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Dietary arginine supplementation mitigates the soybean meal induced enteropathy in juvenile turbot, *Scophthalmus maximus* L.

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Abstract

A 12-week feeding trial was conducted to investigate the protective effects of arginine on the intestinal health of juvenile turbot (*Scophthalmus maximus* L.) fed high doses of dietary soybean meal. Sextuple replicates of 30 fish were fed each of four isonitrogenous and isolipidic experimental diets: fish-meal-based diet (FM); FM with 40% fish meal protein replaced by soybean meal protein (SBM); SBM+1% arginine (ARG1) and SBM+2% arginine (ARG2). Turbot-fed SBM showed typical soybean meal-induced enteropathy, characterized by an increase in the thickness of lamina propria, as well as significant decreases in the absorptive surface and activities of intestinal brush border membrane enzymes and Na^+, K^+-ATPase. On the contrary, fish-fed ARG1 showed enhanced intestinal mucosal barrier function in terms of the enhanced gene expression of anti-inflammatory cytokine and barrier-forming tight junction proteins, as well as depressed gene expression of pro-inflammatory cytokines and pore-forming tight junction proteins. Fish-fed AGR2 showed intermediate intestinal performances between SBM and AGR1. Dietary arginine (1%) also significantly regulated the expression of AMP-activated protein kinase α1 (AMPKα1), myosin light chain kinase (MLCK) and nuclear transcription factor-κB p65 (NF-κB p65), and these regulations correlated well with its regulations on intestinal mucosal barrier related genes at all sampling time-points. In conclusion, arginine supplementation (1%) in diet for turbot mitigated the soybean meal-induced enteropathy by enhancing the intestinal mucosal barrier function. The activation of AMPKα1 signalling molecule as well as the suppression of NF-κB p65 and MLCK signalling molecules may mediate the beneficial effects of arginine.

KEYWORDS
arginine, enteropathy, intestinal mucosal barrier, soybean meal, turbot

1 | INTRODUCTION

Soy protein has become a dominating alternative protein source in aqua-feed. However, the use of soybean meal in feed for marine carnivorous fish species is still limited due to that high doses of dietary soybean meal could induce negative impacts on fish growth performance and other physiological processes, especially intestinal health (Hu et al., 2016; Marjara, Chikwati, Valen, Krogdahl & Bakke, 2012; Tacchi et al., 2012). Some countermeasures have been administered for the extensive use of soybean meal in farmed fish, including improving processing technologies and breeding soy-protein-tolerant varieties. Besides these measures, dietary supplementation

with functional additives has also been used to mitigate the soybean meal induced enteropathy (SBMIE) in fish. Glutamine, butyrate and arginine have been reported to be able to protect fish intestine from enteropathy to some extent (Jiang, Hu et al., 2015; Jiang, Shi et al., 2015; Rimoldi et al., 2016).

As an essential amino acid for fish (National Research Council, 2011), arginine is also the precursor of several bioactive substances, such as creatine, polyamines and nitric oxide, regulating the energy metabolism and immune response (Evoy, Lieberman, Fahey & Daly, 1998; Morris, 2004; Wu et al., 2009). A number of recent studies have demonstrated that arginine could be used in mammals to mitigate enteropathy. In weaned pigs, arginine suppressed inflammatory response in lipopolysaccharide-treated ileum by increasing the numbers of IgA-secreting cells, $CD8^+$ and $CD4^+$ T cells and preventing the elevation of mast cell numbers (Zhu, Liu, Xie, Huang & Hou, 2013). Additionally, arginine alleviated the intestinal inflammatory response in rats with intestinal ischaemia and reperfusion (Taha et al., 2016). In vitro study with Caco-2 cells also showed that arginine prevented the methotrexate-induced barrier disruption by increasing the expression of tight junction proteins (Beutheu, Ghouzali, Galas, Déchelotte & Coëffier, 2013). A growing amount of information has been available about the use of arginine as a functional nutrient in fish (Andersen et al., 2015; Azeredo et al., 2015; Cheng, Buentello & Gatlin, 2011; Coutinho et al., 2016; Fournier et al., 2003; Wang, Liu et al., 2015; Zhang et al., 2017). A study with juvenile Jian carp (*Cyprinus carpio* var. Jian) showed that arginine could inhibit the intestinal inflammatory response induced by lipopolysaccharide (Jiang, Shi et al., 2015). Another study on juvenile Jian carp showed that dietary arginine deficiency led to the abnormal expression of intestinal tight junction proteins (Wang et al., 2016). Following these studies, the present study was aimed at investigating the regulatory effects of dietary arginine supplementation on fish intestinal health.

As it was shown, arginine may mitigate the enteropathy mainly by improving the intestinal mucosal barrier function (including the immune and physical barriers), which is the frontline defence against enteropathy (Salim & Söderholm, 2011; Swidsinski et al., 2007). It has been suggested that the triggering of nuclear transcription factor-κB (NF-κB) stimulated the expression of inflammatory cytokines and modulated the inflammatory response in intestine (Niklasson, Sundh, Fridell, Taranger & Sundell, 2011; Ren et al., 2014; Rogler et al., 1998; Yeh, Yeh, Glass & Granger, 2000). The myosin light chain kinase (MLCK) was known to regulate myosin II regulatory light chain (MLC) phosphorylation and then regulate the intestinal tight junction assembly (Marchiando et al., 2011; Turner, 2009). Also, previous studies had proved that MLCK had connection with the inflammatory cytokines or AMP-activated protein kinase (AMPK) signalling pathway (Horman et al., 2008; Miao et al., 2016; Su et al., 2013; Tinsley, Hunter & Childs, 2009). Therefore, to elucidate the mechanisms involved in the regulation of fish intestinal function by arginine, effects of arginine on the signalling molecules such as NF-κB, MLCK and AMPK, which are potentially involved in intestinal mucosal barrier function, were also evaluated in the present study. The present study was conducted with turbot, which is a typical carnivorous marine fish and sensitive to the soy protein. Results of the present study would be beneficial to comprehensively understanding the functional role of arginine in regulating fish intestinal health.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

