Condensed tannins decreased the growth performance and impaired intestinal immune function in on-growing grass carp (*Ctenopharyngodon idella*)

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Abstract

The present study investigated the effects of condensed tannins (CT) on intestinal immune function in on-growing grass carp (*Ctenopharyngodon idella*). A total of 540 healthy grass carp were fed six diets containing different levels of CT (0, 10·00, 20·00, 30·00, 40·00 and 50·00 g/kg diet) for 70 d and then challenged with *Aeromonas hydrophila* for 14 d. The results showed that, compared with the control group, dietary CT (1) induced intestinal histopathological lesions and aggravated enteritis; (2) decreased lysozyme and acid phosphatase activities, complement 3 (C3), C4 and IgM contents and down-regulated the *Hepcidin*, liver-expressed antimicrobial peptide (*LEAP*)-*2A*, *LEAP-2B*, *Mucin2* and *β-defensin-1* mRNA levels in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) ($P < 0·05$); (3) down-regulated the mRNA levels of anti-inflammatory cytokines transforming growth factor (*TGF*)-*β1*, *TGF-β2* (not in MI and DI), *IL-4/13A* (not *IL-4/13B*), *IL-10* and *IL-11* partly correlated with target of rapamycin (TOR) signalling; and (4) up-regulated the mRNA levels of pro-inflammatory cytokines interferon-*γ2*, *IL-1β*, *IL-6*, *IL-8* (not in PI), *IL-12p35*, *IL-12p40*, *IL-15* and *IL-17D* partly related to NF-κB signalling in the intestine of on-growing grass carp. Overall, the results indicated that CT could impair the intestinal immune function, and its potential regulation mechanisms were partly associated with the TOR and NF-κB signalling pathways. Finally, based on the percentage weight gain and enteritis morbidity, the maximum allowable levels of CT for on-growing grass carp (232·22–890·11 g) were estimated to be 18·6 and 17·4 g/kg diet, respectively.

Key words: Condensed tannins: Intestine health: Immunity: Cytokines: NF-κB: Target of rapamycin

Plant materials are widely used in human foods and animal feed-stuffs[1]. However, the large use of plant materials aggravated persistent diarrhoea in children[2], resulted in the poor growth performance in early-weaned pigs[3] and induced enteritis in common carp (*Cyprinus carpio* L.)[4]. These adverse influences may be largely attributed to various antinutritional factors (ANF) in plant materials, such as tannins, gossypol, saponins, protease inhibitors, glycinin and plant lectins[5]. By reports, ANF mostly interfered with digestive and absorptive abilities in animals[6,7].

The digestive and absorptive abilities of animals rely on the intestinal health, which is correlated with intestinal immune function[8]. Surprisingly, except for the study of gossypol from our laboratory[9], the limited literature concerning the influence of ANF on the intestinal immune function of animals merely determined partial cytokines (such as TNF-α, interferon-γ (IFN-γ), IL-1, IL-4, IL-6, IL-8 and IL-17a) and not involved in signalling pathway, lacking of systematicness and depth so far[10–12]. Additionally, the impacts of different ANF on intestinal immunity

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seem to be not yet identical in animals. For instance, our previous studies in the distal intestine (DI) of juvenile Jian carp (*C. carpio* var. Jian) observed that glycinin down-regulated the mRNA level of *IL-1*[7], whereas β-conglycinin had no impact on this gene expression[13]. Accordingly, it can be seen that diverse ANF have distinct effects on the intestinal immunity of animals, so it is more meaningful to conduct a systematic and in-depth exploration on a kind of ANF.

Tannins, in which condensed tannins (CT) are the most typical types, are common kinds of ANF and are widely distributed in plant foods and feeds[14]. The contents of tannins in soyabean, rapeseed, sorghum, barley, faba bean and cottonseed are up to 1·0, 5·9, 72·0, 12·3, 24·0 and 16·0 g/kg, respectively[15,16]. Studies have shown that tannins reduced the weight gain in weanling pigs[17] and Nile tilapia (*Oreochromis niloticus*)[18]. It is generally well known that animal growth is always dependent on intestinal health, which is closely related to intestinal immune function[19]. Zhu *et al.* have reported that intestinal immune function is closely related to the innate immune components such as lysozyme (LZ), acid phosphatase (ACP), complements (e.g. C3 and C4) and antimicrobial peptides (e.g. β-defensin) and adaptive immune components such as IgM in fish[20]. However, only fragmentary research has considered the effects of tannins on those innate and adaptive immune components in animals. One study showed that tannins stimulated arachidonic acid release in bovine tracheal epithelial cells[21]. Ding *et al.* reported that excess arachidonic acid could weaken serum LZ activity in *Macrobrachium nipponense* (De Haan)[22]. Tannins inhibited histamine release in rat peritoneal mast cells[23]. In humans, it was confirmed that low concentrations of histamine could depress the secretion of C3 and C4 in monocytes[24] and reduce β-defensin-3 mRNA levels in keratinocytes[25]. A study reported that tannins could result in a reduction of vitamin A content in rat liver[26], while depressed vitamin A level down-regulated the serum IgM concentration in broilers[27]. The above observations indicate that there might be a possible relationship between tannins and the innate and adaptive immune function in animals, which deserves research.

