF-1 could promote antioxidant gene expressions through Nrf2 signalling pathway in human neuroblastoma SH-SY5Y cells (Wang et al., 2017). These appear that ETSP may be related to the Nrf2 signalling pathway in fish intestine to influence activities of antioxidant enzymes and relative gene expressions, which is valuable for investigation.

This study was a part of a larger study that involved in the determination of the effects of ETSP on the digestive and absorptive ability in fish using the same growth trial as the previous study (Xiao et al., 2017). Our previous study has shown that adding ETSP to the low-protein diet promoted the digestive and absorptive ability of fish, along with the improvement of growth performance and saving of dietary protein (Xiao et al., 2017). Therefore, the objective of this study was to explore the preliminarily mechanism to improve fish digestion ability and absorption function, by examining the effect of ETSP on the intestinal immune, antioxidant ability and major signaling molecules (Nrf2) of juvenile Jian carp. The study will provide further theoretical evidence for the application of ETSP in commercial aquatic feeds.

2 | MATERIALS AND METHODS

2.1 | Preparation of ETSP

Enzymatic soybean protein was based on a certain proportion of dehulled solvent extracted SBM and SPC. Firstly, SBM and SPC were sterilized at 105°C for 30 min, then cooled to 50°C, mixed with calcium hydroxide and alkaline protease and hydrolysed at 1:4 (w/v) in distilled water at 50°C and pH 8.0–9.0 for 10 hr. The intermediate product after enzyme hydrolysis was dried at 90°C for 12 hr.

TABLE 1 Proximate composition and peptide profile of enzyme-treated soy protein

Nutrients	Content
Dry matter (%)	92.0
Crude protein (%)	46.0
Crude lipid (%)	2.5
Crude ash (%)	13.0
Nitrogen-free extract (%)	24.5
Water soluble nitrogen (%)	30.0
Acid soluble nitrogen (peptide content) (%)	29.0
Glycinin (mg/g)	6
β-Conglycinin (mg/g)	3
Molecular weight distribution (% water soluble nitrogen)	
<160 Da	20
161–650 Da	44
650–1000 Da	15
1,000–10,000 Da	21

Proximate composition and peptide profile of ETSP were quoted from Xiao et al. (2017) providing in Table 1.

2.2 | Experimental design and diets

The present study used the same growth trial as our previous study (Xiao et al., 2017). Composition and nutrient content of the basal diet are presented in Table 2. White fishmeal, SBM, cottonseed meal and rapeseed meal were used as the main protein source in all the dietary treatments. Diet 1 was formulated to contain 34% crude protein to meet the protein requirement of juvenile Jian carp (Liu, Feng, Jiang, Liu, & Zhou, 2009). Diet 2 was reduced to 2.0% of dietary protein in total by iso-proportionally reducing the amount of four protein materials based on Diet 1. Diet 1 and diet 2 were established as positive control and negative control respectively. ETSP was at the concentration of 0%, 0%, 1%, 1.5%, 2.0%, 2.5% and 3% in seven diets, respectively. Acid soluble nitrogen was measured with the method of trichloroacetic acid precipitation, as described by Sousa and Malcata (1996). The molecular weight distribution of peptide was determined by matrix-assisted laser desorption ionisation time-of-flight (TOF) mass spectrometry according to Gobom et al. (2002). Zinc, iron, pyridoxine, pantothenic acid, inositol, thiamine and riboflavin were formulated to meet the nutrient requirements of Jian carp according to Tan et al. (2011); Ling et al. (2010); He, Zhou, Feng, Jiang, and Liu (2009); Wen, Zhou, Feng, Jiang, and Liu (2009); Jiang, Feng, Liu, Jiang, and Zhou (2009); Huang et al. (2011); Li, Zhou, Feng, Liu, and Jiang (2010) respectively. Other nutrients met the requirements of common carp according to NRC (2011). The pellets were produced and stored at −20°C.

TABLE 2 Composition of two control diets

Ingredients	Diets (% dry diet)	
	Diet 1 (positive control)	Diet 2 (negative control)
Fishmeal	7.50	6.83
Soybean meal	28.00	25.50
Cottonseed meal	14.00	12.75
Rapeseed meal	17.00	15.48
Flour	19.92	25.86
Enzyme-treated soy protein (ETSP) premix[a]	5.00	5.00
Ca $(H_2PO_4)_2$	1.90	1.90
Carboxyl cellulose (CMC)	2.00	2.00
Soya bean oil	2.50	2.50
Vitamin premix[b]	1.00	1.00
Mineral premix[c]	1.00	1.00
Choline chloride	0.13	0.13
Ethoxyquin	0.05	0.05
Total (%)	100.00	100.00
Nutrients content (%)[d]		
Crude protein	33.85	32.04
Crude lipid	4.30	3.87

[a]Per kilogram of ETSP premix (g/kg): flour, 1,000 g; ETSP, 0 g (diet 1); flour, 1,000 g; ETSP, 0 g (diet 2); flour, 800 g; ETSP, 200 g (diet 3); flour, 700 g; ETSP, 300 g (diet 4); flour, 600 g; ETSP, 400 g (diet 5); flour, 500 g; ETSP, 500 g (diet 6); flour, 400 g; ETSP, 600 g (diet 7). [b]Per kilogram of vitamin premix (g/kg): retinyl acetate (500,000 IU/g), 0.800 g; cholecalciferol (500,000 IU/g), 0.480 g; DL-α-tocopherol acetate (500 g/kg), 20.000 g; thiamine nitrate (900 g/kg), 0.104 g; riboflavin (800 g/kg), 0.625 g; pyridoxine hydrochloride (810 g/kg), 0.755 g; cyanocobalamin (100 g/kg), 0.100 g; niacin (980 g/kg), 2.970 g; D-biotin (200 g/kg), 7.500 g; meso-inositol (990 g/kg), 52.857 g; folic acid (960 g/kg), 0.526 g; ascorbyl acetate (930 g/kg), 7.510 g; calcium-D- pantothenate (900 g/kg), 2.511 g. All ingredients were diluted with corn starch to 1 kg. [c]Per kilogram of mineral premix (g/kg): $CuSO_4.5H_2O$ (250 g/kg Cu), 1.20 g; $MnSO_4{\cdot}H_2O$ (318 g/kg Mn), 4.09 g; KI (38 g/kg I), 2.90 g; $NaSeO_3$ (10 g/kg Se), 2.50 g; $ZnSO_4.7H_2O$ (345 g/kg Zn), 14.11 g; $FeSO_4.7H_2O$ (197 g/kg Fe), 69.70 g. All ingredients were diluted with $CaCO_3$ to 1 kg. [d]All were measured values.

