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Dietary citric acid supplementation alleviates soybean meal-induced intestinal oxidative damage and micro-ecological imbalance in juvenile turbot, *Scophthalmus maximus* L

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Abstract

A 12-week feeding trial was conducted to investigate the potential protective effects of citric acid against soybean meal-induced intestinal oxidative damage and micro-ecological imbalance in juvenile turbot (*S. maximus* L.). Four isonitrogenous and isolipidic experimental diets, that is fish meal-based diet (FM), FM with 40% fish meal protein replaced with soybean meal protein diet (SBM), supplemented with 1.5% citric acid (1.5% CA) or 3% citric acid (3% CA). Results showed that both citric acid diets significantly enhanced the total antioxidative capacity and the gene expression of superoxide dismutase, glutathione peroxidase and heme oxygenase 1, while also decreasing the malondialdehyde content in the distal intestine. Compared to diet SBM, the genes expression of p53, protein kinase C and Caspase-3 were remarkably declined by dietary citric acid supplementation, while the genes expression of proliferating cell nuclear antigen and mucins showed an opposite trend. The structural integrity of the distal intestine in fish fed citric acid was showed in the histological results. Sequencing of bacterial 16s rRNA V4 region showed that the profile of intestinal bacteria was altered by dietary citric acid supplementation, which was supported by the diet-cluster of PCoA and phylogenetic tree. MetaStat analysis indicated that dietary citric acid dramatically reduced the relative abundance of the *Vibrio* genus. In conclusion, dietary citric acid mitigated soybean meal-induced intestinal oxidative damage, beneficially alleviated the micro-ecological imbalance and specifically reduced the relative abundance of the *Vibrio* genus in the distal intestine of juvenile turbot.

KEYWORDS
citric acid, intestinal barrier function, oxidative stress, soybean meal, turbot

1 | INTRODUCTION

Soybean meal is a potential alternative protein source used in aquafeed; however, in some fish such as Atlantic salmon, common dentex, and turbot soy protein could inhibit fish growth in a dose-dependent manner through negatively influencing the intestinal health (Antonopoulou, Chouri, Feidantsis, Lazou, & Chatzifotis, 2017; Bakke-Mckellep et al., 2007; Chen et al., 2018; Reveco, Øverland, Romarheim, & Mydland, 2014). Oxidative stress was proposed as one of the reasons for intestinal barrier dysfunction induced by soybean meal (Jiang et al., 2017; Zhang et al., 2016). During continuous

enteral stress, increased production in reactive oxygen species (ROS) can cause intestinal epithelium apoptosis and the reduction in mucin synthesis (Shao & Nadel, 2005; Yan et al., 2008). The reduction in mucin synthesis exposes the intestinal tissue to intestinal content, which may lead to worsened inflammation (Matés, Segura, Alonso, & Márquez, 2008). Regarding the regulation of redox status on the cell proliferation and cell apoptosis, oxidative stress potentially trigger the p53 protein, which is a transcription factor and could induce a number of different responses including the induction of cell cycle arrest and cell death (Gatz & Wiesmüller, 2006; Horn & Vousden, 2007; Li et al., 2012). In addition, protein kinase C (PKC) could mediate influence of oxidative stress on epithelial mucus secretion, probably through triggering PI3K and MAPK pathways (El Homsi et al., 2007; Lee, Crawley, Hokari, Kwon, & Kim, 2010). The intestinal microbiota serves as a primary stimulus for shaping and modulating intestinal function (Ringø et al., 2016; Zhou, Ringø, Olsen, & Song, 2017). However, the antigenic nature of indigenous bacteria and the sensitive nature of host cells contribute to a precarious relation under stress conditions, which could alter intestinal function and disease susceptibility (Collins & Bercik, 2009).

Several recent studies indicated that organic acids could alleviate oxidative stress status (Abdel-Salam et al., 2014; Ebrahimi et al., 2017; Li, Cui, & Leng, 2015; Romano et al., 2016; Zhang et al., 2016; Zhu, Qiu, Ding, Duan, & Wang, 2014). Besides, organic acids was also reported to beneficially modulate the intestinal microbiota and possess antimicrobial effects against several food-borne pathogens, such as *Escherichia coli*, *Enterobacteria*, *Salmonella* and *Vibrio* (Bosi et al., 1999; Canibe, Højberg, Højsgaard, & Jensen, 2005; Creus, Pérez, Peralta, Baucells, & Mateu, 2007; Partanen & Mroz, 1999; Silva, Vieira, Mouriño, Bolivar, & Seiffert, 2016; Zhou et al., 2009). As a weak organic tricarboxylic acid, citric acid is widely used in the food and drug industry because of its buffering effects, anticoagulant effects and antioxidant capacities. However, limited information has been available about the use of citric acid as an antioxidant in fish. A study with large yellow croaker (*Larimichthys crocea*) showed that citric acid inhibited the intestinal oxidative stress induced by high levels of dietary soybean meal (Zhang et al., 2016). Another study on red hybrid tilapia (*Oreochromis* sp.) suggested that the content of lipid peroxidation in muscle was significantly lowered by dietary sodium citrate treatments (Romano et al., 2016). Our previous studies indicated that dietary citric acid supplementation markedly improved the bioavailability of phosphorus, without compromising intestinal function and health of turbot (Dai et al., 2018). Nevertheless, there was no information reported about the effects of dietary citric acid on gut microbiota of fish under inflammatory stress.

The present study was aimed at evaluating the mitigative effects of dietary citric acid on the susceptibility of intestine to soybean meal in turbot, a typical carnivorous marine fish wildly cultured in the world. Results of the present study would gain novel insight into understanding the role of citric acid in mitigating the adverse effects of plant protein sources on fish gut health.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Procedures for animal care and handling was conducted in strict compliance with relevant provisions regulating the experimentation with live animals, as overseen by the Institutional Animal Care and Use Committee of the Ocean University of China.

2.2 | Diets and feeding management

As shown in Table 1, four isonitrogenous and isolipidic experimental diets were formulated to contain approximately 50% crude protein and 12% crude lipid. A fish meal-based diet (FM) was used as the positive control diet. Diet FM with 40% fish meal protein replaced by soybean meal protein was used as the negative control diet (SBM). Two citric acid-supplemented diets were formulated by incorporating 1.5% (1.5% CA) and 3.0% citric acid (3% CA) into the SBM diet at the expense of α-starch respectively. Yttrium oxide (Y_2O_3, 1 g/kg) was used as an inert tracer in each diet for determining apparent digestibility coefficient (ADC) of dry matter. Diets were extruded with an experimental single-screw feed mill in the form of 3 mm diameter pellets and dried for about 12 hr in a ventilated oven at 50°C. All the experimental diets were stored at −20°C until use.

