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Citric acid mitigates soybean meal induced inflammatory response and tight junction disruption by altering TLR signal transduction in the intestine of turbot, Scophthalmus maximus L

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ABSTRACT

A 12-week feeding trial was conducted to investigate the effect of citric acid on the involvement of TLRs in the soybean meal induced inflammatory response and tight junction disruption in the distal intestine of juvenile turbot (*Scophthalmus maximus* L.). Four isonitrogenous and isolipidic practical diets were formulated: fish meal-based diet (FM); 40% fish meal protein in FM replaced with soybean meal protein (SBM); SBM + 1.5% citric acid and SBM + 3% citric acid. Compared to the FM, diet SBM significantly increased the gene expression of TLRs (TLR2, TLR3, TLR5b, TLR9, TLR21, TLR22) and MyD88, as well as TLR related molecules (NF-κB, IRF-3, p38 and JNK), which were remarkably reduced by dietary citric acid. Similarly, citric acid supplementation in SBM markedly depressed gene expression of pro-inflammatory cytokines (TNF-α and IFN-γ) and pore-forming tight junction protein Claudin-7, and enhanced gene expression of the anti-inflammatory cytokine TGF-β1 and TJ proteins related to the decrease in paracellular permeability (Claudin-3, Claudin-4, Occludin, Tricellulin and ZO-1). Compared to the SBM, the concentration of IgM and C4 in serum was significantly reduced by dietary citric acid. In brief, dietary citric acid could synchronously inhibit TLRs-dependent inflammatory response regulated by NF-κB and IRF3, as well as cause TLRs-dependent tight junction disruption modulated by p38 and JNK. Therefore, citric acid could function on mitigating soybean meal induced enteropathy in the distal intestine of juvenile turbot.

1. Introduction

The intestinal mucosal barrier is a single layer of cells lining the intestine. It mainly consists of the enterocyte membranes and tight junctions (TJs) between enterocytes [1], and forms an active barrier that prevents from translocation of harmful substances and pathogens [2,3]. Dysfunction of the intestinal mucosal barrier manifests as the secretion of inflammatory cytokines and alterations of TJs, which is an important event in the pathogenesis of many intestinal diseases [4]. TJs determine the selective paracellular permeability between intestinal epithelial cells to preserve the mucosal barrier function [5]. Inflammatory cytokines have been implicated in the regulation of enteritis [6–8]. Soybean meal induced enteropathy (SBMIE), which is the major limiting factor for soy protein utilization in fish feed and has been demonstrated by our previous studies on turbot [9], is described as subacute inflammatory response and TJ disruption [10,11]. However, the precise mechanisms involved in these processes, as well as the mechanisms involved in the SBMIE-mitigating effects of functional nutrients, have been rarely known.

Toll-like receptors (TLRs) are a group of type I transmembrane

Abbreviations: TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; IRF-3, interferon regulatory factor-3; NF-κB, nuclear factor-kappa B; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinase; ERK, extracellular regulated kinase; AP-1, activator protein-1; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; TGF-β1, transforming growth factor-beta1; ZO-1, zonula occludens-1; IgM, immunoglobulin M; C3, complement 3; C4, complement 4

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proteins with an extracellular domain that recognizes invading microbial pathogen associated molecular patterns and an intracellular domain that triggers signal transduction in cell [12,13]. It has been found that only subtypes TLR2, TLR3, TLR5M, TLR5S, TLR 9, TLR21 and TLR 22 have corresponding ligands in fish [14]. After specific recognition, the intracellular signal transduction mainly depends on the myeloid differentiation factor (MyD88)-dependent and MyD88-independent pathways. With the exception of TLR3, activation of all other TLRs leads to the recruitment of MyD88, resulting in activating NF-κB and MAPK kinases (p38, ERK, and JNK). The intranuclear NF-κB binds to the specific sites and regulates the expression of genes encoding inflammatory cytokines [15]. The signaling cascade of MAPK kinases regulates the process of TJ disruption *in vitro* [16,17]. One of the MyD88-independent pathways is initiated by TLR3, which culminates in the activation of IRF3 followed by inducing interferon inducible genes [18]. There have been studies indicating that TLR signal transduction is widely involved in inflammatory bowel disease [19,20], although recent studies on fish are still confined to structural and functional features of TLRs [18]. In fish, available evidence indicated that SBMIE was associated with the TLR signal transduction [21], however, the specific mechanism has not been completely investigated. The inhibition of the TLR signal transduction might be a therapeutic strategy for the mitigation of SBMIE in fish.