2.3 | Feeding trial

The procedures used in this study were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. Juvenile Jian carp obtained from the Tong Wei Hatchery (Sichuan, China) were acclimatized to the experimental environment for 4 weeks. A total of 1,400 fish (initial weight 11.96 ± 0.05 g) were randomly distributed into each of 28 experimental aquaria (90 *L* × 30 *W* × 40 *H* cm), which was connected to a closed recirculation water system and oxygen auto-supplemented system. Feeding management was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Animal Nutritional Institute, Sichuan Agricultural University. Each experimental diet was randomly assigned to aquaria in quadruplicate. Fish were fed with respective diets to apparent satiation six times at the first 4 weeks and four times daily at the second 4 weeks according to our laboratory previous study (Zhao et al., 2014). Uneaten feed was removed by siphoning after feeding, dried and weighed to measure feed intake. Each aquarium was supplied with flow-through water at a rate of 1.2 L/min and the water was drained through biofilters to decrease microorganisms, impurities and ammonia concentration in water. The experimental units were under a natural light and dark cycle (approximately 13:11 hr light:dark). Water temperature was 25 ± 2°C, pH 7.0 ± 0.3, dissolved oxygen >5.0 mg/L, $NH4^+$-N < 0.50 mg/L and NO_2-N < 0.05 mg/L.

2.4 | Sample collection and analysis

At the end of the experiment, the intestine of 15 fish from each aquarium were quickly removed, weighed and stored at −70°C until analysis. Intestine samples were homogenized on ice in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at 6,000 *g* for 20 min at 4°C, and then the supernatant was conserved for index analysis. Lysozyme activity was determined according to El-Boshy, El-Ashram, Abdelhamid, and Gadalla (2010). C3, C4, IgM, T-AOC, ASA, AHR were determined using the corresponding kit (Nanjing

Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocols. Lipid peroxidation and protein oxidation were determined based on malondialdehyde (MDA) and protein carbonyl (PC) content, which were measured according to Zhang, Zhu, Cai, and Wu (2008); Armenteros, Heinonen, Ollilainen, Toldr, and Estevez (2009) respectively. The protein concentration of samples was determined using the method of Bradford (1976) using the Coomassie Brilliant Blue dye binding technique with bovine serum albumin as the standard. Superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione-S-transferase (GST) and glutathione reductase (GR) activities were determined by the method of Meng, Chen, Xu, Huang, and Wang (2013); Zhang et al.(2008); Aebi (1984); Lushchak, Mota, and Hermes-Lima (2001); Lora et al. (2004) respectively. Glutathione (GSH) content was assayed as described by Vardi et al. (2008).

2.5 | Real-time PCR analysis

Total RNA was extracted from the intestine using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's instructions followed by DNase I treatment. RNA quality and quantity were assessed using agarose gel electrophoresis (1%) and spectrophotometry analysis of 260/280 ratios. cDNA synthesis was performed at 37°C, 15 min; 85°C, 5 s using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China) with 2 uL of total RNA. Real-time PCR was performed for IL-1, TNF-α, TGF-β, IL-10, CuZnSOD, MnSOD, CAT, GR, GPx1α, Keap1, Nrf2 and housekeeping gene (β-Actin) according to standard protocols with the primer sequences and optimal annealing temperatures indicated in Table 3. Real-time PCR was performed for these genes according to standard protocols. The expression levels of these genes were normalized to β-actin and expressed relative to positive control (Diet 1). After verification that the primers amplified with an efficiency of approximately 100%, the target and housekeeping gene amplification efficiency were calculated according to the specific gene standard curves that were generated from 10-fold serial dilutions, the results were analysed using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001).

2.6 | Statistical analysis

The data were presented as mean ± standard deviation. All data were subjected to one-way analysis of variance (ANOVA) followed by the Duncan method to determine significant differences among treatment groups. Differences were considered significant at the level of

TABLE 3 Real-time primer sequences and annealing temperature of target genes and the housekeeping gene

Target gene	Sequences of primers annealing	Annealing temperature	Accession number
IL-1β 2-1	Forward 5'-TCCTCCCGTCCTGCTCTTG-3' Reverse 5'-TTGTAATCCGTGCCCGTCTC-3'	60.6	AJ401030.1
IL-1β 2-2	Forward 5'-TGCTTGTACCCAGTCTGGTGG-3' Reverse 5'-CTGAAGAAGAGGAGGCTGTCGT-3'	60.5	AJ401031.1
TNF-α	Forward 5'-TGTGTGGTGTCCTGCTGG-3' Reverse 5'-TGGAAAGACACCTGGCTGTA-3'	56.0	AJ311800
TGF-β2	Forward 5'-GGGACATCATCGCCATCT-3' Reverse 5'-TGACATTCTCGGCAGGGT-3'	57.5	U66874
IL-10	Forward 5'-GCCAGCATAAAGAACTCG-3' Reverse 5'-CCAAATACTGCTCGATGT-3'	59.4	AB110780
CuZnSOD	Forward 5'-TGGCGAAGAAGGCTGTTTGT-3' Reverse 5'-TTCACTGGAGACCCGTCACT-3'	60.4	JF342355
MnSOD	Forward 5'-CTGCCTGACCTTCCATACGA-3' Reverse 5'-CCTTAGCCAGTGCCTCTTGATA-3'	62.0	JF411603
CAT	Forward 5'-CTGGAAGTGGAATCCGTTTG-3' Reverse 5'-CGACCTCAGCGAAATAGTTG-3'	54.0	JF411604
GR	Forward 5'-GAGAAGTACGACACCATCCA-3' Reverse 5'-CACACCTATTGAACTGAGATTGAG-3'	56.0	JF411607
GPx1a	Forward 5'-GTGACGACTCTGTGTCCTTG-3' Reverse 5'-AACCTTCTGCTGTATCTCTTGA-3'	60.4	JF411605
Keap1	Forward 5'-CTACAACCCCGAGAGAGACGA-3' Reverse 5'-GGAGGAGATGAAGCTCCAGAC-3'	60.0	JX470752
Nrf2	Forward 5'-TTCCCGCTGGTTTACCTTAC-3' Reverse 5'-CGTTTCTTCTGCTTGTCTTT-3'	60.0	JX462955
β-Actin	Forward 5'-CGTGATGGACTCTGGTGATG-3' Reverse 5'-TCGGCTGTGGTGGTGAAG-3'	60.0	M24113

Note. IL-1: Interleukin 1; IL-10: Interleukin 10; TNF-α: Tumour necrosis factor α; TGF-β: Transforming growth factor beta; SOD: Superoxide dismutase; CAT: Catalase; GR: Glutathione reductase; GPx: Glutathione peroxidase; Keap1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2-related factor 2.

$p < 0.05$. Statistical analyses were done using the SPSS 18.0 (SPSS Inc., Chicago, IL).The relationships between dietary ETSP and immune parameters, antioxidant parameters and gene expression in the intestine were subjected to a linear or quadratic regression model.

3 | RESULTS

3.1 | Effect of adding ETSP to the low-protein diet on the immune factors in fish intestine

The immune factors in the intestine are presented in Table 4. Lysozyme activities, C3, C4 and IgM content were not significantly affected by reducing the dietary crude protein (diet 2) ($p > 0.05$). After adding 1.5% of ETSP to the low-protein diet, lysozyme activities and IgM content achieved maximum value which were even much higher than that of diet 1, and then reduced with the increasing ETSP level ($p < 0.05$).