Disease-free juvenile turbot were purchased from a local farm in Weihai, Shandong Province, China. Prior to the start of feeding trial, fish were fed in fiberglass tanks (1,500 L, 150 fish per tank) and fed the FM diet to apparent satiation twice daily (7:00 a.m. and 7:00 p.m.) for 2 weeks to acclimate the experimental conditions. Then the fish were fasted for 24 hr and weighed. A total of 360 fish (9.57 ± 0.02 g) were randomly assigned to 12 fiberglass tanks (300 L, 30 fish per tank) connected to an indoor flow-through seawater system. The system was supplied with continuous aeration and flow-through seawater at the rate of 4 L/min. Each diet was randomly assigned to three tanks. The fish were fed to apparent satiation twice daily (7:00 a.m. and 7:00 p.m.) for 12 weeks. After feeding, uneaten feeds were collected from the tank outlets 15 min later, then dried and weighed. During the feeding period, water temperature ranged from 15°C to 18°C; pH 7.5–8.0; salinity 30–33; ammonia nitrogen lower than 0.4 mg/L; nitrite lower than 0.1 mg/L; and dissolved oxygen higher than 7.0 mg/L.

2.3 | Sample collection

Prior to the end of feeding trial, faeces were collected 7–8 hr after feeding with a siphon and stored at −20°C pending analyses. At day 84, 6 hr after the last feeding, fish were anaesthetized with eugenol (1:10,000) (purity 99%; Shanghai Reagent Corp, Shanghai, China), and then counted and weighed. Samples were taken only from fish with the intestinal tract filled with digesta, to ensure the intestine exposure to the diets before sampling. The distal intestine is the

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

Diet	FM	SBM	1.5% CA	3% CA
Fish meal[a]	68.00	40.80	40.80	40.80
Soybean meal[a]	0.00	37.90	37.90	37.90
α-Starch[a]	16.00	11.55	10.05	8.55
Fish oil[a]	4.80	6.70	6.70	6.70
Soybean lecithin[a]	0.50	0.50	0.50	0.50
Vitamin premix[b]	1.00	1.00	1.00	1.00
Mineral premix[b]	0.50	0.50	0.50	0.50
Choline chloride[c]	0.30	0.30	0.30	0.30
$Ca(H_2PO_4)_2{\cdot}H_2O$[c]	0.50	0.50	0.50	0.50
Ethoxyquin[c]	0.05	0.05	0.05	0.05
Y_2O_3[c]	0.10	0.10	0.10	0.10
Calcium propionate[c]	0.10	0.10	0.10	0.10
Microcrystalline cellulose[b]	8.15	0.00	0.00	0.00
Citric acid[d]	0.00	0.00	1.50	3.00
Total	100.00	100.00	100.00	100.00
Proximate composition (% dry matter)				
Moisture	2.93	2.89	2.80	3.13
Crude protein	51.93	50.15	50.64	51.06
Crude lipid	9.52	10.29	9.64	10.46
Ash	12.52	11.12	11.25	11.19

Note. CA: citric acid; FM: fish meal-based diet; SBM: soybean meal protein diet.

[a]Fish meal: Peru anchovy fishmeal containing 74.04% crude protein and 9.97% crude lipid; soybean meal: 53.12% crude protein and 2.12% crude lipid; α-starch: prepared from corn starch, purity >95%; soybean lecithin: purity >99%; microcrystalline cellulose: extracted from bagasse, purity >99%. All these ingredients were purchased from Qingdao Seven Great Bio-tech Company Limited (Qingdao, China).

[b]Vitamin premix: purchased from Qingdao Master Bio-tech Company Limited (Qingdao, China), microcrystalline cellulose, 16.473; V_A, 0.032; V_{B1}, 0.025; V_{B2}, 0.045; V_{B6}, 0.02; V_{B12}, 0.01; V_D, 0.035; V_E, 0.24; V_K, 0.01; calcium pantothenate, 0.06; nicotinic acid, 0.2; folic acid, 0.02; biotin, 0.06; inositol, 0.8; V_C phosphate, 2 (g/kg diet); Mineral premix: purchased from Qingdao Master Bio-tech Company Limited, $FeSO_4{\cdot}H_2O$, 0.08; $ZnSO_4{\cdot}H_2O$, 0.05; $CuSO_4{\cdot}5H_2O$, 0.01; $MnSO_4{\cdot}H_2O$, 0.045; KI, 0.06; $CoCl_2{\cdot}6H_2O$ (1%), 0.05; Na_2SeO_3 (1%), 0.02; $MgSO_4{\cdot}7H_2O$, 1.2; calcium propionate, 1000; zeolite, 8.485 (g/kg diet).

[c]Choline chloride: purity >98%; $Ca(H_2PO_4)_2{\cdot}H_2O$: purity >92%; Ethoxyquin: purity >90%; Y_2O_3: purity >99.99%; calcium propionate: purity >98%. All these ingredients were purchased from Sinopharm Chemical Leagent Company Limited (Shanghai, China).

[d]Citric acid: purchased from Sigma–Aldrich Company (USA), purity >99%.

posterior half of the whole intestine as Li, Hu, et al. (2017) described. For the analysis of gene expression, the middle part of the distal intestine (about 0.5 cm in length) from other six randomly selected fish per tank was collected after removing the intestinal content carefully, and then transferred to 1.5 ml sterile tubes (Axygen, USA). Distal intestinal tissue samples taken from two randomly selected fish per tank were fixed at Bouin's fixative solution (Saturated water solution of picric acid: 40% formaldehyde solution: glacial acetic acid = 15:5:1) for histological evaluation. Two distal intestine tissue samples per tank were collected for analysis of enzyme activities. For the analysis of intestinal microbiota, all the procedures were conducted as described by Yang et al. (2018). Briefly, the surface of one randomly selected fish per tank (three fish per treatment) was sterilized by tampon with 70% alcohol. Then, the whole intestinal mucosa layer of these three fish per treatment was carefully scraped from foregut region to hindgut region using sterile rubber spatula, and then transferred to 2 ml sterile tubes (Axygen). The whole sampling procedure was carried under sterile conditions. All samples mentioned above were immediately stored in liquid nitrogen before further processing.