Four isonitrogenous and isolipidic experimental diets were formulated to contain approximately 52% crude protein and 12% crude lipid (Table 1). A fish-meal-based diet (FM) was used as the positive control. A negative control diet (SBM) was also made by replacing 40% fish meal protein in the FM diet with soybean meal protein. Two arginine-supplemented diets were formulated by including 1% (ARG1) and 2% (ARG2) arginine into the SBM diet. As a nonessential amino acid, alanine was added to make the diets isonitrogenous. The content of each amino acid is shown in Table 2 respectively. Yttrium oxide (Y_2O_3, 1 g/kg) was used as an inert tracer in each diet for determining apparent digestibility of dry matter.

Feed ingredients were ground into fine powder to pass through 320 μm mesh and thoroughly blended with fish oil and soybean lecithin, then water was added to produce stiff dough. The dough was pelleted with an experimental single-screw feed mill and the feeds were dried for about 12 hr in a ventilated oven at 50°C. After being dried, feeds were packed in opaque plastic bags and stored at −20°C.

2.2 | Fish, experimental procedure and conditions

Disease-free juvenile turbot were obtained from a commercial farm in Weihai, Shandong Province, China. Prior to the start of feeding trial, fish were fed the FM diet for 2 weeks to acclimate the experimental conditions. Then, the fish were fasted for 24 hr and weighed. A total of 720 fish of 9.59 g initial body weight were randomly assigned to 24 fiberglass tanks (300 L, 30 fish per tank) connected to an indoor flow-through water system. Each diet was randomly assigned to six tanks. Fish were carefully hand-fed till 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, dried and weighed.

During the feeding period, water temperature ranged from 15 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

To investigate the development of SBMIE and its alleviation by the arginine supplementation thoroughly, three out of six tanks were selected randomly for periodical sampling at the 2nd, 4th and 8th week of the feeding trial. Samples were taken only from fish with the intestinal tract filled with digesta, to ensure the intestine exposure to the diets before sampling. As the illustration of

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

Diet	FM	SBM	ARG1	ARG2
Fish meal[a]	68.00	40.80	40.80	40.80
Soybean meal[a]	0.00	37.90	37.90	37.90
α-Starch	16.00	9.55	9.55	9.55
Fish oil	4.80	6.70	6.70	6.70
Soybean lecithin	0.50	0.50	0.50	0.50
Vitamin premix[b]	1.00	1.00	1.00	1.00
Mineral premix[c]	0.50	0.50	0.50	0.50
Choline chloride	0.30	0.30	0.30	0.30
$Ca(H_2PO_4)_2{\cdot}H_2O$	0.50	0.50	0.50	0.50
Ethoxyquin	0.05	0.05	0.05	0.05
Yttrium oxide	0.10	0.10	0.10	0.10
Calcium propionate	0.10	0.10	0.10	0.10
Microcrystalline cellulose	6.15	0.00	0.00	0.00
Arginine[d]	0.00	0.00	1.00	2.00
Alanine[e]	2.00	2.00	1.00	0.00
Total	100.00	100.00	100.00	100.00
Nutrient composition (% dry matter)				
Dry matter	95.68	96.45	95.14	95.62
Crude protein	51.23	51.38	51.65	52.18
Crude lipid	9.40	10.12	9.89	9.68
Ash	12.77	11.00	11.21	11.24

[a]Fish meal: Purchased from Qingdao Seven Great Bio-tech Company Limited (Qingdao, China), crude protein: 74.04%, crude lipid: 9.97%; Soybean meal: Purchased from Qingdao Seven Great Bio-tech Company Limited, crude protein: 53.12%, crude lipid: 2.12%.

[b]Vitamin premix (g/kg diet): 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.

[c]Mineral premix (g/kg diet): $FeSO_4{\cdot}H_2O$, 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; $CoCl_2{\cdot}6H2O$ (1%), 0.05; Na_2SeO_3 (1%), 0.02; $MgSO_4{\cdot}7H_2O$, 1.2; calcium propionate, 1,000; zeolite, 8.485.

[d]Arginine: Purchased from Sigma-Aldrich (USA). Purity >98%.

[e]Alanine: Purchased from Shanghai Macklin Biochemical (Shanghai, China). Purity >99.26%.

TABLE 2 Amino acid composition of experimental diets (% dry matter)

Diet	FM	SBM	ARG1	ARG2
Essential amino acid				
Arginine	2.57	2.76	3.61	4.55
Histidine	1.36	1.21	1.22	1.17
Isoleucine	1.85	1.88	1.89	1.86
Leucine	3.31	3.29	3.28	3.25
Lysine	3.53	3.16	3.11	3.10
Methionine	1.31	0.86	0.94	0.92
Phenylalanine	1.94	2.43	2.00	1.86
Threonine	1.90	1.76	1.80	1.68
Valine	2.18	2.05	2.10	2.07
Non-essential amino acid				
Alanine	4.48	3.98	3.22	2.19
Aspartic acid	4.02	4.19	4.36	4.21
Cysteine	0.38	0.46	0.48	0.45
Glutamate	6.33	7.14	7.28	7.21
Glycine	2.72	2.31	2.28	2.25
Serine	1.74	1.87	1.91	1.78
Tyrosine	1.41	1.67	1.37	1.27

gastrointestinal tract of turbot showed (Li et al., 2017), the distal intestine is the posterior half of the whole intestine. All distal intestinal tissue samples were clipped from the middle part of distal intestines (about 0.5 cm in length). Distal intestinal tissue samples taken from two randomly selected fish per tank were fixed at Bouin's fixative solution for histological evaluation. For gene expression analysis, distal intestinal tissue samples from another four randomly selected fish per tank were taken and frozen in liquid nitrogen immediately and stored at −80°C pending analysis. All fish were fully anaesthetized with eugenol (1:10,000) before handling and euthanized by cervical dislocation before tissue sampling.