3.2 | Effect of adding ETSP to the low-protein diet on the cytokine gene expression in fish intestine

Relative gene expressions of cytokine in the intestine are shown in Figures A1 and A2. Relative expressions of IL-1β (2-1, 2-2) and TNF-α were significantly increased by reducing the dietary crude protein (diet 2) ($p < 0.05$). After adding 1.5% or 2% of ETSP to the low-protein diet (diet 2), all these indexes were significantly decreased ($p < 0.05$), and the data of TNF-α were even much lower than that of diet 1 ($p < 0.05$). Relative expression of TGF-β2 was not affected by different diets ($p > 0.05$). Reducing the dietary crude protein did not significantly affect the relative expressions of IL-10. After adding 1% ETSP to the low-protein diet (diet 2), this index significantly increased ($p < 0.05$), which achieved maximum value when the ETSP level was up to 2%.

3.3 | Effect of adding ETSP to the low-protein diet on the antioxidant parameters in fish intestine

Antioxidant parameters in the intestine are presented in Table 5. MDA was not affected by reducing the dietary crude protein ($p > 0.05$). After adding 1%–3% of ETSP to the low-protein diet (diet 2), MDA was significantly decreased ($p < 0.05$), which was even much lower than that of diet 1 ($p < 0.05$). PC was significantly increased by reducing the dietary crude protein (Diet 2) ($p < 0.05$). After adding 1%–3% of ETSP to the low-protein diet (diet 2), PC was significantly decreased ($p < 0.05$), which had no significant difference with that of diet 1 ($p > 0.05$). T-AOC was not significantly affected by reducing the dietary crude protein (Diet 2) ($p > 0.05$), which was significantly increased by increasing the dietary ETSP level ($p < 0.05$). ASA was nearly at the same level among different diet ($p > 0.05$). The tendency of AHR is nearly the same as T-AOC ($p < 0.05$).

3.4 | Effect of adding ETSP to the low-protein diet on the antioxidant enzymes in fish intestine

Antioxidant enzymes and GSH in the intestine are shown in Table 6. SOD was significantly decreased by reducing the dietary crude protein (diet 2) ($p < 0.05$). After adding 1.5% of ETSP, SOD achieved maximum value which were significantly higher than that of diet 2, and then decreased with the increasing ETSP level ($p < 0.05$). CAT and GST were not significantly affected by reducing the dietary crude protein (Diet 2) ($p > 0.05$). These two indexes achieved maximum value when the ETSP level was up to 1.5%, which was even much higher than that of diet 1 ($p < 0.05$). GPX and GR were nearly at the same level among different diets ($p > 0.05$). SOD was significantly increased by reducing the dietary crude protein (diet 2) ($p < 0.05$), while adding ETSP to the low-protein diet did not affect this index ($p > 0.05$).

3.5 | Effect of adding ETSP to the low-protein diet on the antioxidant enzyme, Nrf2 and Keap1 gene expression in fish intestine

Relative expressions of antioxidant enzymes, Nrf2 and Keap1a in the intestine were presented in Figures A3 and A4. All the relative expressions of antioxidant enzymes were not significantly affected by reducing the dietary crude protein (Diet 2) ($p > 0.05$). After adding

TABLE 4 Immune factors of fish fed diets with graded levels of ETSP[†]

	Dietary ETSP levels (%)						
Item	Diet 1 (0)	Diet 2 (0)	Diet 3 (1.0)	Diet 4 (1.5)	Diet 5 (2.0)	Diet 6 (2.5)	Diet 7 (3.0)
Lysozyme (U/mg protein)	32.54 ± 4.13^{a}	31.65 ± 5.83^{a}	31.91 ± 3.18^{a}	43.21 ± 3.82^{b}	35.56 ± 3.24^{a}	31.54 ± 6.09^{a}	31.79 ± 3.13^{a}
C_3 (mg/g protein)	2.49 ± 0.28^{a}	2.59 ± 0.18^{a}	2.42 ± 0.14^{a}	2.52 ± 0.22^{a}	2.40 ± 0.18^{a}	2.37 ± 0.20^{a}	2.47 ± 0.18^{a}
C_4 (mg/g protein)	5.61 ± 0.81^{a}	5.30 ± 0.56^{a}	5.09 ± 0.67^{a}	5.00 ± 0.39^{a}	5.40 ± 0.50^{a}	4.99 ± 0.75^{a}	5.12 ± 0.84^{a}
IgM (mg/g protein)	2.32 ± 0.24^{ab}	2.19 ± 0.16^{a}	2.19 ± 0.21^{a}	2.47 ± 0.10^{b}	2.23 ± 0.23^{ab}	2.12 ± 0.26^{ab}	2.24 ± 0.11^{ab}

Note. C3: Complement 3; C4: Complement 4; IgM: Immunoglobulin M; ETSP: enzyme-treated soy protein.
[†]Values are mean ± *SD* of four replicates, with five fish in each replicate.
Values within the same row having different superscripts are significantly different ($P < 0.05$).

TABLE 5 Antioxidant parameters in the whole intestine of fish fed diets with graded levels of ETSP[†]

Item	Dietary ETSP levels (%)						
	Diet 1 (0)	Diet 2 (0)	Diet 3 (1.0)	Diet 4 (1.5)	Diet 5 (2.0)	Diet 6 (2.5)	Diet 7 (3.0)
MDA (nmol/mg prot)	9.36 ± 0.63^{b}	9.48 ± 0.21^{b}	8.60 ± 0.39^{a}	8.46 ± 0.63^{a}	8.36 ± 0.62^{a}	8.44 ± 0.83^{a}	8.48 ± 0.69^{a}
PC (nmol/mg prot)	6.23 ± 0.55^{a}	6.99 ± 0.61^{b}	6.07 ± 0.45^{a}	5.71 ± 0.53^{a}	6.00 ± 0.44^{a}	6.19 ± 0.56^{a}	6.27 ± 0.55^{a}
T-AOC (U/mg prot)	1.27 ± 0.07^{ab}	1.16 ± 0.06^{ab}	1.36 ± 0.15^{c}	1.45 ± 0.12^{c}	1.60 ± 0.10^{d}	1.67 ± 0.12^{d}	1.62 ± 0.04^{d}
ASA (U/mg prot)	312.13 ± 30.61^{a}	302.03 ± 30.66^{a}	309.70 ± 27.18^{a}	318.07 ± 28.12^{a}	315.44 ± 19.91^{a}	314.30 ± 15.90^{a}	309.78 ± 29.91^{a}
AHR (U/mg prot)	690.74 ± 54.87^{b}	518.34 ± 44.64^{a}	755.21 ± 67.06^{bc}	798.75 ± 60.35^{c}	782.74 ± 77.37^{c}	783.48 ± 66.60^{c}	743.63 ± 72.89^{bc}

Note. MDA: Malondialdehyde; PC: Protein carbonyl; T-AOC: Total antioxidant capacity; ASA: Anti-hydroxy radical; AHR: Anti-superoxide anion; ETSP: enzyme-treated soy protein.
[†]Values are mean ± *SD* of four replicates, with five fish in each replicate.
Values within the same row having different superscripts are significantly different ($P < 0.05$).