2.4 | Chemical analysis of diets and faeces

Proximate composition of feed ingredients and diets were analysed according to standard methods (AOAC, 1995). Moisture content was determined gravimetrically to constant weight in an oven at 105°C; Crude lipid was determined with ether extraction using a Soxhlet extractor (Extraction SystemB-811; BUCHI, Switzerland); Crude protein was determined with Kjeldahl method (Kjeltec System 2300; FOSS, Sweden); Ash by combustion at 550°C. The concentrations of Yttrium oxide and phosphorus in the diets and faeces were measured with ICP-AES (VISTA-MPX; Varian, USA) after perchloric acid digestion (Cheng et al., 2010).

2.5 | Enzyme activities

The intestinal samples were homogenized in ten volumes (w/v) of ice-cold physiological saline solution and centrifuged at 6,000 *g* for 20 min at 4°C. Enzyme activity was then determined with supernatants. Trypsin activity was determined according to Hummel (1959). Trypsin can catalyze the hydrolysis of arginine ethyl ester in substrate and increase its absorbance value at 253 nm. The enzyme activity can be calculated according to the change of absorbance. Amylase and lipase were determined as described by Furné et al. (2005). For the amylase enzymatic reaction, starch was used as the substrate, and the activity unit of amylase was defined as the quantity of enzyme that produced 1 mmol of maltose ml^{-1} min^{-1}. One unit of lipase activity was defined as the hydrolysis of 1.0 microequivalent of fatty acids from triacylglycerols in 1 hr at pH 7.7 and 37°C. Total antioxidative capacity (T-AOC) in intestine was determined by colorimetric method as described by Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993 using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). The 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was oxidized to green $ABTS^+$ (Green) under the action of oxidants, while the production of $ABTS^+$ was inhibited in the presence of antioxidants. The absorbance of $ABTS^+$ was determined at 734 nm to calculate the T-AOC of the samples. The content of intestinal malondialdehyde (MDA) was measured spectrophotometrically as described by (Janero, 1990). Briefly, MDA can react with thiobarbituric acid (TBA) in high temperature and acidic conditions, forming MDA-TBA adduct (Red) and increase its absorbance value at 535 nm.

2.6 | Intestinal histology

The distal intestine samples were fixed with Bouin's solution. After fixation for 24 hr, the samples were transferred to 70% ethanol and embedded in paraffin after dehydration. Sections of approximately 5 μm were cut and stained with haematoxylin and eosin (H&E). The slides were examined under a light microscope (DP72; Olympus, Tokyo, Japan) equipped with a camera (E600; Nikon, Tokyo, Japan) and CellSens Standard Software (Olympus) for image acquisition. An image analysis software, Image Pro Plus® (Media Cybernetics, Silver Spring, MD, USA) was used to analyse the micrographs from light microscopy. The perimeter ratio (PR) of the internal perimeter (IP) of the intestine lumen to the external perimeter (EP) of the intestine (PR = IP/EP) were determined as described by Dimitroglou et al. (2009).

2.7 | RNA extraction and qPCR

The total RNA of the distal intestine was isolated using an RNAiso Plus (9109; Takara Biotech, Dalian, China). The integrity of RNA was detected by electrophoresis on 1.2% denaturing agarose gel and then the concentration was detected with Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, RNA was reversely transcribed to cDNA with PrimeScript RT reagent Kit with gDNA Eraser (RR047A; Takara Biotech). Specific primers for target genes and housekeeping genes (Table 2) were synthesized by Sangon (Shanghai, China), and the application efficiency was then assessed. All of the real-time PCR analyses were performed using a quantitative thermal cycler (Mastercyclerep realplex, Eppendorf, Germany) with EvaGreen Express 2× qPCR MasterMix (MasterMix-ES; Applied Biological Materials, Canada). The mRNA levels of these genes were normalized to the mRNA level of turbot RPSD and GAPDH, which were used as housekeeping gene. The gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), and the relative expression level of gene in the FM was used as a calibrator.

2.8 | Bacterial DNA extraction and pyrosequencing

Based on the growth performance, 1.5% CA was selected as the representative of citric acid-supplemented group. Total bacterial DNA of intestinal mucosa layer from groups FM, SBM and 1.5% CA was extracted using a QIAamp DNA Stool minikit (Qiagen, Hilden, Germany) according to the modified instruction by Li, Hu, et al. (2017). The integrity of extracted genomic DNA was detected by electrophoresis on 1.2% denatured agarose gel and then assessed by a Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific) to test the concentration. Sequencing was performed at the Novogene Bioinformatics Technology Co., Ltd. Briefly, 16S rRNA was amplified used the V4 region primer set (515F/806R) with the barcode. PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Beijing, China) with the following protocols: 94°C for 2 min (1 cycle), 94°C for 15 s/60°C (with 1°C decremental steps in every cycle) for 30 s/72°C for 30 s (10 cycles), 94°C for 15 s (1 cycle), 94°C for 15 s/51°C for 30 s/72°C for 30 s (30 cycles), and a last step of 72°C for 10 min. PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The library was sequenced on an Illumina HiSeq platform according to the manufacturer's instructions provided by Beijing Novogene Genomics Technology Co. Ltd. (China). Complete data were submitted to the Genome Sequence Archive in BIG Data Center (https://bigd.big.ac.cn/gsa) under accession number CRA000703.

2.9 | Calculations and statistical analysis

The following variables were calculated:

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

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

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

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

$$\text{Apparent digestibility coefficients of dry matter}(\text{ADC}, \%) = 100 \times [1 - (\%\,Y_2O_3\text{in diet})/(\%\,Y_2O_3\text{in feces})]$$

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

Data were firstly subjected to homogeneity test of variance before one-way analysis of variance (ANOVA) using SPSS 22.0 for windows. Tukey's test was used to compare the means among individual treatments. Differences were regarded as significance when $p < 0.05$ and the results are presented as means ± *SEM*.

2.10 | Bioinformatics and statistical analysis

Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence, and then merged using FLASH software (V1.2.7) (Magoč & Salzberg, 2011). The high-quality clean tags were obtained according to the QIIME (V1.9.1) Quality Controlled Process (Caporaso et al., 2010). The total tags were finally obtained using UCHIME algorithm based on the reference database (Gold database) (Edgar, Haas, Clemente, Quince, & Knight, 2011), and then clustered to OTU (operational taxonomic unit) using Uparse (v7.0.1001) based on 97% sequence similarity (Edgar, 2013). Taxonomic assignment was performed using RDP Classifier (V2.2) based on the reference database (Greengenes database) (Desantis et al., 2006). Alpha diversity analysis including observed species and Chao1 for richness estimates and Shannon index for diversity estimates were calculated with QIIME (V1.9.1). Principal coordinate analysis (PCoA) calculated with QIIME (V1.9.1) was used to analyse all the OTUs based on weighted Unifrac distance, and analyse the diversity among samples. UPGMA Clustering, conducted by QIIME (V1.9.1), was performed as a type of