Citric acid (2-hydroxy-1, 2, 3-propane-tricarboxylic acid), a weak organic tricarboxylic acid, is involved in the citric acid cycle in the metabolism of all aerobic organisms as an intermediate, which is widely used in the food and drug industry because of its buffer, anticoagulant and antioxidant capacities [22]. Recent studies have indicated that citric acid has various pharmacological effects of anti-inflammation. In fish, our previous study has shown that citric acid supplementation in diet for turbot has protecting effects on intestinal function [9]. In *in vivo* rat model of myocardial ischemia/reperfusion injury, treatments with citric acid significantly reduced serum levels of TNF-α [23], and the administration of citric acid could attenuate LPS-induced elevation of TNF-α in mice brain [24]. *In vitro*, citrate treatment improved endothelial function by reducing the secretion of the pro-inflammatory cytokine and chemotactic cytokine, as well as by decreasing neutrophil diapedesis under hyperglycaemic conditions [22]. Furthermore, it has been elucidated that organic acids lessen the intestinal permeability and improve the maturation of intestinal mucosa in piglets [25]. However, hardly any studies have illustrated about the impacts of citrate on TLRs in fish or even in mammals. Following our previous studies on intestine-protecting effects of citric acid in turbot, the present study focuses on the effect of citric acid on TLRs, and the involvement of these effects in the soybean meal induced intestinal inflammatory response and TJ disruption in turbot. The results could be beneficial to elucidate the mechanisms involved in the SBMIE-mitigating effects of functional nutrients in fish.

2. Materials and methods

2.1. Experimental diets

Four isonitrogenous (50% crude protein) and isolipidic (12% crude lipid) practical diets were formulated to contain different protein sources: 68% fish meal (FM), 40% fish meal protein in FM replaced by soybean meal protein (SBM) and diet SBM supplemented 1.5% (1.5% CA) and 3% (3% CA) citric acid. All diets were supplemented with vitamins and minerals to fulfill the fishes' requirements for all nutrients. Feed ingredients were ground into fine powder to pass through 320 μm mesh. The ingredients were blended thoroughly and were extruded as pellets with an experimental single-screw feed mill. All the experimental diets were dried for 12 h in a ventilated oven at 50 °C and were packed in opaque plastic bags before being stored at −20 °C until used. The approximate compositions are shown in Table 1.

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

[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: 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: $FeSO_4{\cdot}H_2O$, 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). All these ingredients were purchased from Qingdao Master Bio-tech Company Limited (Qingdao, China).

[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: Purity > 99%; Purchased from Sigma-Aldrich Company (USA).

2.2. Fish, experimental procedure and conditions

The experiment 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. The whole experimental procedure was processed in an indoor flow-through water system in Haiyang, Shandong province, China. Disease-free juvenile turbots of equal size were obtained from a commercial farm in Weihai, Shandong Province, China. Before the experiment, fish were fed with FM diet for two weeks to acclimate the experimental conditions. After fasting for 24 h, turbots (average initial body weight, 9.59 ± 0.01 g) were randomly assigned to 12 fiberglass tanks (300 L, 30 fish per tank). Each diet was randomly assigned to 3 tanks. Fish were hand-fed to apparent satiation twice daily (7:00 a.m. and 7:00 p.m.) for 12 weeks. During the feeding period, daylight was maintained at 12 h each day, water temperature 15 °C–18 °C, pH 7.5–8.0, salinity 30–33‰, ammonia nitrogen < 0.4 mg/L, nitrite < 0.1 mg/L, and dissolved oxygen > 7.0 mg/L.

2.3. Sample collection

At the termination of the experiment, 10 fish from each tank were anesthetized with eugenol (1:10000), and blood was drawn from the caudal vein using a syringe and stored at 4 °C for 4 h. Serum was collected after centrifugation (4000 g, 10 min) and stored at −80 °C as separate aliquots until analysed. The distal intestine tissue samples

were taken from the bloodless fish removed digesta before being frozen in liquid nitrogen and stored at −80 °C.

