frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 11 April 2023
DOI 10.3389/fimmu.2023.1149151

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

RECEIVED 26 January 2023
ACCEPTED 27 March 2023
PUBLISHED 11 April 2023

CITATION
Zhang X, Wang A, Chang E, Han B, Xu J, Fu Y, Dong X and Miao S (2023) Effects of dietary tryptophan on the antioxidant capacity and immune response associated with TOR and TLRs/MyD88/NF-κB signaling pathways in northern snakehead, *Channa argus* (Cantor, 1842). *Front. Immunol.* 14:1149151. doi: 10.3389/fimmu.2023.1149151

Effects of dietary tryptophan on the antioxidant capacity and immune response associated with TOR and TLRs/MyD88/NF-κB signaling pathways in northern snakehead, *Channa argus* (Cantor, 1842)

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Introduction: Dietary tryptophan (Trp) has been shown to influence fish feed intake, growth, immunity and inflammatory responses. The purpose of this study was to investigate the effect and mechanism of Trp on immune system of juvenile northern snakehead (*Channa argus* Cantor, 1842).

Methods: A total of 540 fish (10.21 ± 0.11 g) were fed six experimental diets containing graded levels of Trp at 1.9, 3.0, 3.9, 4.8, 5.9 and 6.8 g/kg diet for 70 days, respectively.

Results and Discussion: The results showed that supplementation of 1.9-4.8 g/kg Trp in diets had no effect on the hepatosomatic index (HSI) and renal index (RI), while dietary 3.9 and 4.8 g/kg Trp significantly increased spleen index (SI) of fish. Dietary 3.9, 4.8, 5.9 and 6.8 g/kg Trp enhanced the total hemocyte count (THC), the activities of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD). Malondinaldehyde (MDA) levels in the blood were significantly decreased by consuming 3.9 and 4.8 g/kg Trp. Fish fed with 3.0 and 3.9 g/kg Trp diets up-regulated interleukin 6 (*il-6*) and interleukin 8 (*il-8*) mRNA levels. The expression of tumor necrosis factor α (*tnf-α*) was highest in fish fed with 3.0 g/kg Trp diet, and the expression of interleukin 1β (*il-1β*) was highest in fish fed with 3.9 g/kg Trp diet. Dietary 4.8, 5.9 and 6.8 g/kg Trp significantly decreased *il-6* and *tnf-α* mRNA levels in the intestine. Moreover, Trp supplementation was also beneficial to the mRNA expression of interleukin 22 (*il-22*). Additionally, the mRNA expression levels of target of rapamycin (*tor*), toll-like receptor-2 (*tlr2*), toll-like receptor-4 (*tlr4*), toll-like receptor-5 (*tlr5*) and myeloid differentiation primary response 88 (*myd88*) of intestine were significantly up-regulated in fish fed 1.9, 3.0 and 3.9 g/kg Trp diets, and down-

regulated in fish fed 4.8, 5.9 and 6.8 g/kg Trp diets. Dietary 4.8 and 5.9 g/kg Trp significantly increased the expression of inhibitor of nuclear factor kappa B kinase beta subunit (*ikkβ*) and decreased the expression of inhibitor of kappa B (*iκbα*), but inhibited nuclear transcription factor kappa B (*nf-κb*) mRNA level. Collectively, these results indicated that dietary 4.8 g/kg Trp could improve antioxidant capacity and alleviate intestinal inflammation associated with TOR and TLRs/MyD88/NF-κB signaling pathways.

KEYWORDS

tryptophan, antioxidant capacity, TOR signaling pathway, TLRs/MyD88/NF-κB signaling pathway, northern snakehead *Channa argus*

1 Introduction

Amino acids play a significant role in regulating growth, immunity and intestinal health of animals (1, 2). As the lowest concentration of essential amino acids in most common protein sources (3), dietary tryptophan (Trp) has been found to influence the feed intake, growth, immunity and inflammatory responses of fish (4–12), while the regulatory mechanisms need to be further studied.

When fish are subjected to oxidative stress, the content of reactive oxygen species (ROS) in fish increases (13, 14), which can attack macromolecules such as proteins and nucleic acids in the organism, leading to oxidative damage (15). In addition, the lipid peroxidation of ROS with polyunsaturated fatty acids (PUFA) in the cell membrane, and the end products of peroxidation, such as malondialdehyde (MDA), have toxic effects on cells (16, 17). Total antioxidant capacity (T-AOC), superoxide dismutase (SOD) and catalase (CAT) are important components of the antioxidant defense system in fish, which can remove excess ROS in the body, and maintain normal physiological and life activities (16, 18). Supplementation of amino acids in the diet has improved antioxidant capacity and reduced oxidative stress in several fish (19–21). Study has been demonstrated that dietary Trp improved the activity of plasma antioxidant enzymes in juvenile blunt snout bream (*Megalobrama amblycephala*) (22). Dietary Trp has been reported to prevent the increase of MDA content in grass carp (*Ctenopharyngodon idella*) (23). However, the effect of dietary Trp on the enzymatic and nonenzymatic antioxidant capacity of northern snakehead, *Channa argus* (Cantor, 1842) remains to be investigated.

Intestine performs multiple functions, including digestion and absorption of nutrients, recognition of external factors, and signal transduction related to innate and adaptive immunity (24). As is well-known, intestinal cytokines are closely related to intestinal health (25). According to the research, the production of pro-inflammatory cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α), play a significant role in the development of intestinal inflammation (26). Accumulating evidence indicates that amino acids have powerful regulatory roles in cell signaling and mRNA translation (27, 28). Trp was reported to exert beneficial regulatory function in mucosal growth or maintenance, as well as alleviation of intestinal inflammation by 5-hydroxytryptophan (5-HT) (29). Target of rapamycin (TOR) and nuclear factor-kappa B (NF-κB) signaling pathways have been considered to have momentous functions in cell proliferation, differentiation, growth, and metabolism (30, 31). In addition, a large number of studies have shown that various amino acids can regulate intestinal inflammation through the TOR or NF-κB signaling pathway (32–34). Glutamine was found to attenuate intestinal inflammation dependent on its function *via* the mechanistic target of rapamycin (mTOR) and NF-κB signaling pathways (35, 36). Studies have shown that when the TLRs/MyD88/NF-κB signal pathway is inhibited, the inflammatory response in *Oreochromis niloticus* decreases (37). However, there are few reports on the effects and mechanisms of dietary Trp on immunoregulation of *Channa argus*, which need to be further investigated.