TABLE 2 Primers used for real-time PCR analysis

Target gene	Forward primer (5'–3')	Reverse primer (5'–3')	GenBank no.
SOD	AAACAATCTGCCAAACCTCTG	CAGGAGAACAGTAAAGCATGG	HS029499.1
GPx	CCCTGATGACTGACCCAAAG	GCACAAGGCTGAGGAGTTTC	HS032063.1
HOX-1	GGGCTTTCGTTCTTCTCCTT	AGACGACGTTGGATTGGTTC	FE949297.1
PCNA	CTCGTACCGCTGCGACAG	CAGAGGGCATCTTCACCAC	EU711051.1
Caspase-3	TTCTGCCATTGTCTCTGTGC	GCCCTGCAACATAAAGCAAC	JU391554.1
Caspase-7	TCTGCAATGTCCTCAACGAG	TTGCGACCATGTAGTTGACC	JU373310.1
p53	TCAGTATTTTGAAGACCAGCACACA	GTCATCTCGGAGCCCAACTG	EU711045.1
Mucin-2	GTTGGTGCAGCCGCATAG	CACTGGACGCTGGGAATG	KU238186.1
Mucin-18	TTGTCCCTGACCAAGTGATG	ACAAAGCCTGTCCAAGATCG	JU370277.1
PKC	ACATCGCCCCCGAAATACTC	GAGCTCCCAACGATGTCGAA	MG835613
GAPDH	CAGTGTATGAAGCCAGCAGAG	GGTCGTATTTGTCCTCATTAACTC	AY008305.1
RPSD	AACACAGGAAGCAGCAGAAC	ACGGCAGTGATGGTCTCTC	DQ848899.1

Note. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GPx: glutathione peroxidase; HOX-1: heme oxygenase 1; PCNA: proliferating cell nuclear antigen; PKC: protein kinase C; RPSD: RNA polymerase II subunit D; SOD: superoxide dismutase.

hierarchical clustering method to interpret the distance matrix using average linkage. Metastats analysis was performed to identify the bacterial taxa differentially represented between groups at genus or higher taxonomy levels (White, Nagarajan, & Pop, 2009).

3 | RESULTS

3.1 | Growth performance and feed utilization

The growth performance and feed utilization results are presented in Table 3. The survival of fish, ranging from 96.67% to 98.89%, showed no significant difference ($p > 0.05$) among dietary groups. Compared to group FM, reduced SGR, FE and ADC of dry matter and phosphorus were observed in the group SBM, while the FI showed a significant ($p < 0.05$) decline in the group SBM. No significant difference in FI was observed among groups SBM, 1.5% CA and 3% CA. However, compared to the group SBM, a significant ($p < 0.05$) increase in FE were observed in the group 1.5% CA, while the ADC of dry matter was significantly ($p < 0.05$) increased by both 1.5% CA and 3% CA.

3.2 | Intestinal digestive enzyme activities, oxidative stress and antioxidative responses

Compared to the group FM, high doses of dietary soybean meal significantly decreased ($p < 0.05$) the activities of all tested digestive enzymes (Trypsin, Lipase and Amylase) in distal intestine. Compared to the group SBM, citric acid-supplemented diets significantly ($p < 0.05$) increased the activities of these enzymes (Table 4).

Fish fed the SBM diet had significantly lower T-AOC ($p < 0.05$), but T-AOC was significantly ($p < 0.05$) higher in groups 1.5% CA and 3% CA. The MDA content was significantly increased ($p < 0.05$) by fish meal replacement by soybean protein, while the citric acid supplementation ($p < 0.05$) reduced the MDA content (Table 5).

3.3 | Intestinal histology

Compared to the group FM, widened intestinal folds, widened lamina propria within the intestinal folds and a profound infiltration of mixed leucocytes in the lamina propria were observed in the distal intestine of turbot fed SBM, while less morphological changes were observed in the distal intestine of turbot fed citric acid (Figure 1).

As shown in Figure S1, compared to the group FM, turbot fed SBM displayed significantly ($p < 0.05$) decreased PR of distal intestine; however, the PR of the distal intestine of turbot in citric acid-supplemented groups was significantly higher ($p < 0.05$) than that in the SBM group.

3.4 | Expression of intestinal antioxidant enzymes, cell proliferation, cell apoptosis and mucus synthesis related genes

The gene expression of intestinal antioxidant enzymes is presented in Figure 2. Replacement of fish meal by soybean meal reduced ($p < 0.05$) the gene expression of the SOD, GPx and HOX-1, while the gene expression of these antioxidant enzymes was increased significantly ($p < 0.05$) by dietary inclusion of citric acid.

As shown in Figure 3, compared with the group FM, high doses of dietary soybean meal significantly down-regulated ($p < 0.05$) the gene expression of mucin-2 and mucin-18, and up-regulated ($p < 0.05$) the gene expression of PKC in the distal intestine. Meanwhile, the change in gene expression of mucin-2, mucin-18 and PKC showed opposite trends.

The gene expression of PCNA was remarkably ($p < 0.05$) declined, while the gene expression of Caspase-3, Caspase-7 and p53 was significantly increased ($p < 0.05$) by SBM compared to FM. Compared to SBM, the gene expression of PCNA was significantly elevated ($p < 0.05$) by dietary citric acid supplementation. Furthermore, diets with citric acid supplementation significantly reduced the gene expression of Caspase-3 and p53 (Figure 4).

TABLE 3 Effects of dietary citric acid on the survival, growth and feed utilization of juvenile turbot fed high doses of dietary soybean meal (means ± *SEM*, $n = 3$)[a]

Diet	FM	SBM	1.5% CA	3% CA
Survival rate (SR, %)	98.89 ± 1.11	96.67 ± 1.92	97.78 ± 1.11	96.67 ± 3.34
Initial weight (W_0, g)	9.64 ± 0.01	9.52 ± 0.02	9.57 ± 0.04	9.54 ± 0.07
Final weight (W_t, g)	62.21 ± 1.66[a]	48.69 ± 1.40[b]	55.25 ± 1.42[ab]	50.48 ± 1.72[b]
Specific growth rate (SGR, % day^{-1})	2.22 ± 0.03[a]	1.94 ± 0.04[b]	2.09 ± 0.03[ab]	1.98 ± 0.05[b]
Feed intake (FI, % day^{-1})	1.48 ± 0.02[b]	1.67 ± 0.01[a]	1.61 ± 0.02[a]	1.63 ± 0.05[a]
Feed efficiency (FE)	1.17 ± 0.01[a]	0.96 ± 0.01[c]	1.04 ± 0.02[b]	1.00 ± 0.05[bc]
ADC of dry matter (%)	62.77 ± 0.42[a]	49.03 ± 0.64[c]	56.06 ± 0.39[b]	55.30 ± 0.52[b]

Note. ADC: apparent digestibility coefficients; CA: citric acid; FM: fish meal-based diet; SBM: soybean meal protein diet.
[a]Mean values in the same row with different superscript letters are significantly different ($p < 0.05$).