1% or 1.5% of ETSP to the low-protein diet, all the relative expressions were significantly increased, and only the relative expressions of SOD and GR were even higher than that of diet 1 ($p < 0.05$). Keap1a was decreased by adding ETSP, whereas Nrf2 achieved maximum value when the ESTP level was at 1.5%.

4 | DISCUSSION

This study used the same growth trial as in our previous study, which observed that adding optimal level of ETSP to the low-protein diet improved the growth of juvenile Jian carp (the final mean weight and *SD* of groups 1–7 are 86.67 ± 2.70^{b}, 82.22 ± 2.01^{a}, 86.81 ± 2.43^{b}, 89.69 ± 2.61^{b}, 87.58 ± 2.27^{b}, 86.53 ± 3.79^{b}, 85.39 ± 2.12^{ab} g respectively), and promoted digestive and absorptive ability referring to TOR signalling in juvenile fish, showing that 1% of ETSP (only provided 0.46% crude protein) can save 2% of dietary crude protein (Xiao et al., 2017). Our laboratory's previous study showed that the digestion and absorption function mainly rely on the intestinal health in aquatic animals, which has been found to be correlated with the intestinal immunity and structure (Zhao et al., 2014). Therefore, the present study investigates the effects of ETSP on the intestinal immunity and the structure of fish as well as its potential mechanisms, to provide further evidence that ETSP can save fish dietary protein.

4.1 | The effect of low dietary protein on intestinal immune response and antioxidant status of fish

In the current study, compared with the normal protein diet (34%), the low-protein diet (32%) increased the PC content and decreased the AHR and SOD activity. The low-protein diet (32%) also increased the relative gene expression of the pro-inflammatory cytokines such as IL-1 and TNF-α. Zhang, Zhao et al. (2018) also reported that the low-protein diet decreased the activities of CAT, SOD and lysozyme in plasma of *Pelteobagrus fulvidraco* larvae. These results suggested that the low-protein diet may disturb the inflammatory response and increase the oxidative damage in intestine, so as to affect the intestinal health status of fish. The negative impact may be the further reason why the low-protein diet (32%) impressed the growth performance of fish.

4.2 | Adding ETSP to the low-protein diet affect the intestinal immune response and antioxidant status of fish

4.2.1 | ETSP regulated the intestinal immune response of fish

Lysozyme, complement factors and IgM are the first line of the intestinal mucosal immune and play important roles against microbial invasion in fish (Rombout, Abelli, Picchietti, Scapigliati, & Kiron, 2011). The present results indicated that adding ETSP to the low-protein diet increased the contents of C3, C4 and IgM in intestine. Furthermore,

TABLE 6 Antioxidant enzymes in the whole intestine of fish fed diets with graded levels of ETSP[†]

	Dietary ETSP levels (%)						
Item	Diet 1 (0)	Diet 2 (0)	Diet 3 (1.0)	Diet 4 (1.5)	Diet 5 (2.0)	Diet 6 (2.5)	Diet 7 (3.0)
SOD (U/mg prot)	72.26 ± 6.82^{bc}	61.65 ± 5.43^{a}	69.07 ± 5.58^{ab}	78.89 ± 7.37^{c}	74.76 ± 5.80^{bc}	72.85 ± 6.71^{bc}	67.07 ± 5.54^{ab}
CAT (U/mg prot)	30.52 ± 2.51^{ab}	29.61 ± 2.78^{a}	35.14 ± 2.92^{cd}	41.58 ± 3.88^{e}	37.88 ± 3.45^{d}	33.65 ± 3.34^{bc}	31.48 ± 2.50^{abc}
GPX (U/mg prot)	21.66 ± 2.07^{a}	21.64 ± 1.06^{a}	23.04 ± 2.03^{a}	23.82 ± 1.41^{a}	22.56 ± 1.74^{a}	22.24 ± 2.00^{a}	22.02 ± 2.19^{a}
GST (U/mg prot)	27.78 ± 2.12^{a}	26.73 ± 2.39^{a}	28.52 ± 1.92^{ab}	30.86 ± 3.00^{b}	29.75 ± 1.34^{ab}	28.49 ± 2.33^{ab}	28.10 ± 2.60^{ab}
GR (U/g prot)	59.80 ± 4.08^{a}	59.75 ± 2.23^{a}	61.19 ± 4.44^{a}	62.15 ± 2.27^{a}	60.78 ± 4.96^{a}	60.16 ± 4.73^{a}	58.81 ± 2.70^{a}
GSH (mg/g prot)	5.27 ± 0.52^{a}	5.89 ± 0.52^{b}	6.22 ± 0.46^{b}	5.90 ± 0.39^{b}	5.80 ± 0.40^{ab}	5.78 ± 0.45^{ab}	5.62 ± 0.50^{ab}

Note. SOD: Superoxide dismutase; CAT: Catalase; GR: Glutathione reductase; GPx: Glutathione peroxidase; GST: Glutathione-S-transferase; GSH: Glutathione; ETSP: enzyme-treated soy protein.

[†]Values are mean ± *SD* of four replicates, with five fish in each replicate.

Values within the same row having different superscripts are significantly different ($P < 0.05$).

when ETSP level was up to 1.5% or 2%, these indexes were even much higher than that of the high-protein diet. The improvement of immune ability in fish intestine by ETSP may be correlated with the low molecular weight peptides in it. The ETSP used in this trail containing more than 29% peptides, especially rich for the low molecular weight peptides between 161 and 650 Da. Studies indicated that small peptides with low molecular weight (between 550 and 650 Da) released from soy protein could stimulate immunomodulatory activity such as lymphocyte proliferation and macrophage phagocytosis in mice spleen cells and peritoneal exudate cells (Kong, Guo, Hua, Cao, & Zhang, 2008), while the B lymphocyte and macrophage are the main production cells of IgM and complements in vertebrate respectively (Ribatti, 2018). This information suggested that ETSP could improve the intestinal immune ability possibly relating with the low molecular weight peptides in it, which needs further investigation.