C. argus is one of the main economic species in China, due to its rich edible and medicinal value (38). The production of *C. argus* in 2021 was about 548,500 tons (39). To our knowledge, limited information about the nutritional immunity of *C. argus* is available (40, 41). In our previous research, based on the second-degree polynomial regression analysis of specific growth rate and feed efficiency against dietary Trp levels, the optimum dietary Trp requirements for *C. argus* were respectively estimated to be 4.6 and 4.5 g/kg (42). Therefore, in the present study, we investigated the effects of different dietary Trp levels on the antioxidant capacity and intestinal health of *C. argus*, and discussed its possible mechanisms of immune regulation by analyzing the expressions of related genes of the signaling pathways.

2 Materials and methods

2.1 Experimental diets

The formulation and proximate composition of the basal diet are shown in Table 1. Fish meal, poultry by-product meal and mixed L-amino acids were chosen as the main protein sources, fish

TABLE 1 Formulation and proximate analysis of the basal diet (g/kg diet).

Ingredient	Content
Fish meal (70.2% crude protein)	105.00
Poultry by-product meal (66.5% crude protein)	200.00
Wheat meal (12.0% crude protein)	150.00
Gelatin (97.5% crude protein)	40.00
Yeast powder (58.2% crude protein)	30.00
Wheat bran (18.6% crude protein)	110.00
Fish oil	25.00
Soybean oil	30.00
Vitamin mixture [a]	15.00
Mineral mixture [b]	15.00
Amino acid mixture [c]	150.00
Calcium dihydrogen phosphate	10.00
Choline chloride	10.00
Cellulose	99.00
Dimethyl-β-propiothetin	1.00
Composition (% dry weight basis)	
Moisture	7.00
Crude protein	48.56
Crude lipid	10.38
Crude ash	10.04
Carbohydrate[1]	24.02
Gross energy[2], kJ/g	19.72

a.Vitamin premix (mg/kg diet): vitamin B_1, 25 mg; vitamin B_2, 45 mg; vitamin B_{12}, 10 mg; vitamin B_6, 20 mg; vitamin K, 10 mg; vitamin A, 32 mg; vitamin C, 2000 mg; vitamin D_3, 5 mg; vitamin E, 240 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 60 mg; calcium pantothenate, 60 mg; micocrystalline cellulose 12273 mg.
b Mineral premix (mg/kg diet): inositol, 800 mg; $MgSO_4{\cdot}H_2O$, 1200 mg; $CuSO_4{\cdot}5H_2O$, 10 mg; $FeSO_4{\cdot}H_2O$, 80 mg; $ZnSO_4{\cdot}H_2O$, 50 mg; $MnSO_4{\cdot}H_2O$, 45 mg; $CoCl_2{\cdot}6H_2O$, 50 mg; $Ca(IO_3)_2$, 60 mg; Na_2SeO_3, 20 mg; zeolite powder, 12685 mg.
c Amino acid mixture (g/kg diet): arginine, 9.98 g; histidine, 3.37 g; isoleucine, 9.91 g; leucine, 18.60 g; lysine, 23.52 g; methionine, 6.27 g; cysteine, 10.27 g; phenylalanine, 7.57 g; threonine, 10.79 g; valine, 8.48 g; aspartic acid, 28 g; glycine, 14 g.
1 Carbohydrate (%) = 100 - (% crude protein + % crude lipid + % moisture + % ash).
2 Calculated based on 17.2 kJ g^{-1} carbohydrate; 23.6 kJ g^{-1} protein and 39.5 kJ g^{-1} lipid according to the method described in a previous study (43).

oil and soybean oil were chosen as the main lipid sources. 1.0, 2.0, 3.0, 4.0 and 5.0 g/kg diet Trp (Sinopharm Chemical Reagent Co., Ltd, SHH, CHN) were added into the basal diet to produce five experimental diets, respectively. Trp supplement is balanced with glutamate, accounting for 1% of the diet. Prior to the addition of oil and water, all raw ingredients were ground through a 246-micron sieve, then were fully mixed and extruded into particles with diameter of 2.0 × 3.0 mm. Finally, all diets were dried at 40°C and stored at -20°C until use.

Amino acid contents of diets were analyzed with automatic amino acid analyzer (L-8900, Hitachi, Japan). The amino acid profile of the basal diet is presented in Table 2. The final Trp content in six diets are 1.9, 3.0, 3.9, 4.8, 5.9 and 6.8 g/kg, respectively.

TABLE 2 The amino acid profile of the basal diet (% dry weight).

Amino acid	Amino acid composition of basal diet
Essential amino acids (EAAs)	
Lysine	4.29
Methionine	0.99
Arginine	2.89
Phenylalanine	2.18
Histidine	1.01
Isoleucine	2.04
Leucine	3.93
Threonine	2.21
Valine	2.10
Tryptophan	0.19
Non-essential amino acids (NEAAs)	
Aspartic acid	5.06
Glutamic acid	4.65
Serine	1.22
Glycine	4.15
Alanine	2.01
Tyrosine	0.87
Cysteine	1.10

2.2 Fish and culturing conditions

Healthy *C. argus* were purchased from a commercial hatchery in Guangdong, China. Before the trial, fish were acclimated to experimental conditions in the greenhouse in Yangzhou University with a water-recirculating system, and fed with the basal diet for two weeks. Then, 540 fish with initial weight of 10.21 ± 0.11 g were randomly assigned to 18 cages (1 m × 1 m × 80 cm) with 30 fish in each cage, and three cages of fish were randomly provided for each experimental diet. During the feeding trial, all fish were fed at 8:00 and 17:00 daily at apparent satiation level. Feed consumption and fish mortality were recorded every day. The water dissolved oxygen was 6.2-6.6 mg/L and the temperature was 25.5-28.5°C. All experimental protocols were approved by the Animal Care Advisory Committee of Yangzhou University.