2.4. Real-time quantitative PCR

Total RNA of six distal intestine samples (three biological replicates for each dietary group) were extracted using RNAiso Plus (Takara Biotech, Dalian, China), following the manufacturer's protocol. Degradation and contamination of total RNA were analysed by 1.2% denatured agarose gel electrophoresis. The RNA purity and concentrations were assessed by a Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, USA). Then the amount of 1 μg RNA per sample was reversely transcribed to the first-strand cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara Biotech, Dalian, China) according to the instructions.

Real-time quantitative PCR (RT-PCR) assays were performed using EvaGreen Express 2 × qPCR MasterMix (Applied Biological Materials, Canada). Reactions were performed in a 20 μL volume containing 10 μL of Evagreen qPCR Master Mix (2 ×), 1.0 μL of cDNA, 0.8 μL of each primer (10 μM), and 7.4 μL of sterile nuclease-free water. RPSD and GAPDH were used as the housekeeping genes, whose mRNA levels in the distal intestine are stable among all the samples [26]. Specific primers (Table 2) for target genes and housekeeping genes were designed using Primer premier 5.0 and assessed to determine the application efficiency. Real-time quantitative PCR was conducted in a quantitative thermal cycler (Eppendorf, Germany). The amplification protocol was as follows: 95 °C for 10 min followed by 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 mRNA relative expression levels were studied by RT-PCR method: $2^{-\Delta\Delta CT}$ [27].

2.5. Immune parameters

Serum lysozyme activity was measured by the self-contrasted method using Lysozyme assay kit (Nanjingjiancheng, Nanjing, China). Briefly, a reaction mixture of 0.2 ml of serum and 2 ml of 0.25 mg/ml *Micrococcus lysodeikticus* (provided in the Lysozyme assay kit) suspension was incubated at 37 °C for 2 min. The absorbance value was detected under a 530-nm wave length. The serum immunoglobulin M (IgM), complement 3 (C3) and complement 4 (C4) concentrations were determined according to the usage manual of commercially available Fish Immunoglobulin M (IgM) Elisa kit, Fish Complement Component 3 (C3) Elisa kit and Fish Complement Component 4 (C4) Elisa kit, respectively (Nanjingjiancheng, Nanjing, China).

2.6. Calculations and statistical analysis

Data were firstly subjected to homogeneity test of variance before one-way analysis of variance (ANOVA) using SPSS 22.0. 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 (standard error of the mean).

3. Result

3.1. Gene expression of toll like receptors

Compared with FM, gene expression of TLR2, TLR3, TLR5b, TLR9, TLR21 and TLR22 was significantly increased in turbots fed with SBM

Table 2
Primers used for real-time PCR analysis.