The other three tanks per group were retained for terminal sampling. Total number and body weight of fish in each tank were counted and measured. Following the sampling method mentioned above, two distal intestine tissue samples per tank were for histological evaluation after weighing and measuring length; six distal intestine tissue samples per tank were collected for gene expression analysis; and two distal intestine tissue samples per tank were collected for enzyme activity analysis. Faeces from the distal intestine was collected quantitatively after the sampling procedures mentioned above, frozen in liquid nitrogen and then stored at −80°C pending analyses.

2.4 | Chemical analysis of diets and faeces

Standard methods (AOAC, 1995) were used for analysing experimental diets. Moisture content was determined gravimetrically to constant weight in an oven at 105°C. Crude lipid was determined by ether extraction using Soxhlet (Extraction SystemB-811; Buchi, Switzerland). Crude protein was determined by Kjeldahl method with a Kjeltec System (2300, FOSS, Sweden) using boric acid to trap released ammonia. The amino acid compositions in experimental diets were determined by Amino Acid Analyzer (L-8900; Hitachi, Japan) after acid hydrolysis in 6 N HCl for 24 hr at 110°C (Ma et al., 2013). Ash by combustion at 550°C. The concentration of yttrium oxide in the diets and faeces was measured using ICP-AES (VISTA-MPX; Varian, America) following the method of Cheng et al. (2010).

2.5 | Intestinal brush border membrane enzymes and Na^+, K^+-ATPase activities

Activities of maltase (Mal), alkaline phosphatase (AKP), and Na^+, K^+-ATPase were determined spectrophotometrically according to the

usage manual of commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China).

2.6 | Intestinal histology

The distal intestine, fixed in the Bouin's solution for 24 hr, was 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 height of microvilli (hMV) over 50 μm distance, and the perimeter ratio (PR) between the internal perimeter (IP) of the intestine lumen and the external perimeter (EP) of the intestine (PR = IP/EP) were determined as described by Hu et al. (2015) and Dimitroglou et al. (2009). The thickness of the lamina propria (tLP) over 200 μm distance was determined as shown in Figure.1 (10 measurements per fish, 2 fish per tank).

2.7 | Real-time quantitative PCR

To extract and purify the total RNA, distal intestine tissue (10 samples per group) was ground to powder in liquid nitrogen and added to RNAiso Plus (9109; Takara Biotech, Dalian, China). The integrity of RNA was detected by electrophoresis using 1.2% denatured agarose gel and then assessed by a Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, USA) to test the concentration. The first-strand cDNA was synthesized by reversely transcribing 1 μg total RNA using PrimeScript RT reagent Kit with gDNA Eraser (RR047A; Takara Biotech) according to the instructions.

RNA polymerase II subunit D and GAPDH were used as the housekeeping genes, whose mRNA levels in the distal intestine are stable among all the samples. Specific primers for target genes and housekeeping genes (Table 3) were synthesized by Sangon (Shanghai, China), and then assessed to determine the application efficiency. Real-time PCR was conducted in a quantitative thermal cycler (Mastercyclerep realplex, Eppendorf, Germany). The amplification was performed in a total volume of 20 μL, containing 10 μL of EvaGreen Express 2 × qPCR MasteMix (MasterMix-ES; Applied Biological Materials, Canada), 1 μL of the cDNA product, 0.6 μL of each primer (10 mM) and 9.5 μL of diethylpyrocarbonate-treated water. The qRT-PCR conditions were as follows: 95°C for 10 min and then 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 40 s. When PCR amplification was finished, melting curve analysis was performed to verify that only one PCR product was present in each of these reactions. 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.

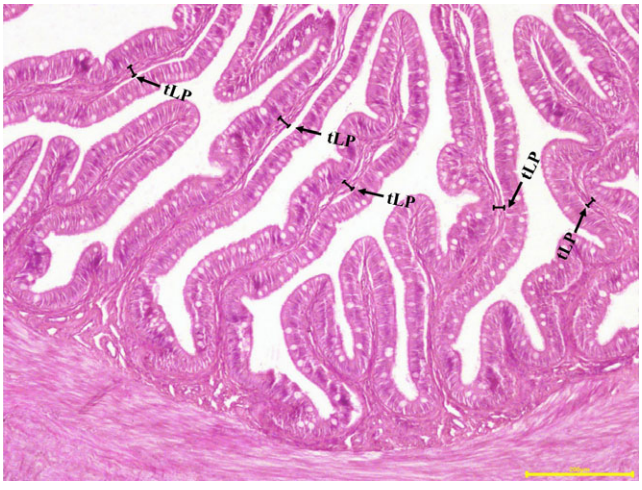

FIGURE 1 The measuring position and method of the thickness of lamina propria (tLP). Staining: H& E. Scale bar = 200 μm [Colour figure can be viewed at wileyonlinelibrary.com]

2.8 | Calculations and statistical analysis

The following variables were calculated:

Survival (%) = 100 × final amount of fish/initial amount of fish

Specific growth rate (SGR, % per day) = 100 × ($\ln W_t - \ln W_0$)/t

Feed intake (FI, % per day) = 100 × feed consumed × 2/($W_0 + W_t$)/t

Feed efficiency ratio (FER) = ($W_t - W_0$)/feed consumed

Apparent digestibility coefficients of dry matter (ADC, %) = 100 × [1 − (% Y_2O_3 in diet)/(%Y_2O_3 in faeces)]

Condition factor (CF, %) = 100 × (body weight, g)/(body length, cm)^3.