2.3 Sample collection and analysis

After 70 days feeding trial, all *C. argus* were fasted for 24 h, then anaesthetized by MS-222 solution (250 mg/L, Sigma). Liver, head kidney and spleen samples were collected from 10 fish in each cage and weighed to calculate the hepatosomatic index (HSI), renal index (RI) and spleen index (SI). Blood samples were collected in heparin sodium-anticoagulant tubes from 10 fish in each cage and divided into two parts, one part was prepared for the determination of

malondinaldehyde (MDA) value and the antioxidant enzymes activity, including superoxide dismutase (SOD), catalase (CAT) and total antioxidant capacity (T-AOC), another part was prepared for the determination of total hemocyte count (THC). The whole intestine samples were collected from 5 fish in each cage for determination of the relative mRNA levels of genes, including tumor necrosis factor α (*tnf-α*), interleukin 1β (*il-1β*), interleukin 6 (*il-6*), interleukin 8 (*il-8*), interleukin 22 (*il-22*), target of rapamycin (*tor*), toll-like receptor-2 (*tlr2*), toll-like receptor-4 (*tlr4*), toll-like receptor-5 (*tlr5*), myeloid differentiation factor 88 (*myd88*), inhibitor of nuclear factor kappa B kinase beta subunit (*ikkβ*), inhibitor of kappa B (*iκbα*), nuclear transcription factor kappa B (*nf-κb*).

The proximate composition of the ingredients and diet, including the moisture, crude protein, crude lipid and crude ash were determined using standard procedures of the AOAC (44). The content of amino acids in ingredients and diet was determined by the method of Rajendra (45). THC was measured and calculated according to Sierra et al. (46). MDA value and the antioxidant enzymes activity were measured using commercial kits provided by Jian Cheng Bioengineering Institute, Nanjing, China. The relative mRNA level of genes was analyzed by RT-*q*PCR method according to Miao et al. (47). The cDNA sequences of the relevant genes were queried in the NCBI, and all primers were designed using Primer Premier 6. All specific primers for genes are provided in Table 3. *β-actin* was chosen as the internal reference gene based on the preliminary tests and Norm Finder algorithms (48, 49).

2.4 Calculations and statistical analysis

Hepatosomatic index (HSI), spleen index (SI), and renal index (RI) were calculated as following:

$$HSI\ (\%) = 100 \times \frac{w_h}{w_b}$$

$$SI\ (\%) = 100 \times \frac{w_s}{w_b}$$

$$RI\ (\%) = 100 \times \frac{w_r}{w_b}$$

W_b: fish weight (g); W_h: liver weight (g); W_s: spleen weight (g); W_r: head kidney weight (g)

After homogeneity variance tests by SPSS 18.0 (SPSS Inc., Chicago, IL, USA), the data (means ± S.D.) were subjected to a one-way analysis of variance (ANOVA) by Tukey's multiple comparison test to assess the significant differences among the treatments at $P < 0.05$. In addition, to determine if the effect was linear and/or quadratic, a follow-up trend analysis using orthogonal polynomial contrasts was performed (50).

3 Results

3.1 Organ index

The effects of dietary Trp on organ index and THC of *C. argus* are shown in Figure 1. The HSI and RI in fish fed the 1.9, 3.0, 3.9 and 4.8 g/kg Trp diets were significantly higher than that in fish fed the 5.9 g/kg Trp diet ($P < 0.05$), and there was no significant difference among fish fed the 1.9, 3.0, 3.9 and 4.8 g/kg Trp diets ($P > 0.05$). The SI and HSI of fish increased at first and then decreased as Trp content increased. Fish fed 3.9 and 4.8 g/kg Trp diets had the highest SI. There were significantly negative linear and positive quadratic trends between the dietary Trp levels and the dependent variables including HSI, SI and RI ($P < 0.05$).

TABLE 3 Primers sequence for RT-*q*PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′–3′)
β-actin	CACTGTGCCCATCTACGAG	CCATCTCCTGCTCGAAGTC
tor	GAGCCTCTCTCATCCTCACCAC	GATTCATTCCTTTCTCTTTAGCCA
tlr-2	CTGGACGAATCATCGAATCACCT	AACTTTGGCTTCCTCTTGGCTCT
tlr-4	GGAGGAGACAGAAGGTGTAGATTTG	AGGTTGTGATCTTGGGCTGAGTG
tlr-5	ACCTCTTCCGCTGTTGTTTCG	AGTGAGCCACCTTCCCTACCA
myd88	TGTCCGAGGTGGAAAGAAGTG	TCAAAGTCGCTCTGGCAGTAG
ikkβ	ATCACAGAGCAACCCCTTTT	CCACTGTAGTTAGGGAAGGA
iκbα	AAAATGTTACCGTGCCAGGAC	ATGTATCACCGTCGTCAGTC
nf-κb	CAGCCAAAACCAAGAGGGAT	TCGGCTTCGTAGTAGCCATG
tnf-α	ACAATACCACCCCAGGTCCCA	ACGCAGCATCCTCTCATCCAT
il-1β	ATGACATGCAATGTGAGCAAAAT	TTAACTCGTATGCTGAATGGTGA
il-6	CATGGAGCACTCAAAGAGGATAG	CTGAGGTGGAGGTAGTGTTGTCG
il-8	GAGTCTGAGCAGCCTGGGAGT	CTGTTCGCCGGTTTTCAGTG
il-22	CAGGCTGTGCAGACGGAGGAAGA	GCGTGGTGATGGTCGTGATAGTGAG