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	GenBank no.
Claudin-3	GCCAGATGCAGTGTAAGGTC	CCGTCCAGGAGACAGGGAT	KU238180.1
Claudin-4	ATGTGGAGTGTGTCGGCTT	AGACCTTGCACTGCATCTG	MF370857
Claudin-7	CTCCATCCTGCAGCTCAACA	GGTGCACTTCATTCCCATGC	MF370858
Occludin	ACTGGCATTCTTCATCGC	GGTACAGATTCTGGCACATC	KU238182.1
Tricellulin	GCCTACATCCACAAAGACAACG	TCATTCCCAGCACTAATACAATCAC	KU238183.1
ZO-1[a]	GAGTTTTCAGCTTCCGTGTT	AGAGAACCTGTCACTGATAGATGC	KU238184.1
IL-1β[a]	ATGGTGCGATTTCTGTTC	CACTTTGGGTCGTCTTTG	AJ295836.2
TNF-α[a]	GGACAGGGCTGGTACAACAC	TTCAATTAGTGCCACGACAAAGAG	AJ276709.1
IFN-γ[a]	GCTTTCCCGATCATCTTCTG	GGTTTCCCAGATTCCCATTC	DQ400686.1
TGF-β1[a]	CTGCAGGACTGGCTCAAAGG	CATGGTCAGGATGTATGGTGGT	KU238187.1
TLR2[a]	AGGAGCCAAGGGAGACCGAT	GGCGCTCATGATGTTGTCC	KU746963.1
TLR3[a]	TGGTGTCGTCGATTCAAAGC	CCAATCCAACTACTCCCCACG	KX216854.1
TLR5a[a]	AGTCTCTTTGGTCTCAGGGC	TTTGGGTAAGACATCGGGCT	KX525706
TLR5b[a]	AACAACTTCCTAGCCTCCCC	CATGTGAAATCCTCCGCTGG	KX525707
TLR8[a]	ACAGATCCTTGAACTCCCCG	TCCAATCCCTCTCCTCCAGA	KX708702.1
TLR9[a]	AAGGCTCTGAGGGGAAAGAC	TTCTTCACAGAGCTGAGGGG	KU746969.1
TLR21[a]	CAGCTGTCATCCTATCACCG	TTGTCATTGCCCTGCGTAG	KU746965.1
TLR22[a]	ACAGAGACTTCGAGCCAGGT	CTTGTTCGGCAGTTTCCTCA	KJ606344.1
MyD88[a]	CCCAATGGTAGCCCTGAGAT	CATCTCGGTCGAACACACAC	KP985236.1
JNK[a]	CTGGTAGAGCAGGTAGGACA	CACAGAAGACACTGGAAGAA	[b]
NF-κB[a]	ACACTGCTGAGCTGAAGATC	CTCTGAGCCCATCAGGGTC	MF370855
ERK[a]	TCAACCACATACTGGGCATCC	TCGAGTCGGCCTTGAAGAA	[b]
p38[a]	GAACGCCCCCAACATCTCTA	CTCGGCTGCTGTTATTCGCT	[b]
AP-1[a]	CAGCTGCGGCTTGAAGTTTT	GTTCTACGACGAAGCCGTGA	[b]
IRF-3[a]	TGGGAGAAGAAACTCATCACA	ACATCTCCATCATCTCTTCCA	HQ424131.1
GAPDH[a]	CAGTGTATGAAGCCAGCAGAG	GGTCGTATTTGTCCTCATTAACTC	AY008305.1
RPSD[a]	AACACAGGAAGCAGCAGAAC	ACGGCAGTGATGGTCTCTC	DQ848899.1

[a] Abbreviations: ZO-1, zonula occludens-1; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TGF-β1, transforming growth factor-β1; TLR2, toll like receptor 2; TLR3, toll like receptor 3; TLR5a, soluble toll like receptor 5; TLR5b, toll like receptor membrane form 5; TLR8, toll like receptor 8; TLR9, toll like receptor 9; TLR21, toll like receptor 21; TLR22, toll like receptor 22; MyD88, myeloid differentiation factor 88; JNK, c-Jun N-terminal kinase; NF-κB, nuclear transcription factor-kappa B; p38, p38 mitogen-activated protein kinase; ERK, extracellular regulated kinase; AP-1, activator protein-1; IRF-3, interferon regulatory factor-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPSD: RNA polymerase II subunit D.

[b] Partial sequences of JNK, ERK, p38 and AP-1 gene in turbot were obtained through online transcriptome data (Accession number: SRP172919).