TABLE 4 Effects of dietary citric acid on activities of intestinal digestive enzymes of juvenile turbot fed high doses of dietary soybean meal (means ± *SEM*, $n = 3$)[a]

Diet	FM	SBM	1.5% CA	3% CA
Trypsin (U/mg protein)	1,788.03 ± 50.69[ab]	1,050.79 ± 19.73[c]	1,504.62 ± 109.23[b]	1,883.37 ± 93.01[a]
Lipase (U/mg protein)	134.11 ± 18.19[a]	45.73 ± 5.74[b]	128.84 ± 18.66[a]	144.61 ± 19.70[a]
Amylase (U/mg protein)	0.18 ± 0.01[a]	0.12 ± 0.01[b]	0.19 ± 0.02[a]	0.15 ± 0.01[a]

Note. CA: citric acid; FM: fish meal-based diet; SBM: soybean meal protein diet.
[a]Mean values in the same row with different superscript letters are significantly different ($p < 0.05$).

TABLE 5 Effects of dietary citric acid on intestinal total antioxidant capacity (T-AOC) and malondialdehyde (MDA) content of juvenile turbot fed high doses of dietary soybean meal (means ± *SEM*, $n = 3$)[a]

Diet	FM	SBM	1.5% CA	3% CA
T-AOC (U/mg protein)	5.30 ± 0.19[a]	3.58 ± 0.11[b]	4.69 ± 0.31[a]	5.20 ± 0.40[a]
MDA (nmol/mg protein)	1.00 ± 0.08[a]	1.70 ± 0.09[b]	0.83 ± 0.07[a]	0.92 ± 0.06[a]

Note. CA: citric acid; FM: fish meal-based diet; SBM: soybean meal protein diet.
[a]Mean values in the same row with different superscript letters are significantly different ($p < 0.05$).

3.5 | Intestinal microbiota

A total number of 585,144 raw reads were generated after assembled, quality screened and trimmed, resulting in identification of 5,395 OTUs under 97% sequence similarity. Rarefaction curves showed that all samples reached the saturation phase, indicating adequate sequencing depth (Figure S2).

Three alpha diversity measures were calculated including observed species, Chao1 and Shannon's diversity index (Figure S3). No significant difference in alpha diversity was observed among all treatments ($p > 0.05$). Figure S4 shows differentially abundant taxa in pairwise comparison of different treatments. The PCoA based on Weighted UniFrac distances and group UPGMA-clustering tree based on Weighted Unifrac distance were used to compare the similarity in the microbial community. The PCoA results showed a clear separation of microbial samples from different experimental groups, indicating dietary citric acid had a strong effect on the overall structure of intestinal microbiota of turbot (Figure 5a). The result of group UPGMA-clustering tree showed that FM showed a higher level of similarity with CA than with SBM (Figure 5b).

At phylum level, the bacterial composition of the FM group was dominated by Proteobacteria and Firmicutes (Figure 6). However, the relative abundance of Bacteroidetes was significantly increased ($p < 0.05$) by dietary soybean meal, and became the most abundant phyla. By contrast with SBM group, dietary citric acid significantly reduced ($p < 0.05$) the relative abundance of Bacteroidetes, maintaining the domination of Proteobacteria and Firmicutes. As shown in Figure 7, the difference of *Vibrio* level between different groups was analysed by MetaStat analysis. Turbot in CA group showed a significantly higher ($p < 0.05$) relative abundance of *Vibrio* than those in FM and SBM.

4 | DISCUSSION

In accordance with previous studies on Atlantic salmon (*Salmo salar* L.) (Krogdahl, Bakke-Mckellep, & Baeverfjord, 2015) and turbot (Gu, Bai, Zhang, & Krogdahl, 2016; Peng et al., 2013), typical histomorphological changes induced by high doses of dietary soybean meal was shown in the experimental turbot of SBM group, including widened intestinal folds, widened lamina propria within the intestinal folds, and a profound infiltration of mixed leucocytes in the lamina propria. However, supplemented citric acid in the soybean meal diet mitigated the intestinal impairment, leading to narrowed

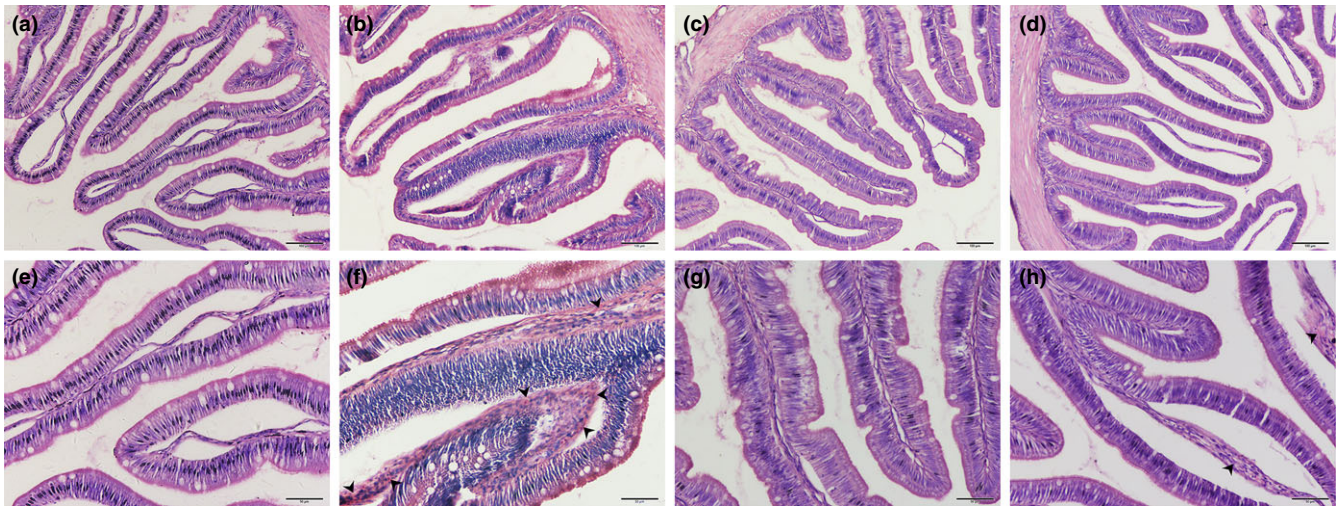

FIGURE 1 Representative histological sections of distal intestine from turbot fed diet FM (a, e), SBM (b, f), 1.5% CA (c, g) and 3% CA (d, h). Scale bar = 100 μm for a–d; scale bar = 50 μm for e–h. Black arrows showed the profound infiltration of mixed leucocytes in the lamina propria. Staining: H&E [Colour figure can be viewed at wileyonlinelibrary.com]