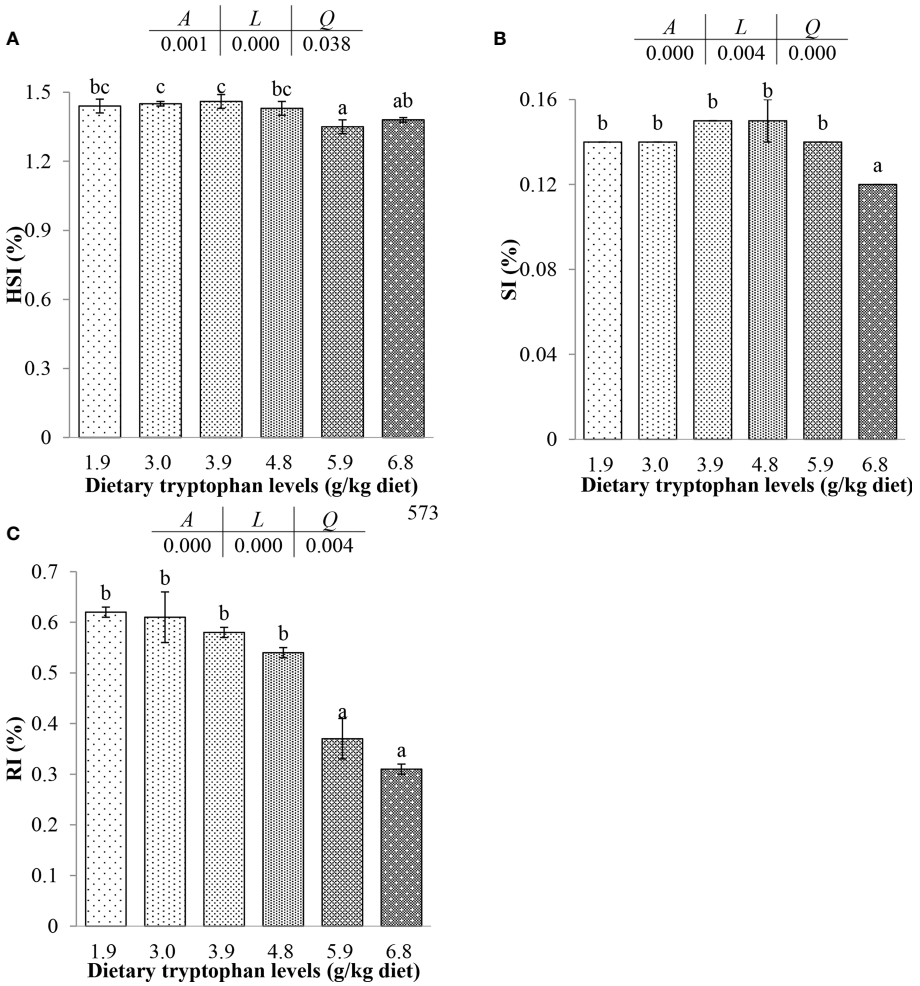

FIGURE 1
Effects of dietary tryptophan with different levels on organ index of *C. argus* (means ± S.D. of three replications). Bars with different letters indicate significantly among the treatments ($P < 0.05$). **(A)** Hepatosomatic index (HSI); **(B)** Spleen index (SI); **(C)** Renal index (RI).

3.2 Total hemocyte count and hematologic antioxidant-related parameters

The effects of dietary Trp level on THC and the hematologic antioxidant-related parameters are shown in Figure 2. The THC levels in *C. argus* increased as Trp levels increased until the dietary Trp level reached 3.9 g/kg, then began to decrease when the dietary Trp level exceeded 4.8 g/kg, and had significantly positive linear and negative quadratic trends with dietary Trp levels ($P < 0.05$).

With the increase of Trp, the activities of SOD, CAT and T-AOC were increased first and then decreased. SOD reached the highest activity in fish fed the diet with 3.9 g/kg Trp, and T-AOC reached the maximum activity in fish fed the 3.9 and 4.8 g/kg Trp diets ($P < 0.05$). However, the highest CAT activity was showed in fish fed the diet with 3.0 g/kg Trp ($P < 0.05$), but there was no significant difference between the fish fed the 1.9 and 3.9 g/kg Trp diets ($P > 0.05$), and the activities of CAT in fish fed the 1.9-3.9 g/kg Trp diets were significantly higher than that in other fish ($P < 0.05$). There were significantly linear and positive quadratic trends between the dietary Trp levels and the dependent variables including SOD, T-AOC and CAT ($P < 0.05$). However, the MDA contents had positive quadratic trend with dietary Trp levels ($P < 0.05$). The MDA contents in fish fed the 3.9 and 4.8 g/kg Trp diets were significantly decreased compared with those in fish fed the 1.9, 3.0 and 6.8 g/kg Trp diets ($P < 0.05$), and there was no significant difference with fish fed the 5.9 g/kg Trp diet ($P > 0.05$).

3.3 Relative mRNA expression of genes related to intestinal inflammatory factors

As shown in Figure 3, dietary Trp level significantly affected the relative expressions of genes related to intestinal inflammatory factors, including interleukins and TNF-α ($P < 0.05$).

The expressions of *tnf-α*, *il-6* and *il-8* increased first and then decreased with dietary 1.9-4.8 g/kg Trp. There were significantly negative linear trends between the dietary Trp levels and the dependent variables including *tnf-α* and *il-6* ($P < 0.05$). For *il-6*