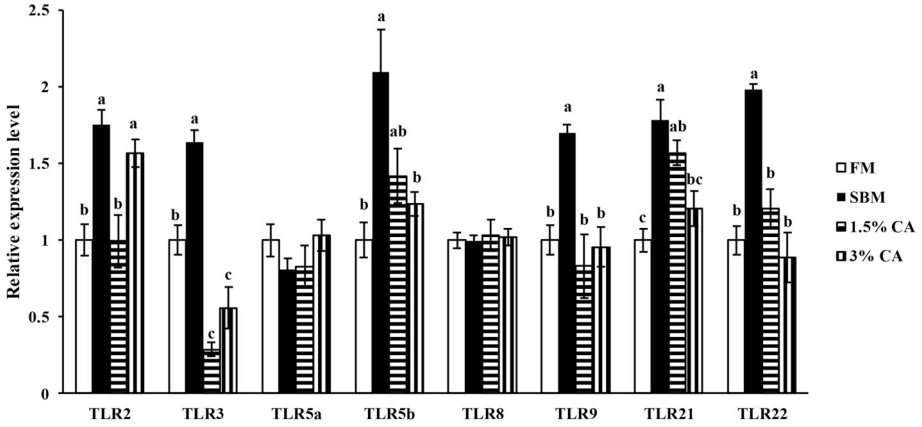

Fig. 1. Relative expression level of TLR2, TLR3, TLR5a, TLR5b, TLR8, TLR9, TLR21 and TLR22 in the juvenile turbot distal intestine. Error bars of columns denote standard error of means (n = 6) and columns with different letters above are significantly different ($P < 0.05$).

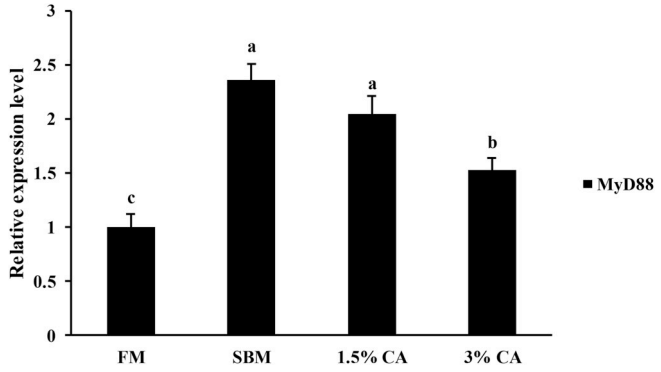

Fig. 2. Relative expression level of MyD88 in the juvenile turbot distal intestine. Error bars of columns denote standard error of means (n = 6) and columns with different letters above are significantly different ($P < 0.05$).

($P < 0.05$). However, both 1.5% CA and 3% CA administration significantly decreased ($P < 0.05$) the expression of TLR3, TLR5b, TLR9 and TLR22, which showed no significant difference with FM group ($P > 0.05$) (Fig. 1).

Besides, citric acid supplementation reduced MyD88 expression compared with SBM group and the lowest level was observed in 3% CA group ($P < 0.05$) (Fig. 2).

3.2. Gene expression of TLR pathway downstream regulatory molecules

The expression of NF-κB, IRF-3, JNK, p38 and AP-1 were significantly up-regulated by SBM compared with FM ($P < 0.05$), whereas the expression of these genes were significantly down-regulated by dietary citric acid ($P < 0.05$). There is no significant difference of ERK among all groups ($P > 0.05$) (Fig. 3).

3.3. Gene expression of cytokines

The expression of pro-inflammatory cytokines TNF-α and IFN-γ were significantly increased in the SBM group compared with the FM group ($P < 0.05$), whereas expression of these genes were significantly decreased by dietary citric acid ($P < 0.05$). The expression of anti-inflammatory cytokine TGF-β1 was significantly increased by citric acid supplementation ($P < 0.05$). However, citric acid supplementation in SBM diet had no significant effect on the expression of IL-1β ($P > 0.05$) (Fig. 4).

3.4. Gene expression of TJ proteins

As shown in Fig. 5, diet SBM increased ($P < 0.05$) the gene expression of pore-forming TJ protein (Claudin-7) and reduced ($P < 0.05$) the gene expression of TJ proteins related to the decrease in paracellular permeability (Claudin-3, Claudin-4, Occludin, Tricellulin and ZO-1). The gene expression of Claudin-7 was decreased ($P < 0.05$) by dietary citric acid, while the gene expression of the other TJ proteins was increased in citric acid treatment groups ($P < 0.05$).