Hepatosomatic index (HSI, %) = 100 × (liver weight, g)/(body weight, g).

Viscerosomatic index (VSI, %) = 100 × (viscera weight, g)/(body weight, g).

Intestinal length index (ILI, %) = 100 × (intestine length, cm)/(total body length, cm).

Intestine somatic index (ISI, %) = 100 × (intestine weight, g)/(body weight, g).

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

Data were analysed by 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 $p < .05$ and the results are presented as means ± *SEM* (standard error of the mean).

3 | RESULTS

3.1 | Growth performance and feed utilization

The survival of experimental fish was greater than 95% in each group and no significant difference was observed among all groups

TABLE 3 Primers used for real-time PCR analysis

Target gene	Sequences of primers (5′–3′)	Annealing temperature (°C)	GenBank no.
IL-1β	F: ATGGTGCGATTTCTGTTC	58	AJ295836.2
	R: CACTTTGGGTCGTCTTTG		
TNF-α	F: GGACAGGGCTGGTACAACAC	58	AJ276709.1
	R: TTCAATTAGTGCCACGACAAAGAG		
IFN-γ	F: GCTTTCCCGATCATCTTCTG	58	DQ400686.1
	R: GGTTTCCCAGATTCCCATTC		
TGF-β1	F: CTGCAGGACTGGCTCAAAGG	58	KU238187.1
	R: CATGGTCAGGATGTATGGTGGT		
Claudin-3	F: GCCAGATGCAGTGTAAGGTC	58	KU238180.1
	R: CCGTCCAGGAGACAGGGAT		
Claudin-4	F: ATGTGGAGTGTGTCGGCTT	58	MF370857
	R: AGACCTTGCACTGCATCTG		
Claudin-7	F: CTCCATCCTGCAGCTCAACA	58	MF370858
	R: GGTGCACTTCATTCCCATGC		
ZO-1	F: GAGTTTTCAGCTTCCGTGTT	58	KU238184.1
	R: AGAGAACCTGTCACTGATAGATGC		
Occludin	F: ACTGGCATTCTTCATCGC	58	KU238182.1
	R: GGTACAGATTCTGGCACATC		
Tricellulin	F: GCCTACATCCACAAAGACAACG	58	KU238183.1
	R: TCATTCCCAGCACTAATACAATCAC		
NF-κB p65	F: ACACTGCTGAGCTGAAGATC	58	MF370855
	R: CTCTGAGCCCATCAGGGTC		
MLCK	F: TGTGCTGGGAAGTTCTACAAAGG	58	MF370856
	R: CAATCTCAGGCTTGTGGTCGTAG		
AMPKα1	F: CATCCTCGGAGACACGCTCGGAG	58	KJ634080.1
	R: CCGGCGAATCTTTCCCACCACAT		
GAPDH	F: CAGTGTATGAAGCCAGCAGAG	58	AY008305.1
	R: GGTCGTATTTGTCCTCATTAACTC		
RPSD	F: AACACAGGAAGCAGCAGAAC	58	DQ848899.1
	R: ACGGCAGTGATGGTCTCTC		

IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ; TGF-β1, transforming growth factor-β1; ZO-1, zonula occludens-1; NF-κB p65, nuclear transcription factor-κB p65; MLCK, myosin light chain kinase; AMPKα1, AMP-activated protein kinase α1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RPSD: RNA polymerase II subunit D.

($p > .05$). Turbot-fed SBM showed significant lower SGR, FE and ADC of dry matter, in spite of a significant increase in the FI ($p < .05$). Compared with SBM, the SGR of turbot was significantly increased by both ARG1 and ARG2, while a significant increase in FE and ADC of dry matter were observed only in group ARG1 ($p < .05$). No significant difference in FI was observed among groups SBM, ARG1 and ARG2 ($p > .05$). Significantly higher HSI, ISI and ILI were observed in fish-fed ARG1 compared to fish-fed SBM ($p < .05$) (Table 4).

3.2 | Intestinal brush border membrane enzymes and Na^+, K^+-ATPase activities

The activities of Mal, AKP and Na^+, K^+-ATPase were significantly depressed by high doses of soybean meal compared with FM ($p < .05$). By contrast with SBM, dietary arginine supplementation increased the activities of intestinal Mal, AKP and Na^+, K^+-ATPase, and the highest activities of these enzymes were observed in group ARG1 ($p < .05$) (Table 5).

3.3 | Intestinal morphology

Typical morphological changes of SBMIE were observed in the distal intestine of turbot fed SBM, such as widened and shortened intestinal folds, widened lamina propria within the intestinal folds and loss of microvilli above the intestinal folds. Compared with SBM, less typical morphological changes of SBMIE were observed in the distal intestine of turbot-fed ARG1 and ARG2 (Figure 2).

Turbot-fed SBM displayed significantly decreased hMV and PR of distal intestine at all sampling time-points ($p < .05$), whereas significantly increased tLP was observed at the 4th, 8th and 12th week ($p < .05$). Turbot in arginine-supplemented groups showed significantly increased hMV and PR at all sampling time-points as well as significant decreased tLP at the 4th, 8th and 12th week in distal intestine compared to group SBM ($p < .05$) (Table 6).

3.4 | Relative mRNA expression of intestinal mucosal immunological barrier-related proteins in the distal intestine

Compared with group FM, turbot in group SBM showed significantly higher gene expression of TNF-α, IL-1β, IFN-γ and NF-κB p65 at the 4th, 8th and 12th week ($p < .05$), and significantly lower expression of TGF-β1 at the 4th and 8th week ($p < .05$). Meanwhile, diets with arginine supplementation (1% and 2%) significantly down-regulated the gene expression of IL-1β, TNF-α, IFN-γ and NF-κB p65 at the 4th, 8th and 12th week, and up-regulated the gene expression of TGF-β1 at the 4th and 8th week compared with SBM ($p < .05$) (Fig. 3).