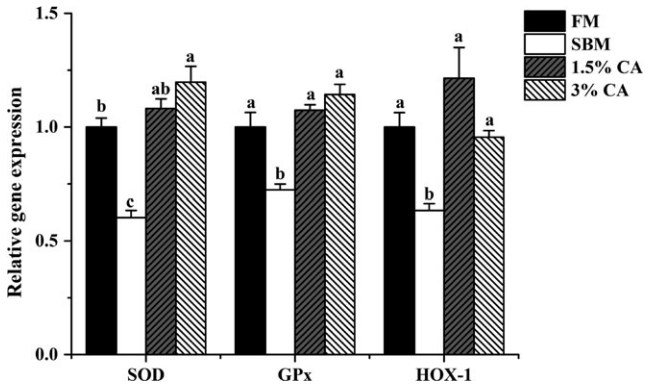

FIGURE 2 Relative mRNA expression of SOD, GPx and HOX-1 in the distal intestine. Error bars of columns denote standard error of means (n = 3) and columns with different letters above are significantly different ($p < 0.05$)

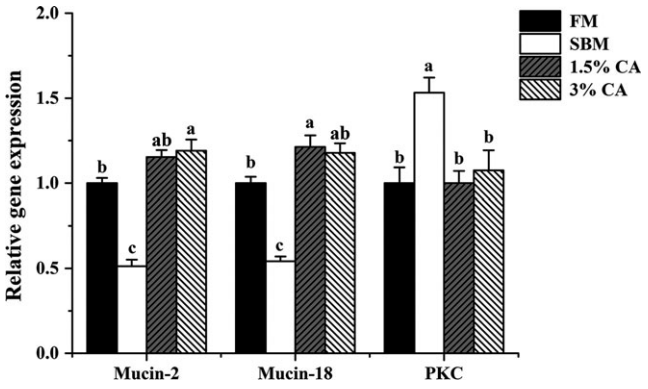

FIGURE 4 Relative mRNA expression of Mucin-2, Mucin-18 and PKC in the distal intestine. Error bars of columns denote standard error of means (n = 3) and columns with different letters above are significantly different ($p < 0.05$)

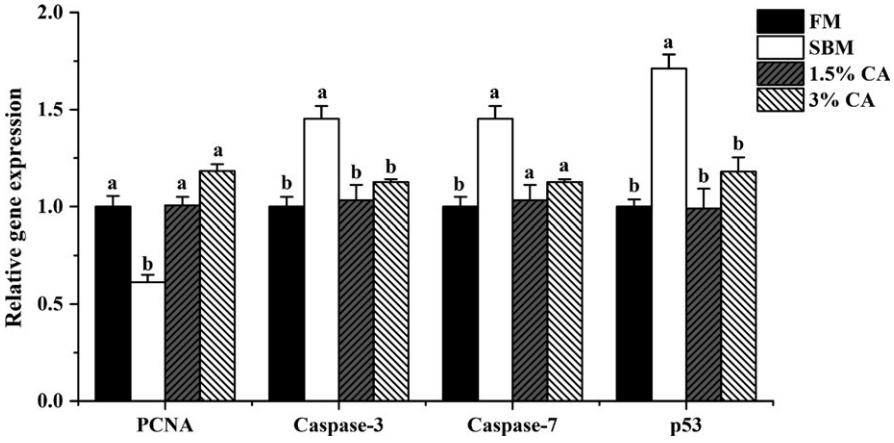

FIGURE 3 Relative mRNA expression of PCNA, Caspase-3, Caspase-7 and p53 in the distal intestine. Error bars of columns denote standard error of means (n = 3) and columns with different letters above are significantly different ($p < 0.05$)

intestinal folds and a superficial infiltration of mixed leukocytes in the lamina propria. Similar results were also observed in the study on broiler chicken showing that dietary citric acid supplementation at 3% exhibited greater impact on morphology of all segments of small intestine (Nourmohammadi & Afzali, 2013). Besides, high inclusions of dietary soybean meal led to significant digestion