3.5. Serum immune parameters

In Table 3, the IgM and C4 concentration were significantly increased by SBM ($P < 0.05$). However, citric acid supplementation in SBM significantly reduced the concentration of IgM and C4 ($P < 0.05$) and no significant difference was observed between FM and CA groups ($P > 0.05$). There was no significant difference of C3 concentration

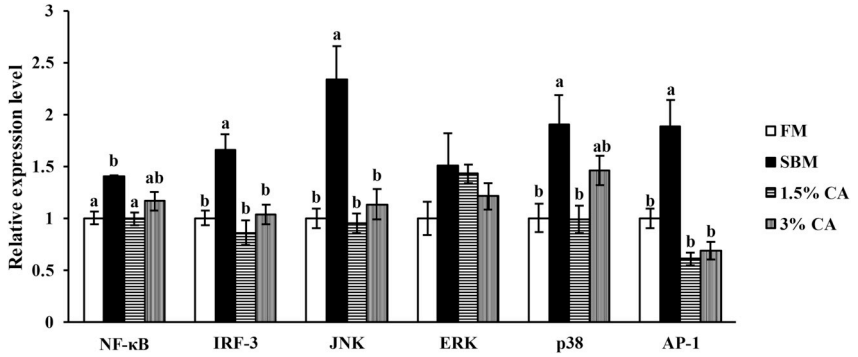

Fig. 3. Relative expression level of NF-κB, IRF-3, JNK, ERK, p38 and AP-1 in the juvenile turbot distal intestine. Error bars of columns denote standard error of means (n = 6) and columns with different letters above are significantly different ($P < 0.05$).

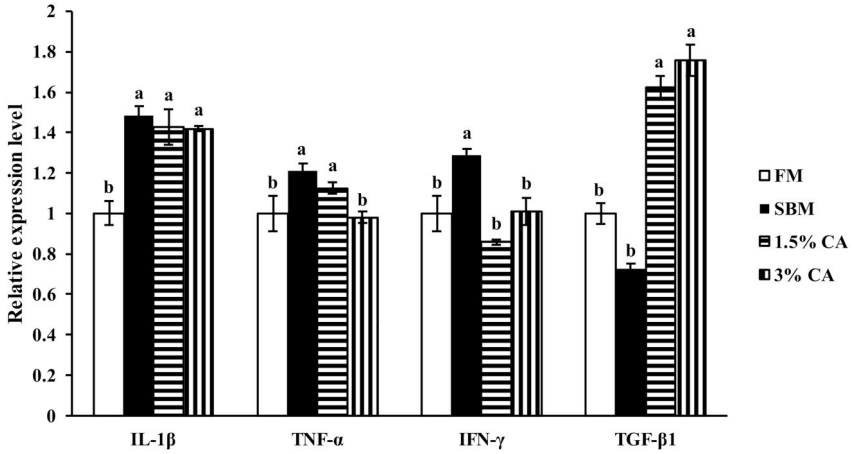

Fig. 4. Relative expression level of IL-1β, TNF-α, IFN-γ and TGF-β1 in the juvenile turbot distal intestine. Error bars of columns denote standard error of means (n = 6) and columns with different letters above are significantly different ($P < 0.05$).

and lysozome activity among all groups ($P > 0.05$).

4. Discussion

As the front line of the organism immune system, the intestinal immune system is confronted with dual challenges of protecting the host from pathogenic infections and coexistence with the intraluminal myriad commensal organisms. TLRs convert the recognition of intestinal pathogen-associated molecules into signals involved in normal gut maintaining inflammation under control [28]. So far, regarding to the subject of citric acid effects on TLRs, no literature has been available. Nevertheless, Nan Yang et al. have verified the down-regulation function of organic acids (chlorogenic acid, caffeic acid, and cichoric acid) on TLR4 both in mice and in human bronchial epithelial cells [29,30]. There has been a similar study that chlorogenic acid could inhibit the expression of MyD88 and TLR4 *in vitro* [31]. In the present study, high doses of dietary soybean meal upregulated the gene expression of mammalian homologous TLRs (TLR2, TLR3, TLR5b, TLR9), fish-specific TLRs (TLR21 and TLR22) [32–34], and downstream signaling molecule MyD88 compared to the FM group. In contrast, the administration of citric acid significantly mitigated the increased gene expression of TLRs related molecules caused by the high doses of dietary soybean meal. These results indicated that the gene expression of TLRs can be suppressed by citric acid in turbot SBMIE.