3.5 | Relative mRNA expression of intestinal mucosal physical barrier-related proteins in the distal intestine

Since the 2nd week, the gene expression of tight junction proteins such as Claudin-3, Claudin-4, Occludin, ZO-1 and Tricellulin, as well as that of AMPKα1, was remarkably ($p < .05$) declined, while the gene expression of Claudin-7 and MLCK was significantly ($p < .05$) increased by SBM compared to FM. However, the gene expression of Claudin-3, Claudin-4, Occludin, ZO-1, Tricellulin and AMPKα1 was significantly ($p < .05$) elevated by dietary arginine since the 2nd week compared to SBM. Compared to SBM, diets with arginine supplementation reduced the gene expression of Claudin-7 and MLCK during the latter half period of the feeding trial (Figure 4).

4 | DISCUSSION

In the present study, after 12-week feeding, the typical symptoms of SBMIE described by Gu, Bai, Zhang and Krogdahl (2016) including significant decrease in the absorptive surface, microvillus atrophy and profounder infiltration of mixed leukocytes in lamina propria of distal intestine were observed in the experimental turbot-fed SBM. Fish in SBM also showed the reduction of the ILI, ISI, intestinal brush border membrane enzymes and Na^+, K^+-ATPase activities. However, fish-fed diet ARG1 showed no intestinal impairment or enteritis symptoms, having well-developed and integrated intestine as fish-fed diet FM. Similar results were also observed in studies with red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), which showed that 1% dietary arginine increased the fold height, enterocyte height and microvilli height when the fish were fed plant-protein-based diets (soybean meal providing 40% dietary protein) (Cheng, Dmi & Buentello, 2012; Cheng et al., 2011). On the other hand, the growth performance of experimental fish confirmed the beneficial effects of arginine on intestinal health. The enhanced growth performance in group ARG1 compared to group SBM could be mainly attributed to the intestinal health enhancing effects of arginine because of the requirement of arginine

TABLE 4 Effects of dietary arginine on the survival, growth, feed utilization and growth of digestive organs of juvenile turbot-fed high doses of dietary soybean meal (means ± *SEM*, $n = 3$)[1]

Diet	FM	SBM	ARG1	ARG2
Survival (%)	98.89 ± 1.11	97.78 ± 1.11	98.89 ± 1.11	98.89 ± 1.11
Initial weight (g)	9.58 ± 0.05	9.55 ± 0.03	9.59 ± 0.03	9.61 ± 0.01
Final weight (g)	57.74 ± 2.93^a	44.35 ± 1.82^b	54.50 ± 1.18^a	51.91 ± 0.78^{ab}
SGR (% per day)	2.14 ± 0.06^a	1.82 ± 0.05^b	2.07 ± 0.02^a	2.01 ± 0.02^a
FI (% per day)	1.45 ± 0.01^b	1.63 ± 0.04^a	1.62 ± 0.02^a	1.67 ± 0.02^a
FE	1.17 ± 0.01^a	0.94 ± 0.02^c	1.03 ± 0.02^b	0.98 ± 0.01^{bc}
CF[2]	5.56 ± 0.05	5.53 ± 0.03	5.81 ± 0.04	5.48 ± 0.05
ADC of dry matter (%)	67.28 ± 0.85^a	48.59 ± 0.66^c	59.61 ± 0.41^b	52.47 ± 0.25^c
HSI[3] (%)	1.33 ± 0.14^a	0.86 ± 0.08^c	1.08 ± 0.12^b	0.99 ± 0.09^{bc}
VSI[4] (%)	5.29 ± 0.06^a	4.76 ± 0.08^b	5.13 ± 0.10^{ab}	5.11 ± 0.17^{ab}
ISI[5] (%)	1.15 ± 0.04^a	0.97 ± 0.04^b	1.19 ± 0.03^a	1.15 ± 0.03^a
ILI[6] (%)	56.22 ± 1.77^a	46.91 ± 1.49^b	56.20 ± 1.86^a	51.99 ± 2.59^{ab}

SGR, specific growth rate; FI, feed intake; FE, feed efficiency; ADC, Apparent digestibility coefficients.
[1]Mean values in the same row with different superscript letters are significantly different ($p < .05$).
[2]Condition factor.
[3]Hepatosomatic index.
[4]Viscerosomatic index.
[5]Intestinal somatic index.
[6]Intestine length index.

TABLE 5 Effects of dietary arginine on activities of distal intestine brush border membrane enzymes and Na^+, K^+-ATPase of juvenile turbot fed high doses of dietary soybean meal (means ± *SEM*, *n* = 6)[1]

Diet	FM	SBM	ARG1	ARG2
Maltase	6.01 ± 0.05^a	4.59 ± 0.13^b	5.51 ± 0.06^a	5.18 ± 0.12^a
Alkaline Phosphatase	63.51 ± 2.44^a	33.63 ± 3.02^c	50.42 ± 1.03^b	41.86 ± 1.60^{bc}
Na^+, K^+-ATPase	56.15 ± 1.25^a	44.27 ± 1.47^b	53.91 ± 0.61^a	52.54 ± 1.36^a

[1]Mean values in the same row with different superscript letters are significantly different ($p < .05$).