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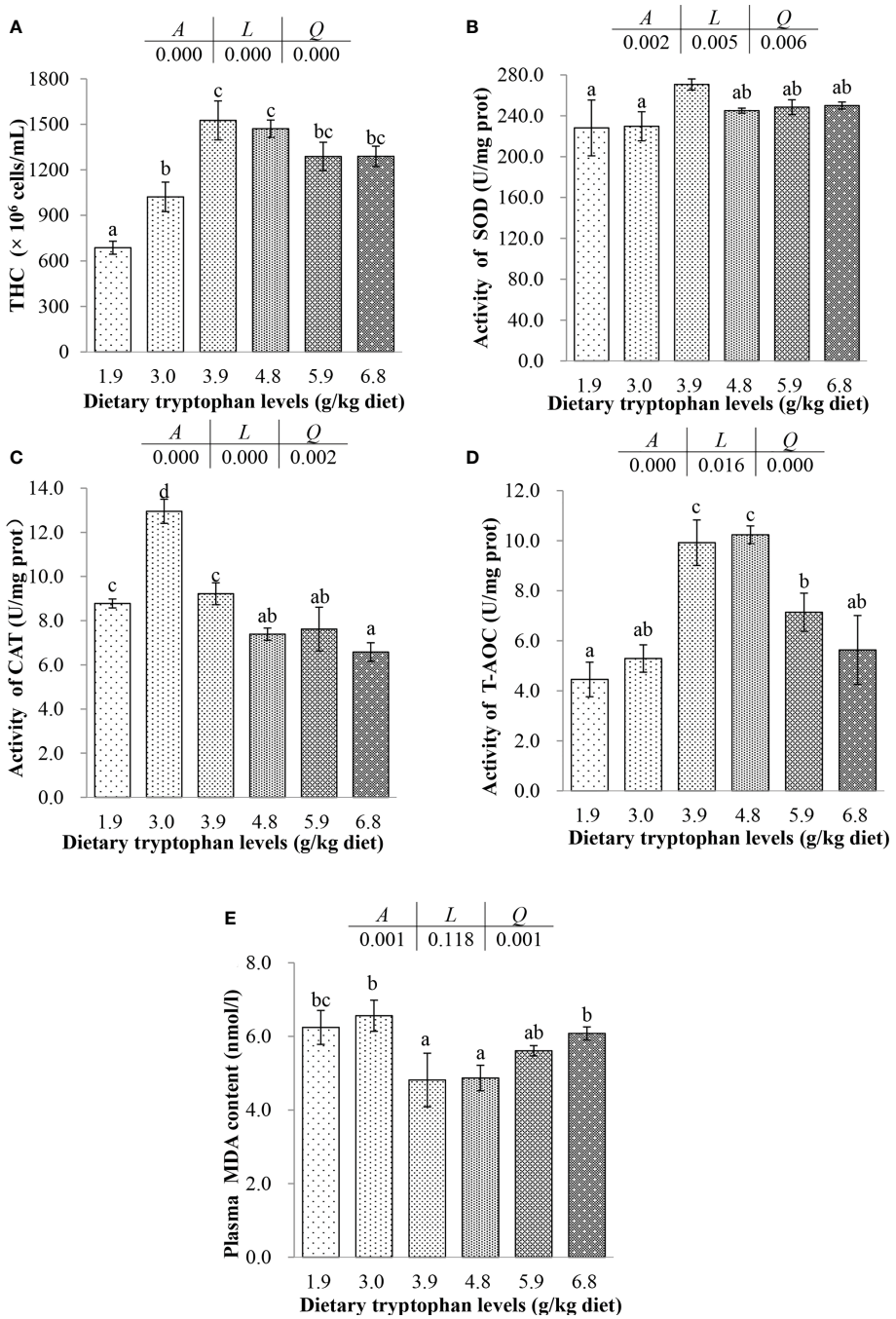

FIGURE 2
Effects of dietary tryptophan different levels on total hemocyte count and hematologic antioxidant parameters of blood in *C argus* (means ± S.D. of three replications). Bars with different letters differ significantly among the treatments ($P < 0.05$). **(A)** Total hemocyte count (THC); **(B)** Superoxide dismutase (SOD); **(C)** Catalase (CAT); **(D)** Total antioxidant capacity (T-AOC); **(E)** Malondialdehyde (MDA).

and *il-8*, the expressions of them in fish fed the 4.8 g/kg Trp diet were significantly lower than those fed with 1.9, 3.0 and 3.9 g/kg Trp diets ($P < 0.05$), and the expressions of them were higher in fish fed the 3.0-3.9 g/kg Trp diets compared with 1.9 g/kg Trp diet group ($P < 0.05$). The expression of *tnf-α* was highest in fish fed 3.0 g/kg Trp diet and significantly decreased in fish fed 3.9 and 4.8 g/kg Trp diets compared to fish fed the basal diet ($P < 0.05$). The expression of *il-1β* in fish fed diets with 3.0, 4.8, 5.9 and 6.8 g/kg Trp showed a decreasing trend compared with the control group, but there was no significant difference except for dietary 6.8 g/kg Trp ($P > 0.05$). There were significantly negative quadratic trends between the dietary Trp levels and the dependent variables including *il-1β*, *il-6* and *il-22* ($P < 0.05$). The higher expression of *il-22* was found in fish fed diets with 3.0-4.8 g/kg Trp ($P < 0.05$), and there was no significant difference between the fish fed diets with 3.0-4.8 g/kg Trp ($P > 0.05$).