Besides the innate and adaptive immune components, cytokines also play vital roles in the intestinal immune function of fish[28]. Cytokines contain pro-inflammatory and anti-inflammatory cytokines which could be modulated by NF-κB[29] and mammalian target of rapamycin (TOR)[30] in humans, respectively. Up to now, excepting inflammatory infiltration was observed in the ileum of sheep[31] induced by tannins, the influences on intestinal inflammatory cytokines and potential signalling molecules in animals have never reported. A study displayed that tannins lessened the butyric acid concentrations in the caecum of rat[32]. It was confirmed that butyrate inhibited the mRNA levels of pro-inflammatory cytokines *IL-1β* and *IL-6* by depressing *NF-kB* in human peripheral blood mononuclear cells[33]. Moreover, Lee *et al.* showed that tannin supplementation decreased plasma Fe concentration in weanling pigs[17]. Our previous study demonstrated that dietary Fe deficiency can down-regulate *TOR* signalling to reduce anti-inflammatory cytokines transforming growth factor-β (*TGF-β*) and *IL-10* mRNA levels in the spleen of grass carp[34]. Based on these observations, we hypothesised that tannins might influence intestinal immune

function of fish associated with the inflammatory cytokines as well as potential NF-κB and TOR signalling.

Taken together, the present study for the first time systematically investigated the influences of tannins on the innate and adaptive immune components, inflammatory cytokines as well as the related signalling pathways (such as NF-κB and TOR signalling), which may partially reveal the impacts of tannins on intestinal immune function and the underlying regulatory mechanisms in animals. Meanwhile, we also determined the maximum allowable level of tannins for on-growing grass carp, which might provide a partial basis for the feed efficiently formulating on-growing grass carp.

Materials and methods

Experimental diets preparation

The formulation of basal diet is shown in Table 1. Fishmeal, casein and gelatin were used as main dietary protein sources. Fish oil and soyabean oil were used as main dietary lipid sources. CT (purity, 78 %; purchased from Guangzhou Senlai Trading Co. Ltd), one of the most widely distributed and abundant tannins in plants[14], were added to the basal diet to provide graded concentrations of 0 (as the control group), 10·00, 20·00, 30·00, 40·00 and 50·00 g/kg diet according to Buyukcapar *et al.*[18], and the amounts of maize starch were reduced to compensate as described by Rasid *et al.*[35]. The prepared diets were air-dried

Table 1. Composition and nutrient content of basal diet

Ingredients	Content (%)	Nutrients	Content (%)
Fishmeal	6·5	Crude protein*	28·59
Casein	20·0	Crude lipid*	5·04
Gelatin	6·0	*n*-3†	1·04
Fish oil	2·44	*n*-6†	0·96
Soyabean oil	1·81	Available P‡	0·40
α-Starch	24·0		
Maize starch	21·24		
Cellulose	5·0		
Ca(H₂PO4)₂	1·47		
Vitamin premix§	1·0		
Mineral premix‖	2·0		
Choline chloride (50 %)	1·0		
DL-Met (99 %)	0·43		
L-Trp (99·2 %)	0·06		
Ethoxyquin (30 %)	0·05		
CT premix¶	7·0		

CT, condensed tannins.
* Crude protein and crude lipid contents were measured values (mean values).
† *n*-3 and *n*-6 were according to Zeng *et al.*[103] and calculated according to the National Research Council (2011).
‡ Available P was according to Wen *et al.*[50] and calculated according to the National Research Council (2011).
§ Per kg of vitamin premix (g/kg): retinyl acetate (500 000 IU/g), 0·39; cholecalciferol (500 000 IU/g), 0·20; DL-α-tocopheryl acetate (50 %), 23·23; menadione (22·9 %), 0·83; thiamine nitrate (98 %), 0·10; calcium-D-pantothenate (98 %), 3·85; pyridoxine hydrochloride (98 %), 0·45; cyanocobalamin (1 %), 0·94; niacin (99 %), 3·44; D-biotin (2 %), 0·75; meso-inositol (98 %), 28·23; folic acid (95 %), 0·17; riboflavin (80 %), 0·73; ascorhyl acetate (95 %), 9·77. All ingredients were diluted with maize starch to 1 kg.
‖ Mineral premix (g/kg): MnSO₄·H₂O (31·8 % Mn), 2·6590; MgSO₄·H₂O (15·0 % Mg), 200·0000; FeSO₄·H₂O (30·0 % Fe), 12·2500; ZnSO₄·H₂O (34·5 % Zn), 8·2460; CuSO₄·5H₂O (25·0 % Cu), 0·9560; KI (74·9 % iodine), 0·0668; Na₂SeO₃ (44·7 % Se), 0·0168. All ingredients were diluted with maize starch to 1 kg.
¶ Per kg of CT premix: the CT was diluted with maize starch to obtain graded levels of CT according to Rasid *et al.*[35].