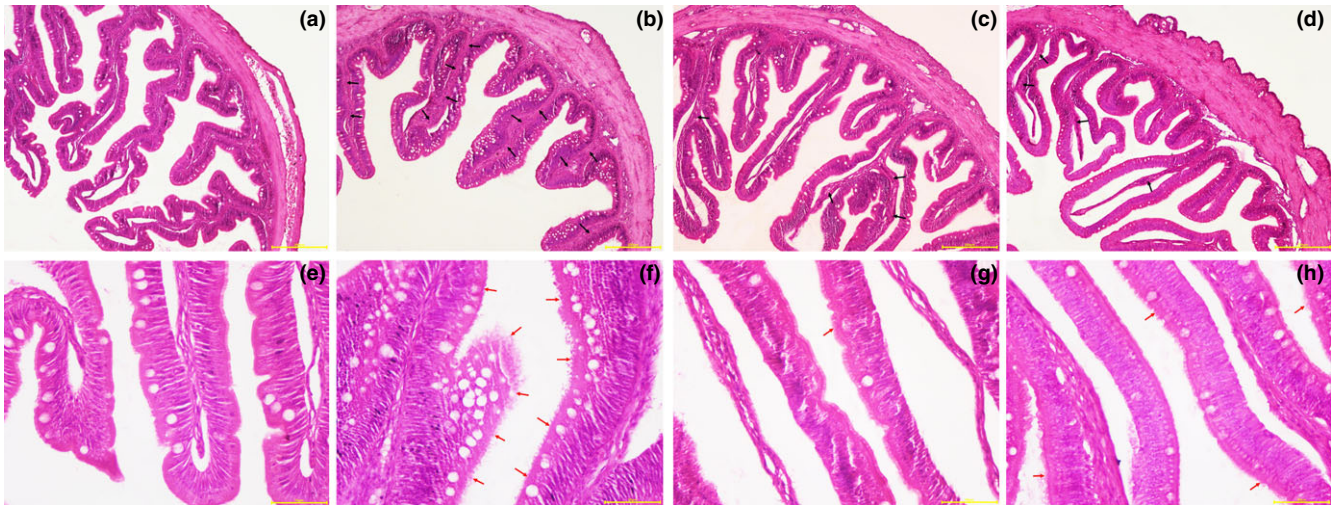

FIGURE 2 Representative histological sections of distal intestine from turbot-fed diet fish meal (a, e), soybean meal protein (b, f), ARG1 (c, g) and ARG2 (d, h). Black arrows show the widening of the lamina propria within the intestinal folds (b, c, d) (Scale bar = 200 μm); Red arrows show the loss of microvilli above the intestinal folds (f, g, h) (Scale bar = 50 μm). Staining: H& E [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 6 Effects of arginine on distal intestine morphometry of juvenile turbot fed high doses of dietary soybean meal at the 2nd, 4th, 8th and 12th week (means ± *SEM*, *n* = 6)[1]

Diet	FM	SBM	ARG1	ARG2
Distal intestine morphometry of juvenile turbot at the 2nd week				
hMV (μm)	3.40 ± 0.10^a	2.69 ± 0.04^c	3.33 ± 0.10^a	2.99 ± 0.05^b
tLP (μm)	19.88 ± 1.41	23.78 ± 0.92	21.85 ± 3.94	21.25 ± 1.23
PR	3.85 ± 0.02^a	3.41 ± 0.13^b	4.14 ± 0.07^a	4.15 ± 0.07^a
Distal intestine morphometry of juvenile turbot at the 4th week				
hMV (μm)	3.59 ± 0.14^a	2.62 ± 0.05^b	3.56 ± 0.08^a	3.31 ± 0.06^a
tLP (μm)	20.15 ± 1.08^b	28.24 ± 0.76^a	20.23 ± 1.49^b	16.72 ± 0.78^b
PR	4.43 ± 0.20^a	4.11 ± 0.16^b	4.51 ± 0.21^a	4.53 ± 0.24^a
Distal intestine morphometry of juvenile turbot at the 8th week				
hMV (μm)	3.71 ± 0.13^a	2.41 ± 0.09^c	3.75 ± 0.0.06^a	3.10 ± 0..6^b
tLP (μm)	19.55 ± 0.52^b	27.67 ± 0.68^a	18.88 ± 0.89^b	19.12 ± 1.72^b
PR	4.67 ± 0.17^a	3.46 ± 0.20^b	4.40 ± 0.28^a	4.77 ± 0.14^a
Distal intestine morphometry of juvenile turbot at the 12th week				
hMV (μm)	4.05 ± 0.06^a	2.62 ± 0.03^d	3.68 ± 0.07^b	3.29 ± 0.04^c
tLP (μm)	27.58 ± 2.04^b	34.08 ± 0.83^a	26.50 ± 1.87^b	25.93 ± 0.60^b
PR	4.98 ± 0.12^a	3.46 ± 0.24^b	4.66 ± 0.12^a	4.92 ± 0.15^a

hMV, The height of microvilli; tLP, The thickness of lamina propria; PR, Perimeter ratio.
[1]Mean values in the same row with different superscript letters are significantly different ($p < .05$).