Normal immune function is also maintained by a balance between the activities of pro- and anti-inflammatory cytokines (Chiu & Yang, 2007). Further, we investigated the relationship between ETSP and intestinal pro- and anti-inflammatory cytokine gene expression in Jian carp. IL-1β and TNF-α are key pro-inflammatory cytokines in fish (Chiu & Yang, 2007). In the present study, we observed that the relative gene expressions of IL-1β and TNF-α were increased by reducing the dietary crude protein level. Whereas adding 1.5% or 2% of ETSP to the low-protein diet, all these indexes decreased which were even much lower than that of the high-protein diet. TGF-β and IL-10 are key anti-inflammatory regulators in the maintenance of immunological homeostasis (Fu, Ye, Lee, & Chiang, 2006). The result indicated that adding 1.5% or much more ETSP to the low-protein diet, the relative gene expressions of IL-10 significantly increased which were even much higher than that of the high-protein diet. These data showed that the inflammatory response was inhibited after adding ETSP, which was even much lower than that of the high-protein diet. That means more nutrients would be needed for growth in normal body status by adding ETSP, which might contribute to the faster growth. The ETSP can lower the relative gene expression of pro-inflammatory cytokines and raise the relative gene expression of anti-inflammatory cytokines, which might be related to the promotion of TOR signalling. Our previous study found that ETSP improved TOR gene expression in the intestine of juvenile Jian carp (Xiao et al., 2017). Meanwhile, research showed that the promotion of mTOR decreased TNF-α production and increased the release of IL-10 in human PBMCs (Weichhart et al., 2008). That means ETSP might regulate the relative gene expression of inflammatory cytokines in fish intestine through TOR signalling. However, the exact mechanism needs further investigation.

4.2.2 | ETSP improved the intestinal antioxidant status of fish

The structural integrity plays an important role for fish intestinal health, which has been associated with its oxidative status (Zhang et al., 2013). MDA and PC contents of intestine were widely used as biomarkers for oxidative damage of lipids and proteins respectively (Chen, Zhou, Feng, Liu, & Jiang, 2009). In this study, MDA and PC decreased when adding 1.5% of ETSP to the low-protein diet, which were even much lower than that of the high-protein diet. This suggested that ETSP could depress the lipid peroxidation and protein oxidation in the intestine of fish. The lipid peroxidation and protein oxidation can be suppressed by non-enzymatic antioxidants such as GSH and antioxidant enzymes such as SOD, CAT, GPx, GST and GR in fish (Martinez-Alvarez, Morales, & Sanz, 2005). The results showed that adding optimal level of ETSP improved the activities of SOD, CAT and GST in fish intestine. The improvement of antioxidant ability in fish intestine by ETSP may be correlated with the antioxidant peptides in it. Zhang, Tong et al. (2018) reported that antioxidant peptides from soybean hydrolysate, especially peptides with the molecular weight between 500 and 1,000 Da, significantly decreased the MDA level, increased the activities of CAT, GPx and GR in human intestinal epithelial Caco-2 cells. While the ETSP used in this trail is also rich for the molecular weight peptides less than 1,000 Da. This information suggested that ETSP could improve the intestinal antioxidant ability possibly relating with the antioxidant peptides in it, which also needs further investigation. An alternative possibility to increased antioxidant enzyme activities may be

correlated to an increase in antioxidant enzymes gene expressions in fish (Zhao et al., 2014). Further, studying the relationship between ETSP and gene expression of antioxidant enzymes in the intestine of Jian carp would be very valuable.

4.2.3 | ETSP regulated intestinal gene expressions of antioxidant enzymes, Nrf2 and Keap1 of fish

Our study showed that adding 1.5% of ETSP to the low-protein diet, the relative gene expressions of all antioxidant enzymes were increased, while CuZnSOD, MnSOD and GR were even much higher than that of the high-protein diet. These results suggested that ETSP could improve these antioxidant enzyme activities through the up-regulation of gene transcription in fish intestine. To date, this is the first report about the relationship between ETSP and antioxidant enzyme gene expressions in terrestrial and fish animal. Furthermore, antioxidant genes transcription is regulated by Nrf2 signalling in terrestrial animals. Thus, we next investigated the effect of ETSP on the major signalling molecules Nrf2 and Keap1 involved in the Nrf2 signalling pathway.

Nrf2 is a key nuclear transcription factor that could bind the antioxidant response element to regulate the transcription of antioxidant genes in terrestrial animal, while Keap1 could retain Nrf2 in the cytoplasm and prohibit the Nrf2 nuclear translocation (Muthusamy et al., 2012). In mice, the up-regulation of Nrf2 gene expression could improve the antioxidant enzyme gene expression such as CuZnSOD, MnSOD, and CAT, and the down-regulation of Keap1 gene could increase the Nrf2 nuclear translocation, resulting in the transcriptional induction of antioxidant gene (Reisman, Yeager, Yamamoto, & Klaassen, 2009). When adding 1.5% of ETSP to the low-protein diet, the relative gene expressions of Nrf2 increased and the relative gene expressions of Keap1 decreased. The regulation of Nrf2 signalling pathway in fish intestine after adding ETSP might be related to the improvement of IGF-1. Jayachandran and Xu (2019) found that ETSP improved the serum IGF-1 in mice. Gui, Liu, Shao, and Xu (2010) found that another plant protein hydrolysate (cottonseed meal protein hydrolysate) replacing the cottonseed meal also improved the serum IGF-1 in crucian carp (*Carassius auratus gibelio*). Study indicated that the IGF-1 could promote antioxidant gene expressions through Nrf2 signalling pathway in human neuroblastoma SH-SY5Y cells (Wang et al., 2017). This information suggested that ETSP might regulate antioxidant gene expressions through Nrf2 signalling pathway in fish intestine. However, the exact mechanism also needs further investigation.

5 | CONCLUSION

In conclusion, the low-protein diet would disturb the intestinal inflammatory response and antioxidant status that might be the further reason for slowing the growth performance when fish fed the low-protein diet. It is the first time to found that adding 1.5% of ETSP to the low-protein diet improved intestinal health via regulating intestinal immunity and antioxidant capacity of fish, and the much better intestinal health might contribute to the improvement of the digestive and absorptive capacities in our previous study. Also, the results of gene expression of CuZnSOD, MnSOD, CAT and GR in the intestine could be partly attributable to the improvement of antioxidant enzyme activities by ETSP in fish. Additionally, dietary ETSP regulates gene expression of Nrf2 and Keap1 in the intestine, which might be a preliminary model of ETSP affecting the gene expressions of antioxidant enzyme. From these data, we provide some further proofs that ETSP can save the fish dietary protein.

ACKNOWLEDGEMENTS

This research was financially supported by the National Basic Research Program of China (973 Program) (2014CB138600), National Natural Science Foundation of China (31672662), National Department Public Benefit Research Foundation (Agriculture) of China (201003020), the Key Fund Project of Sichuan Provincial Department of Education (16ZA0022), Outstanding Talents and Innovative Team of Agricultural Scientific Research (Ministry of Agriculture), Science and Technology Support Program of Sichuan Province of China (2014NZ0003), Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China (2012NC0007; 2013NC0045), the Demonstration of Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China (2015CC0011), Foundation of Sichuan Youth Science and Technology Innovation Research Team (2017TD0002), and Supported by the Earmarked Found for China Agriculture Research System (CARS-45). The authors would like to thank the personnel of these teams for their kind assistance.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ORCID

Xiaoqiu Zhou https://orcid.org/0000-0001-5266-0041

REFERENCES

Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology*, *105*, 121–126.