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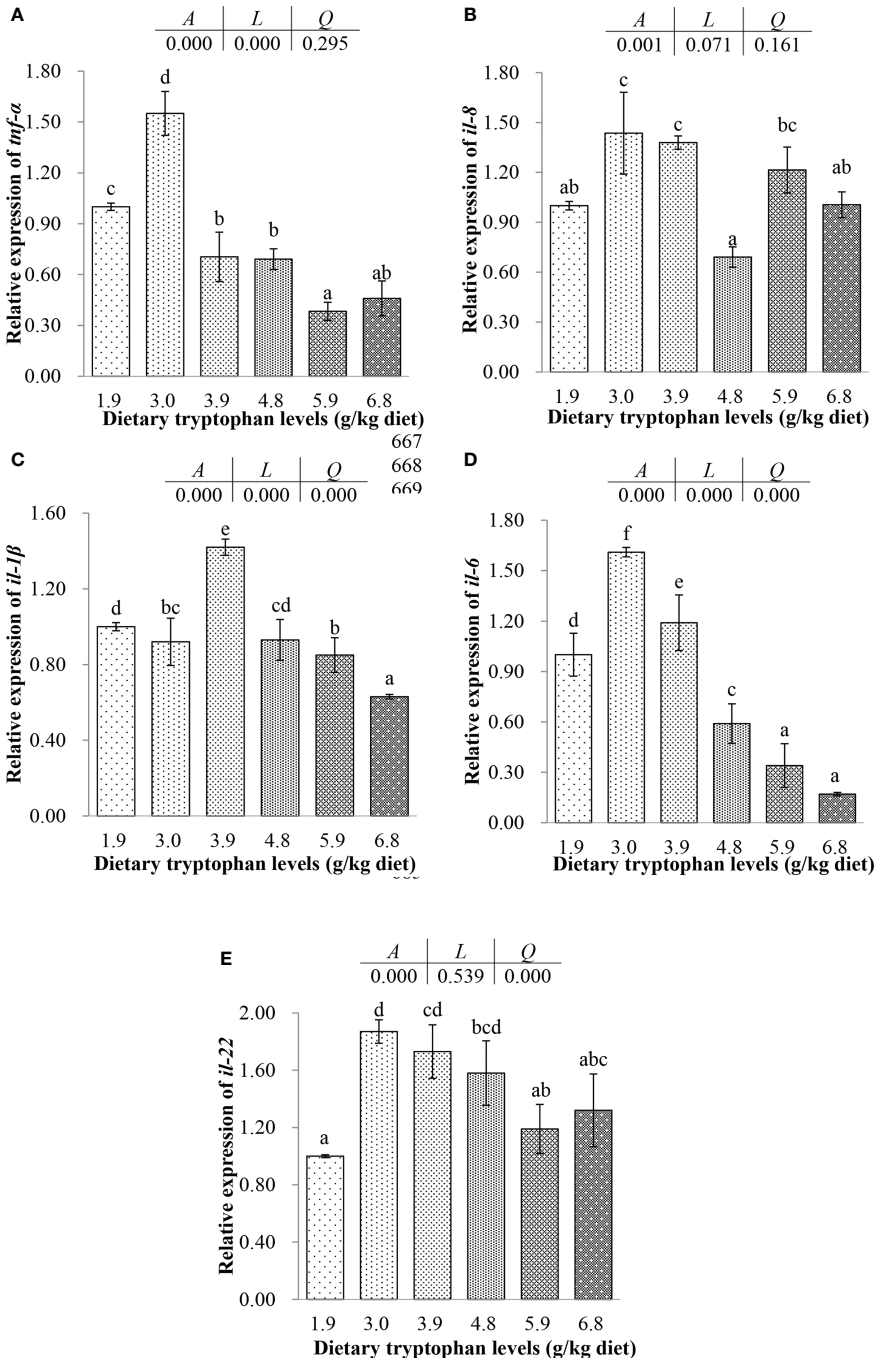

FIGURE 3
Effects of dietary tryptophan with different levels on the relative mRNA expression of genes related to intestinal inflammatory factors of *C argus* (means ± S.D. of three replications). Bars with different letters differ significantly among the treatments ($P < 0.05$). **(A)** Tumor necrosis factor α (*tnf-α*); **(B)** Interleukin 8 (*il-8*); **(C)** Interleukin 1β (*il-1β*); **(D)** Interleukin 6 (*il-6*); **(E)** Interleukin 22 (*il-22*).

3.4 Relative mRNA expression of genes related to the intestinal target of signaling pathways

As shown in Figure 4, there were significantly linear and positive quadratic trends between the dietary Trp levels and the expressions of *tor*, *tlr-2*, *tlr-4*, *tlr-5*, *myd88*, *iκbα* and *ikkβ* ($P < 0.05$).

The expressions of *tor*, *tlr-2*, *tlr-4*, *tlr-5* and *myd88* were all significantly increased with the dietary Trp increasing from 1.9 to 3.9 g/kg, and then decreased ($P < 0.05$). The expressions of *tlr-2*, *tlr-5*, *myd88* and *iκbα* in fish fed the 4.8 g/kg Trp diet were significantly lower than those in fish fed the basal diet ($P < 0.05$). Moreover, the lowest *tor*, *tlr-2*, *tlr-5* and *myd88* gene expressions were found in fish fed the 5.9 and 6.8 g/kg Trp diets ($P < 0.05$). The expression of *ikkβ* increased with increasing dietary Trp up to 4.8 g/kg ($P < 0.05$) and

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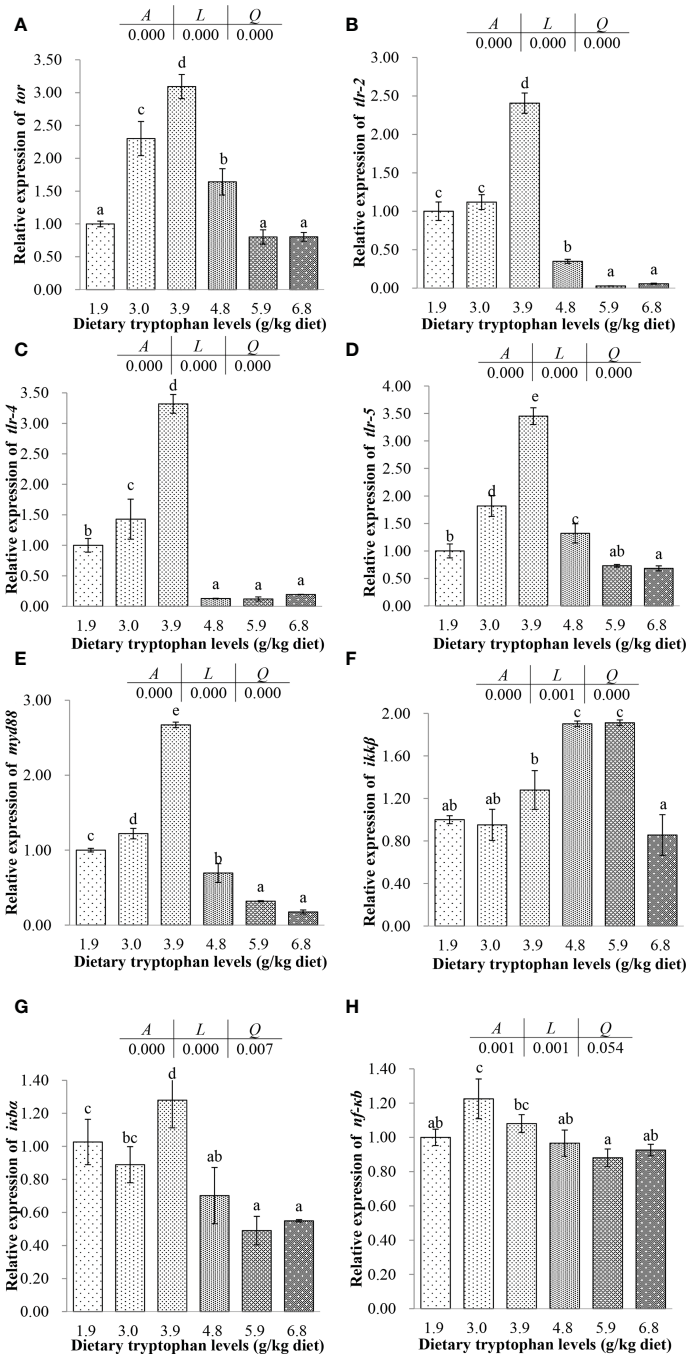