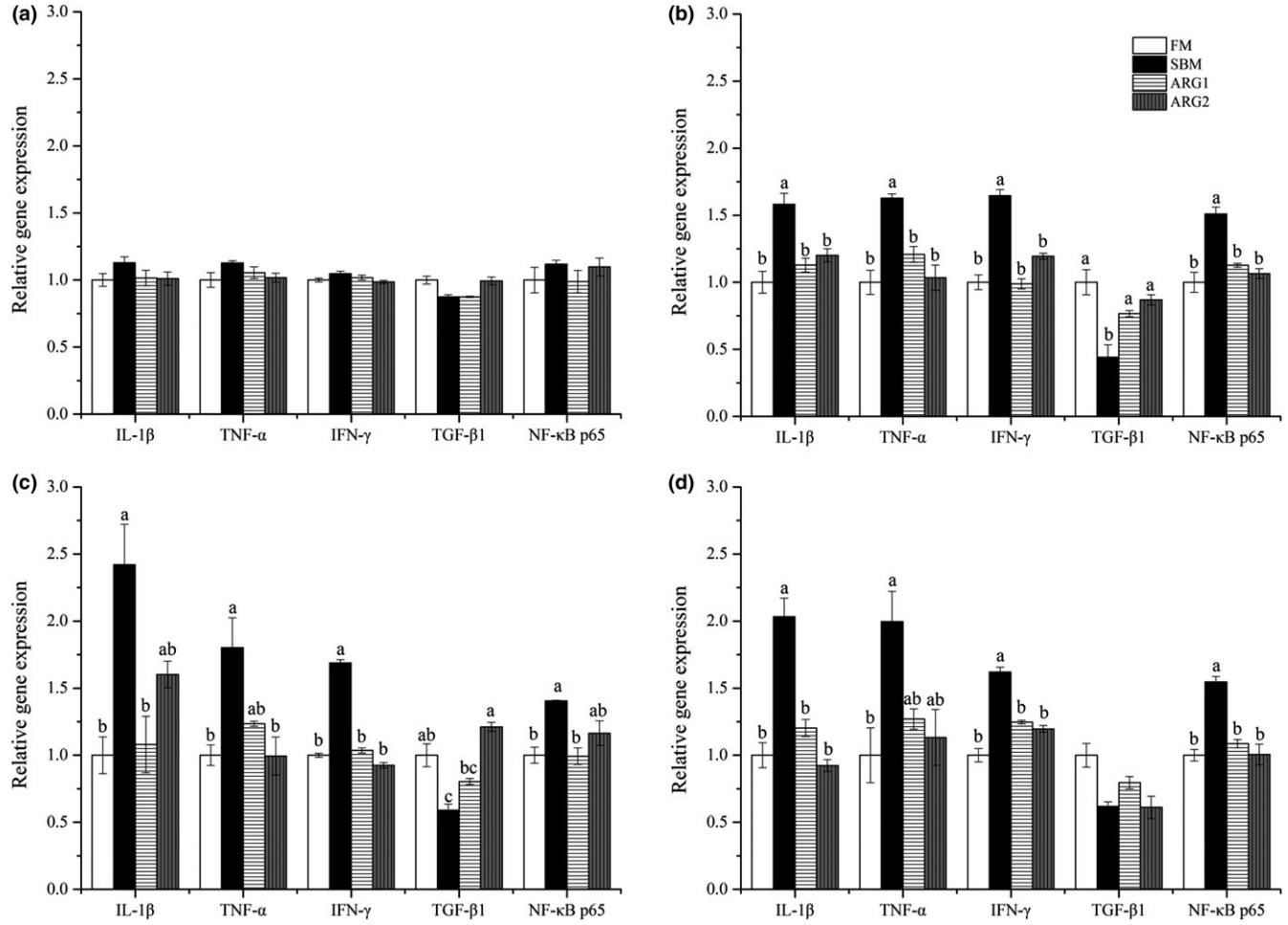

FIGURE 3 Relative mRNA expression of intestinal mucosal immunological barrier-related proteins in the distal intestine at the 2nd week (a), 4th week (b), 8th week (c) and 12th week (d). Error bars of columns denote standard error of means (n = 10) and columns with different letters above are significantly different ($p < 0.05$)

as an essential amino acid was met in all groups (Fournier et al., 2003). In addition, no more benefit was showed in ARG2, and the study by Azeredo et al. (2015) even showed that European seabass (*Dicentrarchus labrax*) immune status and disease resistance could be impaired by excess dietary arginine; therefore, the suitable dosage for arginine supplementation merits closer attention to determine.

In the present study, 1% dietary arginine remarkably suppressed the gene expression of intestinal pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ) and elevated the expression of intestinal anti-inflammatory cytokine (TGF-β1) in the distal intestine of turbot-fed SBM. These results are in accordance with a previous study with LPS-challenged weaned pigs, which showed that dietary arginine suppressed the intestinal inflammatory response in terms of increasing the intestinal immunity and decreasing the infiltration of inflammatory cells in the intestinal mucosa (Zhu et al., 2013). Jiang, Shi et al. (2015) also provided the evidence that dietary arginine alleviated lipopolysaccharide-induced overexpression of intestinal pro-inflammatory cytokines in juvenile Jian carp. As a precursor for NO synthesis in animal bodies, arginine was reported to be involved in NO/ONOO– cycle and regulate the activation of NF-κB (Pall, 2007). In this study, the overexpression of NF-κB p65 induced by soybean meal was significantly down-regulated by dietary arginine supplementation. Consistent with our findings, Jiang, Shi et al. (2015) reported that arginine mitigated inflammatory response in the intestine of juvenile Jian carp by inhibiting the expression of NF-κB p65 signalling molecule. Nevertheless, how arginine blocks the overexpression of NF-κB p65 merits further investigations.

The present results also showed that arginine supplementation enhanced the gene expression of barrier-forming TJ (tight junction) proteins, claudin-3, claudin-4, occludin, ZO-1 and tricellulin (Chasiotis & Kelly, 2011; Kwong & Perry, 2013), but supressed the gene expression of pore-forming TJ protein (claudin-7) in the distal intestine of turbot-fed SBM (Günzel & Yu, 2013). Similarly, Wang, Feng et al. (2015) showed that arginine alleviated the disorder of TJ mRNA expression in the gills of juvenile Jian carp. In all epithelium, TJ proteins assembly and disassembly is a dynamic process involving endocytosis, migration and recycling (König et al., 2016). MLCK are known to phosphorylate MLC and thus have the potential to regulate the tight junction permeability (Marchiando et al., 2011; Turner, 2009). In the present study, it was proved that arginine showed