Armenteros, M., Heinonen, M., Ollilainen, V., Toldr, F., & Estevez, M. (2009). Analysis of protein carbonyls in meat products by using the DNPH-method, fluorescence spectroscopy and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). *Meat Science*, *83*, 104–112. https://doi.org/10.1016/j.meatsci.2009.04.007

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Chen, J., Zhou, X. Q., Feng, L., Liu, Y., & Jiang, J. (2009). Effects of glutamine on hydrogen peroxide-induced oxidative damage in intestinal epithelial cells of Jian carp (*Cyprinus carpio* var.

Jian). *Aquaculture*, *288*, 285–289. https://doi.org/10.1016/j.aquaculture.2008.10.053

Chiu, S. C., & Yang, N. S. (2007). Inhibition of tumor necrosis factor- a through selective blockade of pre-mRNA splicing by shikonin. *Molecular Pharmacology*, *71*, 1640–1645. https://doi.org/10.1124/mol.106.032821

Delcenserie, V., Martel, D., Lamoureux, M., Amiot, J., Boutin, Y., & Roy, D. (2008). Immuno- modulatory effects of probiotics in the intestinal tract. *Current Issues in Molecular Biology*, *10*, 37–54.

El-Boshy, M. E., El-Ashram, A. M., Abdelhamid, F. M., & Gadalla, H. A. (2010). Immuno-modulatory effect of dietary Saccharomyces cerevisiae, beta-glucan and lami-naran in mercuric chloride treated Nile tilapia (*Oreochromis niloticus*) and experimentally infected with Aeromonas hydrophila. *Fish & Shellfish Immunology*, *28*, 802–808. https://doi.org/10.1016/j.fsi.2010.01.017

Fu, C. L., Ye, Y. L., Lee, Y. L., & Chiang, B. L. (2006). Effects of overexpression of IL-10, IL-12, TGF-β and IL-4 on allergen induced change in bronchial responsiveness. *Respiratory Research*, *7*, 72. https://doi.org/10.1186/1465-9921-7-72

Gobom, J., Mueller, M., Egelhofer, V., Theiss, D., Lehrach, H., & Nordhoff, E. (2002). A calibration method that simplifies and improves accurate determination of peptide molecular masses by MALDI-TOF MS. *Analytical Chemistry*, *74*, 3915–3923. https://doi.org/10.1021/ac011203o

Gui, D., Liu, W. B., Shao, X. P., & Xu, W. N. (2010). Effects of different dietary levels of cottonseed meal protein hydrolysate on growth, digestibility, body composition and serum biochemical indices in crucian carp (*Carassius auratus gibelio*). *Animal Feed Science and Technology*, *156*, 112–120. https://doi.org/10.1016/j.anifeedsci.2010.01.012

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: Effects of global demand and supplies of fishmeal. *Aquaculture Research*, *41*, 770–776. https://doi.org/10.1111/j.1365-2109.2009.02349.x

He, L. X., Wang, J. B., Sun, B., Zhao, J., Lin, L., Xu, T., … Li, Y. (2017). Suppression of TNF-α and free radicals reduces systematic inflammatory and metabolic disorders: Radio-protective effects of ginseng oligopeptides on intestinal barrier function and antioxidant defense. *The Journal of Nutritional Biochemistry*, *40*, 53–61. https://doi.org/10.1016/j.jnutbio.2016.09.019

He, W., Zhou, X. Q., Feng, L., Jiang, J., & Liu, Y. (2009). Dietary pyridoxine requirement of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Nutrition*, *15*, 402–408.

Hoyle, I., Shaw, B. J., & Handy, R. D. (2007). Dietary copper exposure in the African walking catfish, *Clarias gariepinus*: Transient osmoregulatory disturbances and oxidative stress. *Aquatic Toxicology*, *83*, 62–72. https://doi.org/10.1016/j.aquatox.2007.03.014

Huang, H. H., Feng, L., Liu, Y., Jiang, J., Jiang, W. D., Hu, K., … Zhou, X. Q. (2011). Effects of dietary thiamin supplement on growth, body composition and intestinal enzyme activities of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Nutrition*, *17*, 233–240. https://doi.org/10.1111/j.1365-2095.2010.00756.x

Jayachandran, M., & Xu, B. J. (2019). An insight into the health benefits of fermented soy products. *Food Chemistry*, *271*, 362–371. https://doi.org/10.1016/j.foodchem.2018.07.158

Jiang, W. D., Feng, L., Liu, Y., Jiang, J., & Zhou, X. Q. (2009). Growth, digestive capacity and intestinal microflora of juvenile Jian carp (*Cyprinus carpio* var. Jian) fed graded levels of dietary inositol. *Aquaculture Research*, *40*, 955–962. https://doi.org/10.1111/j.1365-2109.2009.02191.x

Kokou, F., & Fountoulaki, E. (2018). Aquaculture waste production associated with anti-nutrient presence in common fish feed plant ingredients. *Aquaculture*, *495*, 295–310. https://doi.org/10.1016/j.aquaculture.2018.06.003

Kong, X. Z., Guo, M. M., Hua, Y. F., Cao, D., & Zhang, C. M. (2008). Enzymatic preparation of immunomodulating hydrolysates from soy proteins. *Bioresource Technology*, *99*, 8873–8879. https://doi.org/10.1016/j.biortech.2008.04.056

Li, W., Zhou, X. Q., Feng, L., Liu, Y., & Jiang, J. (2010). Effect of dietary riboflavin on growth, feed utilization, body composition and intestinal enzyme activities of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Nutrition*, *16*, 137–143.

Ling, J., Feng, L., Liu, Y., Jiang, J., Jiang, W. D., Hu, K., … Zhou, X. Q. (2010). Effect of dietary iron levels on growth, body composition and intestinal enzyme activities of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Nutrition*, *16*, 616–624. https://doi.org/10.1111/j.1365-2095.2009.00699.x

Liu, Y., Feng, L., Jiang, J., Liu, Y., & Zhou, X. Q. (2009). Effects of dietary protein levels on the growth performance, digestive capacity and amino acid metabolism of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Research*, *40*, 1073–1082.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*, 402–408.

Lora, J., Alonso, F. J., Segura, J. A., Lobo, C., Márquez, J., & Matés, J. M. (2004). Antisense glutaminase inhibition decreases glutathione antioxidant capacity and increases apoptosis in Ehrlich ascitic tumour cells. *European Journal of Biochemistry*, *271*, 4298–4306. https://doi.org/10.1111/j.1432-1033.2004.04370.x

Lushchak, V. I., Lushchak, L. P., Mota, A. A., & Hermes-Lima, M. (2001). Oxidative stress and antioxidant defenses in goldfish (*Carassius auratus*) during anoxia and reoxygenation. *American Journal of Physiology-regulatory Integrative and Comparative Physiology*, *280*, 100–107.