Previous studies in juvenile turbot indicated that the pathogenesis of SBMIE was mediated by the release of inflammatory cytokines (TNF-α, IL-1β, IFN-γ and TGF-β1) [11,35]. It has been clearly demonstrated that NF-κB and the family of IRF proteins regulate the release of inflammatory cytokines in a complex way with interrelated roles of the transcription factor binding sites [36,37]. The NF-κB mediated signaling pathway is the downstream signal transduction pathway involved in the SBMIE from MyD88-dependent TLR mediated activations, and regulates gene expressions of a number of key cytokines [37]. The present results showed that the expression of NF-κB was suppressed by dietary citric acid. The decreased TLRs mRNA levels in citric acid administrated groups may reduce NF-κB, thereby selectively mediate the activation of key inflammatory mediators. In addition, IRF-3, a crucial transcription factor during innate immune responses, could also been triggered by the TLR mediated activations of MyD88-independent signal transduction. Moreover, it has been illustrated that citric acid participates in regulating the expression of IFN-γ, which could be induced by IRF-3 [18,38]. Interestingly, the results indicated that the expression of IFN-γ showed a significant increase at the stage of IRF-3 mRNA over-expression in the SBM group, both of which could be attenuated by citric acid. Therefore, it is considered that citric acid blocks the IRF-3 mediating over-expression of IFN-γ to some extent. Over all, the present study suggests that citric acid inhibits the expression of NF-κB and IRF-3, and the TLR-stimulating signal transduction pathway related to inflammatory cytokines in SBMIE.

In the light of previous studies, it has been showed that the intercellular spaces dilatation and the inefficient regulation of the junctions between cells make a direct contribution to the development of enteropathy [39,40]. TJs consist of a series of proteins including transmembrane protein Occludin, Tricellulin and members of the Claudin

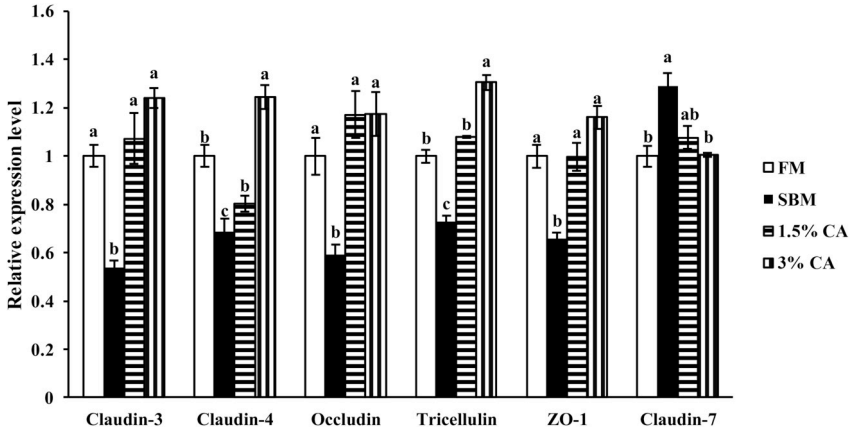

Fig. 5. Relative expression level of Claudin-3, Claudin-4, Occludin, tricelluin, ZO-1 and Claudin-7 in the juvenile turbot distal intestine. Error bars of columns denote standard error of means (n = 6) and columns with different letters above are significantly different ($P < 0.05$).

Table 3
Effects of dietary citric acid on concentration of serum C3, C4, IgM and activity of serum lysozome of juvenile turbot.

	FM	SBM	1.5% CA	3% CA
C3 (mg/ml)	0.4984 ± 0.05	0.5889 ± 0.03	0.5725 ± 0.04	0.5664 ± 0.05
C4 (mg/ml)	0.0839 ± 0.04^{b}	0.1869 ± 0.03^{a}	0.0920 ± 0.02^{b}	0.1553 ± 0.01^{ab}
IgM (mg/ml)	1.1600 ± 0.08^{b}	1.7879 ± 0.27^{a}	0.5792 ± 0.10^{b}	1.4062 ± 0.39^{b}
Lysozome (μg/ml)	46.2500 ± 3.30	54.2708 ± 6.04	66.7709 ± 10.10	45.9375 ± 1.14

Abbreviations: C3, complement 3; C4, complement 4; IgM, immunoglobulin M.
Values are mean ± SEM of three replicates.
Values within the same line with different letters are significantly different ($P < 0.05$).