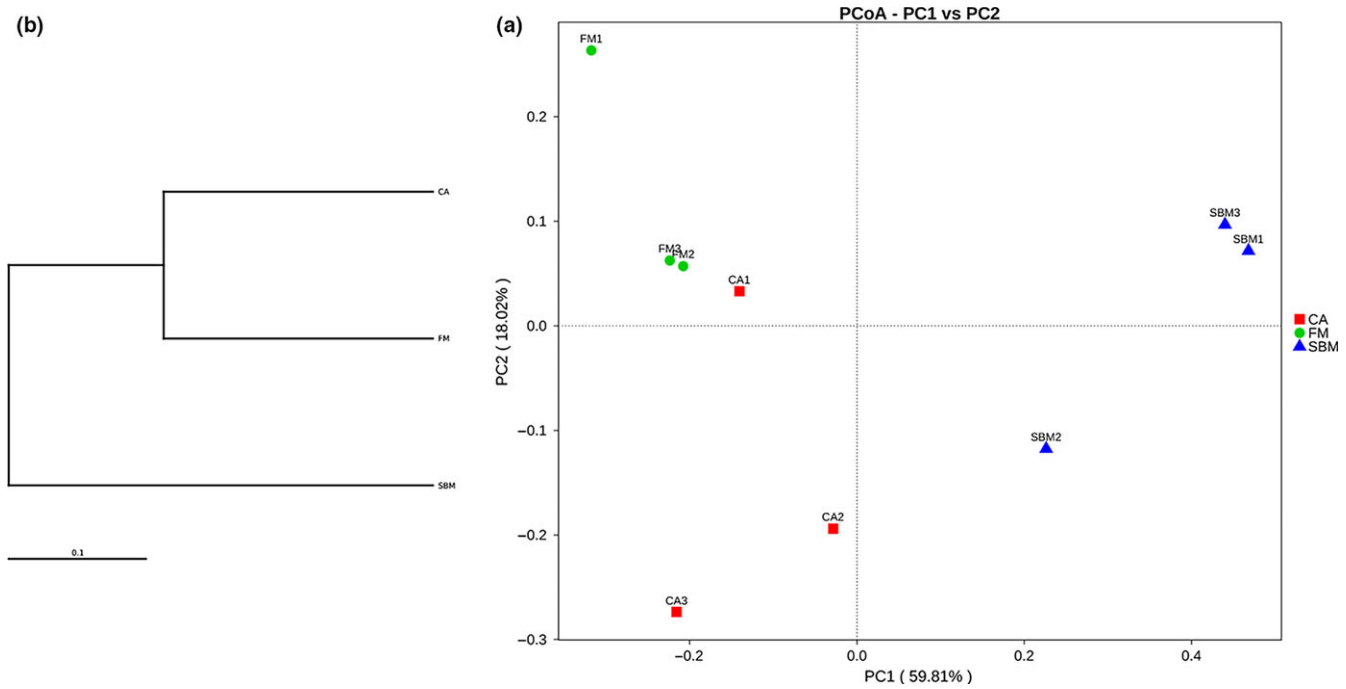

FIGURE 5 Beta diversity of intestinal microbiota of juvenile turbot. Principal coordinate analysis (PCoA) of dissimilarity between bacterial communities based on Weighted UniFrac distances (a). Samples UPGMA-clustering tree based on Weighted Unifrac distance (b) [Colour figure can be viewed at wileyonlinelibrary.com]

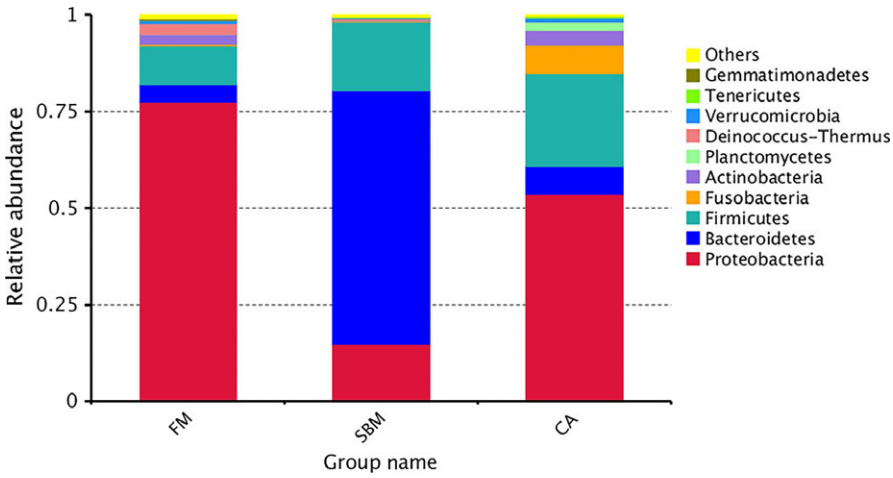

FIGURE 6 Taxonomy classification of reads at phylum taxonomic level. Only top 10 most abundant (based on relative abundance) bacterial phyla are shown in the figures. Other phyla was all assigned as "Others" [Colour figure can be viewed at wileyonlinelibrary.com]

inhibitory effects in terms of suppression on digestive enzymes activities, ADC value of dry matter, and FE, which was in accordance with the study on juvenile Amur Sturgeon (*Acipenser schrenckii*) (Xu, Wang, Zhao, & Luo, 2012). The higher feed intake recorded in the SBM group could be a compensation for the lower digestibility. When the feed efficiency ratio was worse, the fish need to consume more feed to maintain the normal energy supply and growth. Similar and more prominent results were also observed in other studies by Refstie, Førde-Skjærvik, Rosenlund, and Rørvik (2006) and Li, Hu, et al. (2017). Dietary citric acid supplementation significantly enhanced the intestinal digestion ability and feed efficiency in this study, which was in accordance with the previous studies on juvenile red drum (*Sciaenops ocellatus*), tilapia (*Oreochromis niloticus* × *Oreochromis aureus*), and red sea bream (*Pagrus major*) showing that dietary citric acid significantly increased intestinal digestive enzyme activities and feed efficiency (Castillo, Rosales, Pohlenz, & Gatlin, 2014; Hossain, Pandey, & Satoh, 2007; Li, Li, & Wu, 2009).

In the present study, dietary citric acid also reduced intestinal oxidative damage in terms of reducing MDA content and increasing antioxidative capacity. Similar results were observed in the study on large yellow croaker-(*Larimichthys crocea*) fed high doses of dietary plant protein, which showed that dietary citric acid alleviated intestinal oxidative damage (lipid peroxidation and oxidative carbonyl

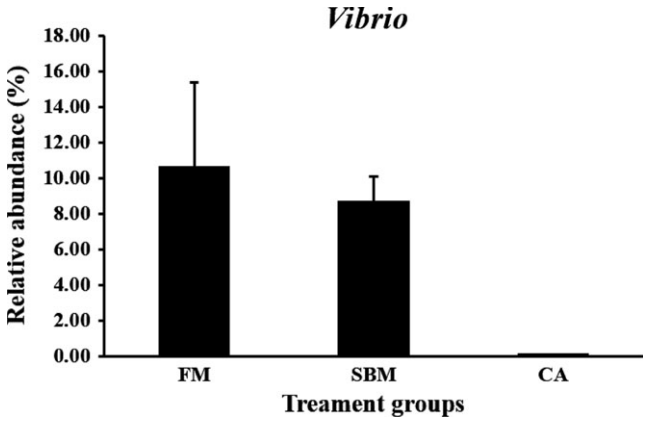

FIGURE 7 The relative abundance of *Vibrio* genus in the distal intestine from juvenile turbot fed the experimental diets

protein) through increasing intestinal T-AOC and activities of SOD and Cu-Zn SOD (Zhang et al., 2016). Another study on mice showed that ingestion of citric acid significantly attenuated LPS-induced lipid peroxidation (in terms of MDA) in the brain tissue through increasing glutathione peroxidase (GPx) and paraoxonase 1 (PON1) activities (Abdel-Salam et al., 2014). The increasing intestinal oxidative stress could cause the peroxidation of key biomolecules involved in various pathological processes including intestinal mucosal injury and digestive malfunctions (Ames, 1983; Halliwell, 1999; Jiang et al., 2016). Reducing intestinal oxidative damage seemed an important way by which citric acid protects fish intestinal health.