Martinez-Alvarez, R. M., Morales, A. E., & Sanz, A. (2005). Antioxidant defenses in fish: Biotic and abiotic factors. *Reviews in Fish Biology Fisheries*, *15*, 75–88. https://doi.org/10.1007/s11160-005-7846-4

Meng, Q., Chen, J., Xu, C., Huang, Y., & Wang, Y. (2013). The characterization, expression and activity analysis of superoxide dismutases (SODs) from Pro-cambarus clarkia. *Aquaculture*, *406*, 131–140.

Moure, A., Domınguez, H., & Parajo, J. C. (2006). Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochemistry*, *41*, 447–456. https://doi.org/10.1016/j.procbio.2005.07.014

Muthusamy, V. R., Kannan, S., Sadhaasivam, K., Gounder, S. S., Davidson, C. J., Boeheme, C., … Rajasekaran, N. S. (2012). Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium. *Free Radical Biology and Medicine*, *52*, 366–376. https://doi.org/10.1016/j.freeradbiomed.2011.10.440

Niklasson, L., Sundh, H., Fridell, F., Taranger, G. L., & Sundella, K. (2011). Disturbance of the intestinal mucosal immune system of farmed Atlantic salmon (Salmo salar), in response to long-term hypoxic conditions. *Fish & Shellfish Immunology*, *31*, 1072–1080.

NRC (National Research Council), Nutrient requirements of fish and shrimp (2011). *The National Academies Press Washington*. USA: DC.

Reisman, S. A., Yeager, R. L., Yamamoto, M., & Klaassen, C. D. (2009). Increased Nrf2 activation in livers from Keap1-knockdown mice increases expression of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. *Toxicological Science*, *108*, 35–47. https://doi.org/10.1093/toxsci/kfn267

Ribatti, D. (2018). Immunology in the twentieth century. *From Basic Science to Clinical Application*, 17–36.

Rombout, J. H., Abelli, L., Picchietti, S., Scapigliati, G., & Kiron, V. (2011). Teleost intestinal immunology. *Fish & Shellfish Immunology*, *31*, 616–626. https://doi.org/10.1016/j.fsi.2010.09.001

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides*, *31*, 1949–1956. https://doi.org/10.1016/j.peptides.2010.06.020

Singh, B. P., Vij, S., & Hati, S. (2014). Functional significance of bioactive peptides derived from soybean. *Peptides*, *54*, 171–179. https://doi.org/10.1016/j.peptides.2014.01.022

Sousa, M. J., & Malcata, F. X. (1996). Influence of pasteurization of milk and addition of starter cultures on protein breakdown in ovine

cheeses manufactured with extracts from flowers of *Cynara cardunculus*. *Food Chemistry*, *57*, 549–556. https://doi.org/10.1016/S0308-8146(96)00190-2

Tan, L. N., Feng, L., Liu, Y., Jiang, J., Jiang, W. D., Hu, K., … Zhou, X. Q. (2011). Growth, body composition and intestinal enzyme activities of juvenile Jian carp (*Cyprinus carpio* var. Jian) fed graded levels of dietary zinc. *Aquaculture Nutrition*, *17*, 338–345. https://doi.org/10.1111/j.1365-2095.2010.00793.x

Tuckova, L., Novotna, J., Zuzana Flegelova, P. N., Kveton, T., Jelınkova, L., Zıdek, Z., … Tlaskalova-Hogenova, H. (2002). Activation of macrophages by gliadin fragments: Isolation and characterization of active peptide. *Journal of Leukocyte Biology*, *71*, 625–631.

Vardi, N., Parlakpinar, H., Ozturk, F., Ates, B., Gul, M., Cetin, A., … Otlu, A. (2008). Potent protective effect of apricot and b-carotene on methotrexate-induced intestinal oxidative damage in rats. *Food and Chemical Toxicology*, *46*, 3015–3022.

Wang, Z., Xiong, L. u., Wang, G., Wan, W., Zhong, C., & Zu, H. (2017). Insulin-like growth factor-1 protects SH-SY5Y cells against β-amyloid-induced apoptosis via the PI3K/Akt-Nrf2 pathway. *Experimental Gerontology*, *87*, 23–32. https://doi.org/10.1016/j.exger.2016.11.009

Weichhart, T., Costantino, G., Poglitsch, M., Rosner, M., Zeyda, M., Stuhlmeier, K. M., … Säemann, M. D. (2008). The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity*, *29*, 565–577. https://doi.org/10.1016/j.immuni.2008.08.012

Wen, Z. P., Zhou, X. Q., Feng, L., Jiang, J., & Liu, Y. (2009). Effect of dietary pantothenic acid supplement on growth, body composition and intestinal enzyme activities of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture Nutrition*, *15*, 470–476.

Xiao, W. W., Jiang, W. D., Feng, L., Liu, Y., Wu, P., Jiang, J., … Zhou, X. Q. (2017). Supplementation of enzyme-treated soy protein saves dietary protein and promotes digestive and absorptive ability referring to TOR signaling in juvenile fish. *Fish Physiology and Biochemistry*, *43*, 1657–1675. https://doi.org/10.1007/s10695-017-0400-1

Yin, B., Liu, H. Y., Tan, B. P., Dong, X. H., Chi, S. Y., Yang, Q. H., … Chen, L. Q. (2018). Cottonseed protein concentrate (CPC) suppresses immune function in different intestinal segments of hybrid grouper (♀ *Epinephelus fuscoguttatus*×♂ *Epinephelus lanceolatu*) via TLR-2/MyD88 signaling pathways. *Fish and Shellfish Immunology*, *81*, 318–328. https://doi.org/10.1016/j.fsi.2018.07.038

Zhang, J. X., Guo, L. Y., Feng, L., Jiang, W. D., Kuang, S. Y., & Liu, Y. (2013). Soybean β-con-glycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. *PLoS ONE*, *8*, e58115.

Zhang, J., Zhao, N. N., Sharawy, Z., Lia, Y., Ma, J., & Lou, Y. A. (2018). Effects of dietary lipid and protein levels on growth and physiological metabolism of *Pelteobagrus fulvidraco* larvae under recirculating aquaculture system (RAS). *Aquaculture*, *495*, 458–464. https://doi.org/10.1016/j.aquaculture.2018.06.004

Zhang, Q. Z., Tong, X. H., Sui, X. N., Wang, Z. J., Qi, B. K., Li, Y., & Jiang, L. Z. (2018). Antioxidant activity and protective effects of Alcalase-hydrolyzed soybean hydrolysate in human intestinal epithelial Caco-2 cells. *Food Research International*, *111*, 256–264. https://doi.org/10.1016/j.foodres.2018.05.046

Zhang, X. D., Zhu, Y. F., Cai, L. S., & Wu, T. X. (2008). Effects of fasting on the meat quality and antioxidant defenses of market-size farmed large yellow croaker (*Pseudo-sciaena crocea*). *Aquaculture*, *280*, 136–139. https://doi.org/10.1016/j.aquaculture.2008.05.010

Zhao, J., Feng, L., Liu, Y., Jiang, W. D., Wu, P., Jiang, J., … Zhou, X. Q. (2014). Effect of dietary isoleucine on the immunity, antioxidant status, tight junctions and microflora in the intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Fish & Shellfish Immunology*, *41*, 663–673.