FIGURE 4
Effects of dietary tryptophan with different levels on the relative mRNA expression of genes related to the TOR and NF-κB signaling pathways of *C argus* intestine (means ± S.D. of three replications). Bars with different letters differ significantly among the treatments ($P < 0.05$). **(A)** Target of rapamycin (*tor*); **(B)** Toll-like receptor-2 (*tlr-2*); **(C)** Toll-like receptor-4 (*tlr4*); **(D)** Toll-like receptor-5 (*tlr5*); **(E)** Myeloid differentiation factor88 (*myd88*); **(F)** Inhibitor of nuclear factor kappa B kinase beta subunit (*ikkβ*); **(G)** Inhibitor of kappa B (*iκbα*); **(H)** Nuclear factor-κ-gene binding (*nf-κb*).

decreased with increasing dietary Trp up to 6.8 g/kg ($P < 0.05$). The expression of *iκbα* showed a positive linear except for that in the fish fed diet with the 3.9 g/kg Trp. At the same time, the expressions of *iκbα* were lower in the fish fed with 4.8, 5.9 and 6.8 g/kg Trp diets ($P < 0.05$), compared with the fish fed with basal diet. The expressions of *nf-κb* in the fish fed with 4.8, 5.9 and 6.8 g/kg Trp diets were lower than those in fish fed the basal diet, but there was no significant difference ($P > 0.05$). The expression of *nf-κb* was highest in the fish fed with 3.0 g/kg Trp diet ($P < 0.05$), and had significantly positive linear trend with dietary Trp levels ($P < 0.05$).

4 Discussion

The organ index usually reflects the development of organs and the general nutritional status of animals (51). The liver, spleen and

kidney are the main immune organs of fish, which can reflect the immune state of fish to some extent (52). In the present study, HSI, SI and RI decreased significantly in linear and quadratic curves with dietary Trp increasing, but there was no significant difference in fish fed with 1.9-4.8 g/kg Trp diets. Sharf et al. (53) showed that HSI and viscera somatic index of fingerling *Channa punctatus* decreased with the increase of dietary 0.9-9.1 g/kg Trp. However, studies have shown that dietary supplemented with 0.4-0.6 g/kg Trp could significantly increase SI of ducks (54). Carrillo-Vico et al. (55) also proved that melatonin (the metabolic product of Trp) could positively stimulate the development of spleen. In the present study, although dietary 3.9 or 4.8 g/kg Trp couldn't significantly improve the development of spleen, it has shown a significantly negative quadratic trend, which may be due to the differences in culturing time and breeding subjects. These results provide a hint that the optimum supplementation of Trp in feed was between 3.9 and 4.8 g/kg, which may be beneficial to the development of spleen and had no negative effect on the liver and head-kidney.

The function of blood is closely related to maintaining the stability of various physiological environments in the fish, such as eliminating invading bacteria, phagocytosis of foreign body particles and participating in immune response (56, 57). In this study, the THC in *C. argus* increased at first and then decreased with the increase in dietary Trp level, and there was a significant linear and quadratic relationship. Studies have shown that changes of THC are related to the health status of spleen, liver and other hematopoietic organs (58). This phenomenon was also observed in this study, when inclusion of 3.9-4.8 g/kg Trp in the diets, the SI of *C. argus* showed an upward trend, accompanied by the highest THC. In aquatic animals, antioxidant systems served as the first line of defense against oxidative damage (59). SOD, CAT, T-AOC are commonly used to evaluate the antioxidant capacity and immune response of aquatic animals (11, 32, 59, 60). The present study showed that the activities of SOD and T-AOC in blood were significantly increased when fish fed with 3.9-4.8 g/kg Trp. These results are similar to the findings in the liver of pigs (61), intestine of young grass carp (32). Meanwhile, MDA levels in tissues can be used to estimate lipid peroxidation (62). In this study, the MDA contents in the blood of *C. argus* were significantly decreased with dietary 3.9-4.8 g/kg Trp. Similar results also have been observed in hybrid catfish (*Pelteobagrus vachelli*♀ ×*Leiocassis longirostris*♂) (11). In this study, the activity of CAT in blood was highest in fish feed with 3.0 g/kg Trp diet. However, dietary 4.0 g/kg Trp significantly increased the activity of CAT in juvenile blunt snout bream (22). The reason may be caused by different kinds of fish. These findings suggested that dietary 3.9 and 4.8 g/kg Trp can enhance the antioxidant capacity of *C. argus* by increasing of SOD and T-AOC activities and decreasing the contents of MDA in the blood.