The mucus layer coating the intestinal tract is the front line of innate host defense (Kim & Ho, 2010), which is essential for the maintenance of intestinal mucosal homeostasis (Mcguckin, Eri, Simmsc, Florin, & Dphil, 2009; Moal & Servin, 2006). Prolonged oxidative stress causes the qualitative alteration in mucus layers due to both altered synthesis and secretion of mucins (Dharmani, Srivastava, Kissoonsingh, & Chadee, 2009; Kim & Ho, 2010; Liévinle & Servin, 2006; Moncada, Kammanadiminti, & Chadee, 2003). The present results showed that high inclusions of dietary soybean meal suppressed the gene expression of mucins (mucin-2 and mucin-18), but significantly enhanced gene expression of mucins was observed in the citric acid-supplemented groups. This highlighted the positive roles of dietary citric acid in maintaining the intact intestinal mucus layer. Previous studies on colonic epithelial cells (El Homsi et al., 2007) and human airway epithelial cells (Lee et al., 2010; Shao & Nadel, 2005) showed that the expression of secreted and membrane-associated mucins was modulated by targeting PKC pathways. In the present study, the gene expression of PKC was significantly reduced by citric acid supplementation, indicating the PKC signalling molecule may be involved in the maintenance of mucins synthesis by dietary citric acid.

Similar to previous studies on Atlantic salmon and common dentex (*Dentex dentex*) (Antonopoulou et al., 2017; Bakke-Mckellep et al., 2007), high inclusions of dietary soybean meal dramatically suppressed the epithelial cell proliferation and promoted epithelial cell apoptosis in the present study. Nevertheless, the fish in citric acid-supplemented groups showed changes in the opposite trend. The present results were in accordance with previous studies on hypoxia/reoxygenation-induced cardiomyocytes, which showed that citric acid significantly down-regulated the gene expression of cleaved Caspase-3 and decreased cell apoptotic rate (Tang et al., 2013). Abdel-Salam et al. (2014) also provided evidence that citric acid significantly decreased the hepatic Caspase-3 immunoreactivity in lipopolysaccharide-treated mice. Involved in removal of intracellular ROS and repair of oxidative damage, p53 was reported to inhibit the cell proliferation and drive a temporary cell cycle arrest (Bensaad et al., 2006; Budanov, Sablina, Feinstein, Koonin, & Chumakov, 2004; Gatz & Wiesmüller, 2006; Sablina et al., 2005; Yoon, Nakamura, & Arakawa, 2004). On the other hand, when the stress-induced damage is too severe for the cell to recover, p53 initiates programmed cell death (Horn & Vousden, 2007; Oda et al., 2000). Consistent with the previous studies showing that soybean meal induced higher expression of p53 protein in mid jejunum mucosa of young rats (Godlewski, Slazak, Zabielski, Piastowska, & Gralak, 2006), the gene expression of p53 was also significantly elevated in the group SBM in the present study. Considering the suppressed gene expression of p53 in citric acid-supplemented groups, the present study suggested that citric acid might mediate intestinal epithelial cell apoptosis and proliferation by inhibiting the gene expression of p53, which may consequently alleviate the intestinal oxidative damage induced by soybean meal. Moreover, increased mitotic activity and decreased apoptosis in the distal intestine by dietary citric acid supplementation may account for the reversal of intestinal absorptive surface (PR).

Regarding the intestinal microbiota, the present results showed that intestinal bacterial community of turbot fed the FM diet was overly dominated by Proteobacteria (77.41%) and Firmicutes (10.17%) at the phylum level, which was consistent with previous results on turbot intestinal microbiome (Li, Yang, et al., 2017; Xing et al., 2013; Yang et al., 2018). Maintaining the microecological balance contributes to normal intestinal function, especially for the development and maturation of the immune system and improvement of host digestion by providing enzymes (Gómez & Balcázar, 2008; Ray, Ghosh, & Ringø, 2012). In the present study, the relative abundance of Proteobacteria showed a significant decrease in the group SBM. Similarly, Desai et al. (2012) showed that that 30% soybean meal inclusion in rainbow trout (*Oncorhynchus mykiss*) diets led to a reduction in Proteobacteria. In contrast, dietary citric acid significantly increased the relative abundance of Proteobacteria, and maintained its dominant position in the intestinal microbial community. Moreover, the relative abundance of Bacteroidetes was significantly increased and became the most dominant phylum in the group SBM, while dietary citric acid significantly decreased the abundance of Bacteroidetes. Considering the UPGMA-clustering tree results showing that the structure and composition of the gut microbiota in the CA group showed a higher similarity with the FM group (compared with the SBM group) likely indicates that dietary citric acid alleviated the dysbiosis of the microbiota and maintained intestinal microecological balance. Notably, at the genus level, the relative abundance

of *Vibrio* was significantly diminished in the CA group compared with the FM and SBM groups. Under intensive pisciculture conditions, *Vibrio* abound in turbot skin, turbot gut and turbot-associated seawater (Cerdà-Cuéllar & Blanch, 2004; Montes et al., 2003; Montes, Farto, Pérez, Armada, & Nieto, 2006). Nevertheless, within *Vibrio* genus, *Vibrio anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. salmonicida* were deemed as potential pathogenic microbe which could lead to vibriosis (Austin et al., 2010; Colwell & Grimes, 1984; Egidius, 1987; Gatesoupe, Lambert, & Nicolas, 2010; Lee, 1995). Dietary citric acid specifically reduced the relative abundance of the *Vibrio* genus in the distal intestine of juvenile turbot and might inhibit the opportunistic pathogens of *Vibrio* genus, which is in accordance with the studies by (He et al., 2017; Silva et al., 2016). Regarding the potential antimicrobial effects of organic acid, it is now generally accepted that the bacteriostatic and bactericidal effects are mainly due to the ability of organic acids to penetrate bacterial in nondissociated form and then dissociate within the pH neutral cytoplasm (Booth & Stratford, 2003; Cherrington, Hinton, Mead, & Chopra, 1991), resulting in the consumption of its own energy to retain osmotic balance (Salmond, Kroll, & Booth, 1984), disturbing cell metabolism and enzyme activity, and eventually leading to cell death (Luckstadt, 2008). More studies are needed to investigate the microbiota regulating effects of citric acid in fish.

5 | CONCLUSIONS

Results presented in this study suggested that moderate levels of citric acid supplementation in diet could alleviate the soybean meal-induced qualitative alteration in mucus layers, preventing intestinal lesions. In addition, dietary citric acid favourably modulated the balance of indigenous gut microbiota in juvenile turbot, including reducing potentially pathogenic *Vibrio* spp. Therefore, it seemed that citric acid had promising potential as a dietary supplement to increase the utilization efficiency of plant protein sources in marine fish, while further studies are needed to determine the suitable dosage used in specific fish species. The precise mechanisms through which citric acid alleviates intestinal health of fish need to be further elucidated by future studies.

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