How to cite this article: Xiao W, Jiang W, Feng L, et al. Effect of dietary enzyme-treated soy protein on the immunity and antioxidant status in the intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquac Res*. 2019;50:1411–1421. https://doi.org/10.1111/are.14016

APPENDIX

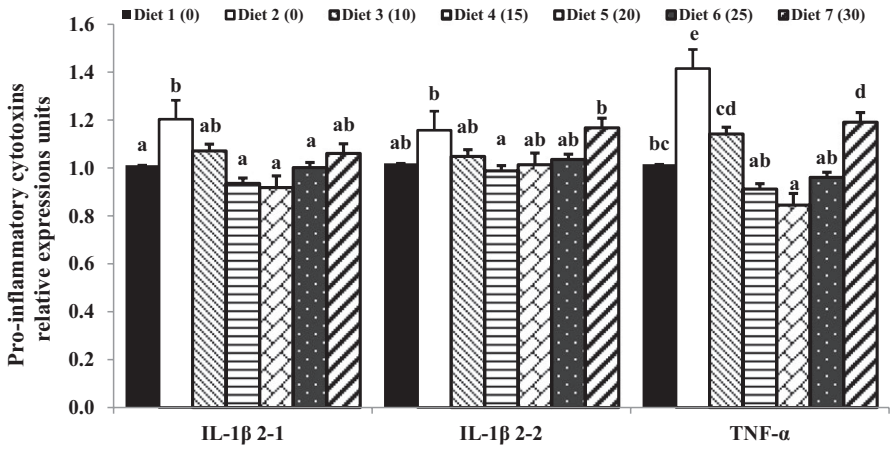

FIGURE A1 Relative expressions of pro-inflammatory cytokines in the intestine of fish fed diets with graded levels of enzyme-treated soy protein (values are mean ± *SD* of four replicates, with five fish in each replicate). IL-1: Interleukin 1; TNF-α: Tumour necrosis factor α

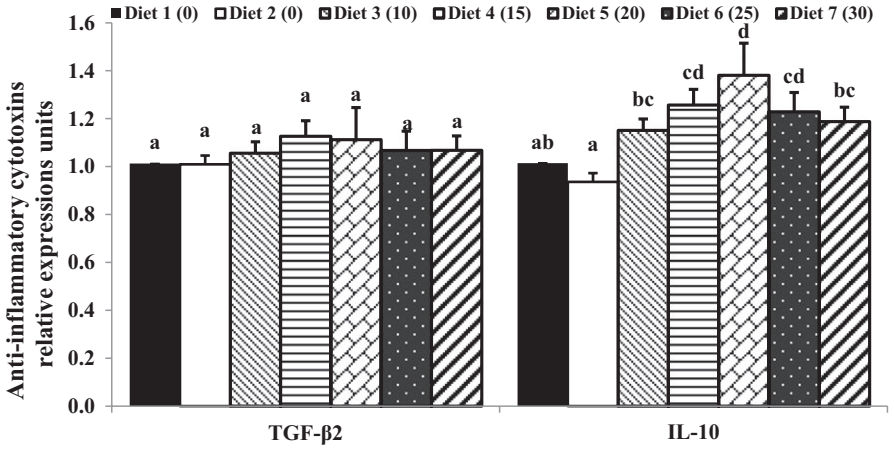

FIGURE A2 Relative expressions of anti-inflammatory cytokine in the intestine of fish fed diets with graded levels of enzyme-treated soy protein (values are mean ± *SD* of four replicates, with five fish in each replicate). TGF-β: Transforming growth factor beta; IL-10: Interleukin 10

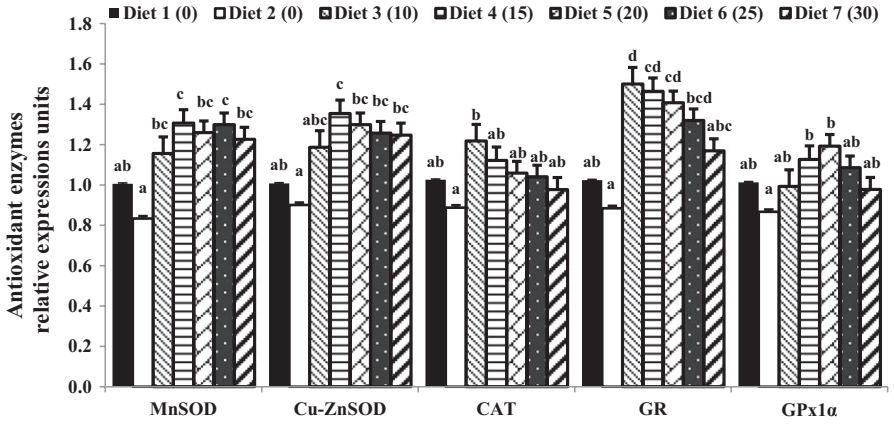

FIGURE A3 Relative expressions of antioxidant enzymes in the intestine of fish fed diets with graded levels of enzyme-treated soy protein (values are mean ± *SD* of four replicates, with five fish in each replicate). SOD: Superoxide dismutase; CAT: Catalase; GR: Glutathione reductase; GPx: Glutathione peroxidase

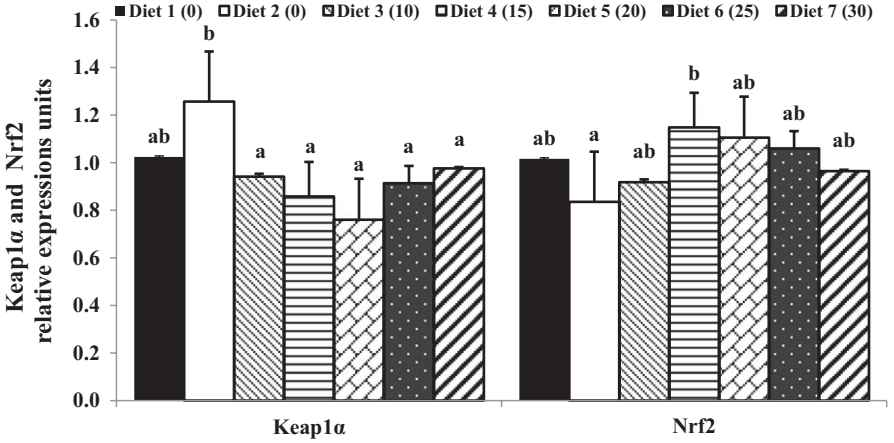

FIGURE A4 Relative expressions of Nrf2 and Keap1a in the intestine of fish fed diets with graded levels of enzyme-treated soy protein (values are mean ± *SD* of four replicates, with five fish in each replicate). Keap1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2-related factor 2