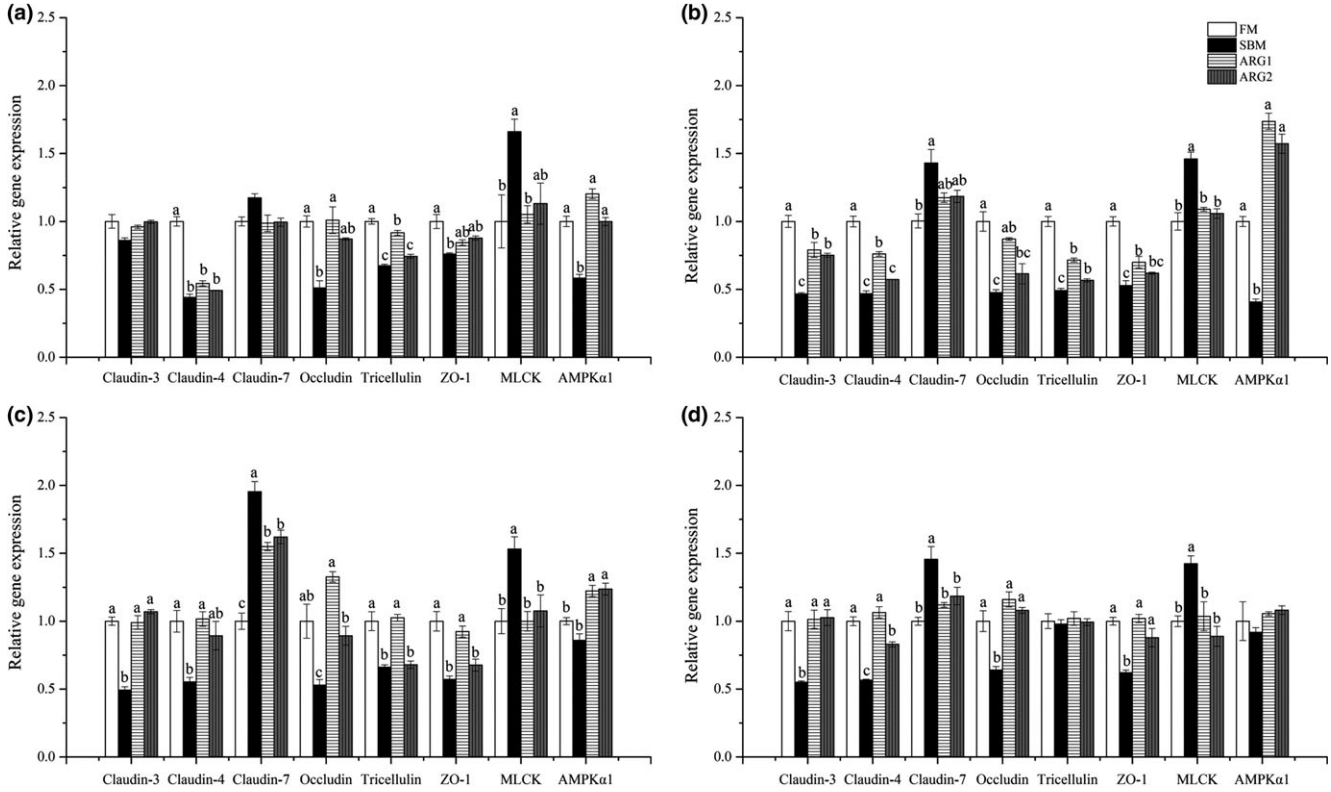

FIGURE 4 Relative mRNA expression of intestinal mucosal physical barrier-related proteins in the distal intestine at the 2nd week (a), 4th week (b), 8th week (c) and 12th week (d). Error bars of columns denote standard error of means (n = 10) and columns with different letters above are significantly different ($p < .05$)

beneficial effects on the gene expression of TJ proteins via suppressing the gene expression of MLCK. Also, in this study, the expression of AMPKα1 was significantly up-regulated by dietary arginine, in line with the studies on humans and terrestrial animals, which showed that dietary arginine supplementation enhanced the gene expression of AMPK (Jobgen, Fried, Fu, Meininger & Wu, 2006). Moreover, the expression of AMPKα1 showed a negative correlation with MLCK, and the enhanced AMPKα1 gene expression by dietary arginine was followed by the suppressed gene expression of MLCK. This was in accordance with the study by Horman et al. (2008) which showed that AMPK modulated the MLC phosphorylation by inactivating MLCK. Therefore, we may draw a conclusion that dietary arginine inhibited MLC phosphorylation by increasing the expression of AMPKα1 and then ameliorate TJ assembly.

König et al. (2016) pointed out that the inflammatory response occurring in the intestinal epithelium is associated with a clearly damaged intestinal mucosal physical barrier, raising a question in the relationship between them. Studies by Slater et al. (2017) and Su et al. (2009) showed that intestinal mucosal physical barrier dysfunction caused immune activation and contributed to development of enteropathy. In this study, the gene expression of MLCK and most barrier-forming TJ proteins significantly changed at the 2nd week, whereas inflammatory response induced by soybean meal significantly displayed till the 4th week. Based upon these results, a reasonable conjecture was established, that is, the disrupted intestinal mucosal physical barrier might be an important contributing factor to the development of SBMIE in turbot. As such, enhancing the reassembly of tight junctions may be a possible mode of action by which dietary arginine supplementation mitigated the SBMIE in turbot.

In conclusion, our findings provided evidences that moderate levels of arginine supplementation in diet helped prevent the turbot from the SBMIE. Regulation of NF-κB p65, AMPKα1 and MLCK signalling molecules contributed to the beneficial effects of arginine on turbot intestinal barrier function. The discovery opens new possibilities in further promoting utilization efficiency of plant protein sources in diets for marine fish, but further studies are needed to determine the suitable dosage for arginine application in specific fish species. Precise mechanisms involved in the regulation of fish intestinal health by arginine also need to be elucidated by more efforts.

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