Intestinal cytokines, such as interleukins (ILs) and tumor necrosis factors (TNFs), are important components of the fish mucosal immune system (63). Pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8 can promote the occurrence of inflammatory reactions (64, 65), and anti-inflammatory cytokines such as IL-22 can promote host immune defense against bacterial pathogens (66). In the present study, when dietary Trp reached 4.8 g/kg, the relative expressions of *tnf-α*, *il-1β*, *il-6* and *il-8* in intestine of *C. argus* decreased, whereas the relative expression of *il-22* increased significantly. Trp have been shown to have a similar effect in other study (67). The TOR signaling pathway plays a critical role in the immune system of monocytes (68). And the TOR signaling pathway may improve the innate immune system of fish and human by regulating the transcription of cytokines (69, 70). In the present study, we observed that the expressions of *tor*, *il-1β*, *il-6* and *il-8* were significantly increased when the dietary Trp was up to 3.9 g/kg, but the opposite results were observed when dietary Trp reached 4.8 g/kg. Meanwhile, recent studies have shown that dietary Trp may up-regulate anti-inflammatory factors and down-regulate pro-inflammatory factors of fish partly by regulating the transcription of TOR (5, 22). These results demonstrated that the optimum dietary Trp level could alleviate intestinal inflammation partly by down-regulating the expressions of *tnf-α*, *il-1β*, *il-6* and *il-8*, and up-regulating the expression of *il-22* in fish intestine *via* regulating the expression of *tor*. However, the underlying mechanism by which dietary Trp attenuates inflammatory responses through the TOR signaling pathway remains to be further studied.

Furthermore, the NF-κB translocates to the nucleus and upregulates the expression of genes linked with inflammation, cell survival, proliferation, invasion, and angiogenesis (71), and mediated the proinflammatory action of TOR (70). The increased expression of IKK complex (including IKKα, IKKβ and IKKγ) promotes the phosphorylation and degradation of IκBα, which in turn activates NF-κB, which suppresses the relative expression of anti-inflammatory cytokines and up-regulates the relative expression of pro-inflammatory cytokines (72). In the present study, the relative expression of *ikkβ* increased and the expression of *iκbα* decreased significantly with dietary Trp up to 5.9 g/kg, but there was no difference in *nf-κb*. These immune responses may be caused by multiple pathways regulating NF-κB (73), such as TLRs/MyD88/NF-κB signaling pathway. TLRs (including TLR2, TLR4 and TLR5) are transmembrane proteins that can recognize a variety of related molecules (such as lipopolysaccharide, sodium urate crystal, viral double-stranded RNA, etc.) and cause inflammatory immune response. TLRs can activate the MyD88-dependent pathways, thus activating NF-κB and resulting in the release of inflammatory mediators and cytokines (74, 75). In the present study, the relative expressions of *tlr2* and *tlr4* showed a certain positive correlation with that of *myd88* when dietary Trp 1.9-4.8 g/kg. At the same time, dietary 4.8 g/kg Trp inhibited the relative expression of *nf-κb*, which is consistent with the relative expressions of *tnf-α*, *il-1β*, *il-6* and *il-8*. Li et al. (52) also observed similar results in the kidney of juvenile blunt snout bream fed with 2.8 or 4.0 g/kg Trp. These results implied that the optimum dietary Trp may regulate inflammatory cytokines through the TLRs/MyD88/NF-κB signaling pathway.

In conclusion, the present study provided evidence that dietary 3.9-4.8 g/kg Trp could improve total hemocyte count, antioxidant

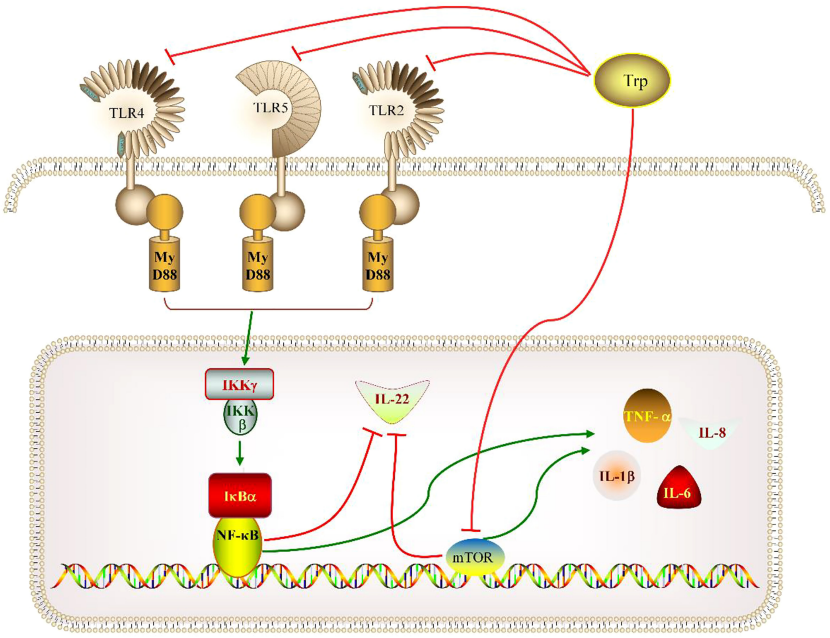

FIGURE 5
Possible mechanisms of the mechanisms of optimal dietary tryptophan promoted intestinal health *via* TOR and TLRs/MyD88/NF-κB signaling pathways.

enzyme activity in blood, and decrease the content of MDA in blood, but have no effect on the development of liver, spleen and head-kidney. Furthermore, dietary 4.8 g/kg Trp could alleviate intestinal inflammation partly by down-regulating the expression of *tnf-α*, *il-1β*, *il-6* and *il-8* and up-regulating the expression of *il-22* in fish intestine *via* the TOR and TLRs/MyD88/NF-κB signaling pathways (Figure 5).

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Animal Care Advisory Committee of Yangzhou University.

Author contributions

XZ: data curation and writing-original draft. AW: writing-reviewing and editing. EC: software. BH: investigation and formal analysis. JX and YF: formal analysis. XD: writing-reviewing and editing. SM: conceptualization, methodology and funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This research was financially supported by grant from National Natural Science Foundation of China (31972799) and National Key R. & D. Program of China (2018YFD0900400).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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