superfamily, junctional adhesion molecules, and cytosolic proteins, such as ZO-1 [4,40]. There have been studies characterizing Claudin-3 and Claudin-4 chiefly as barrier-forming TJ proteins, whereas Claudin-7 acts as pore-forming TJ protein [5,41,42]. *In vitro*, separate knockdown of Occludin and Tricellulin both induces the increase in paracellular permeability [43,44]. Regarding the regulatory role of ZO-1 on TJ assembly, Tsukita et al. [45] found the assembly arrearage of other TJ proteins in the absence of ZO-1. In accordance with our previous results, high doses of dietary soybean meal led to the disruption of intestinal tight junction, characterized by up-regulated gene expression of Claudin-7 and down-regulated expression of Claudin-3, Claudin-4, Occludin, Tricellulin, and ZO-1 [35]. In all epithelium, TJ proteins assembly and disassembly is a dynamic process involved in endocytosis, migration, and recycling [4]. The MAPK kinases mediated signaling pathway, triggered by MyD88, is one of the main downstream signal transduction pathway from a range of TLRs [46–48]. It has been demonstrated that in the time of TJ unrest, the level of p38 activated form was induced, proving that p38 participated in TJ dynamics [49]. Moreover, recent studies indicate that JNK may play a role in regulation of TJ integrity in different epithelial cells [16,17]. The physiological relevance of p38 and JNK activity in the soybean meal induced TJ disruption was confirmed in the present study. Increasing p38 and JNK expression induced by SBM were attenuated by citric acid administration, which was associated with enhanced gene expression of Claudin-3, Claudin-4, Occludin, Tricellulin, and ZO-1, as well as depressed gene expression of Claudin-7. It was assumed that the transcriptional suppression of p38 and JNK by inhibited TLR signal transduction led to reduced disruption of TJs. These results suggest that in SBMIE of turbot, citric acid may inhibit the expression of p38 and JNK, as well as TLR-stimulating signal transduction pathways related to TJ proteins.

There have been studies showing that cytokines are involved in the regulation of intestinal TJs [50,51]. IFN-γ is the first cytokine that has been demonstrated to increase the epithelial permeability in T84 human derived intestinal epithelial monolayers [52]. According to numerous results, Rana Al-Sadi et al. [51] drew the conclusion that IFN-γ modulated intestinal barrier may through induction of macropinocytosis of TJ proteins. Subsequently, TNF-α and IL-1β have been proven to have stimulating effect on intestinal epithelial permeability [53,54], while TGF-β1 has been shown to have opposite effects by raising transepithelial electrical resistance [55]. In the present study, cytokines induced by upstream molecules in TLR signaling pathways may regulate another kind of TLR downstream molecules — TJ proteins, resulting in disruption of the intestinal mucosal barrier in SBM group in comparison with FM and CA groups. Therefore, citric acid supplementation may relieve the intestinal inflammation by preserving TJ relative stability.

Previous study in rainbow trout suggested that the intestinal mucosal integrity injury could up-regulate serum immune parameters, such as immunoglobulins [56]. As the vital component of the non-specific humoral immune system, complement is commonly depicted as a linear cascade of separate pathways, and the classical pathway is often referred to as antibody-dependent because it is strongly initiated by IgM or IgG clusters [57]. In the present study, the serum IgM and C4 concentration were remarkably increased in turbots fed with SBM. This was in agreement with a previous study, which showed the significant increased serum IgM level in turbots fed with soy protein *in vivo* [58]. The result that dietary citric acid reducing IgM and C4 concentration dovetailed with the intestinal mucosal barrier in turbots fed with SBM. Thus, it might indicate that citric acid administration has protective effects on the specific humoral immune system in turbots suffering from SBMIE.

In conclusion, our findings provided evidences that citric acid supplementation in diet was beneficial for turbots to mitigate SBMIE. Citric acid displayed anti-inflammatory function via the TLR-mediated activation of NF-κB and IRF-3 signaling pathways. Besides, citric acid contributed to the maintenance of TJ proteins via the TLR-mediated p38 and JNK pathways. These results provide new insights into the mechanisms involved in the SBMIE-mitigating effects of functional nutrients in fish. Further investigation is needed to clarify the precise mechanisms by which citric acid inhibit the soybean meal induced overexpression of TLR-related genes.

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