using an electrical fan at room temperature and then stored at –20°C until being used.

Feeding trial and sample collection

The procedures used in the present study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. The on-growing grass carp was obtained from fishery (Sichuan, China). According to the normal feeding and swimming of fish and microscopic examination to observe no diseases and parasites, fish were acclimatised to the experimental environment for 2 weeks before experiment, as our laboratory study of Feng et al.[36]. Subsequently, a total of 540 healthy fish (initial mean weight 232·67 (SD 0·60) g) were randomly assigned to eighteen experimental cages (1·4 length × 1·4 width × 1·4 height, m), resulting in three cages per treatment and thirty fish per cage, as described by Tian et al.[37]. Each cage was equipped with a disc of 1 m diameter in the bottom to collect the uneaten feed. In the feeding trial, fish were fed with their respective diets four times per d for 70 d, mainly according to the great differences of feed intake (FI) among the different treatments and referring to the literatures of tannin on fish[18,38]. According to Cai et al.[39], 30 min after feeding, uneaten feed was collected, dried using an electrical fan at room temperature and weighed to calculate the FI. During the experimental period, the dissolved O_2 was higher than 6·0 mg/l. The temperature was measured to be 28·1 (SD 2·0)°C using the thermometer with a precision of 0·1°C. The pH value was estimated to be 7·4 (SD 0·3) using a commercial test kit by the colorimetric method and pH indicator paper with a precision of 0·1. The experiment was conducted under a natural photoperiod (approximately 14 h light–10 h dark).

Fish in each cage were weighed at the origination and termination of the feeding trial to determine the percentage weight gain (PWG), specific growth rate and feed efficiency (FE). After that, seven fish were randomly selected from each treatment and anaesthetised in a benzocaine (50 mg/l) bath as described by Geraylou et al.[40]. After weighing and measuring the fish body, they were killed and their intestines were quickly separated, measured and weighed for calculating the intestinal length index (ILI) and intestinal somatic index (ISI) (The precisions of electronic scale and ruler are 0·01 g and 0·1 cm, respectively.). According to the description of Stroband[41] and Stroband et al.[42], the whole intestine was divided into the proximal intestine (PI), mid intestine (MI) and DI, of which the PI is started from the ending of intestinal bulb to the last second bend, the MI is from the ending of PI to the last bend and the DI is from the last bend to the anus. And then, the intestine samples were frozen in liquid N_2 and stored at –80°C for later analysis as described by Ji et al.[43]. Intestinal histological samples from three fish in each group were douched with physiological saline and preserved in 4 % paraformaldehyde for histological examination according to Wang et al.[44]. At the same time, the intestinal content of six fish was collected from each group for measuring the counts of Aeromonas spp., Escherichia coli, Lactobacillus spp. and Bifidobacterium spp., according to Spanggaard et al.[45]. These intestinal micro-organisms were enumerated depending on their respective colony characteristics in the selective medium[46–49] (Aeromonas spp., shiny, smooth and round on tryptone soya agar; E. coli, black centre and metallic sheen on eosin-methylene blue agar; Lactobacillus spp., white protrusion on Lactobacillus selective agar; Bifidobacterium spp., cream and soft texture on Bifidobacterium selective medium).

Digestibility trial and sample collection

The digestibility trial was conducted on the basis of our laboratory's previous study[50]. During the digestibility trial, according to the growth performance after the 70 d' feeding trial, only the 0, 20·00 and 50·00 g/kg CT diet treatments were used for the apparent digestibility coefficient (ADC) measurements of nutrients. According to Che et al.[51], the three group diets were all added to the inert marker chromic oxide (Cr_2O_3) with 5 g/kg dry diet, measured with an electronic scale with 0·01 g precision. A total of 270 healthy fish (initial mean weight 202·67 (SD 0·02) g) were randomly assigned to nine above-mentioned experimental cages (three cages per treatment and thirty fish per cage, one ADC per cage). According to Burel et al., the adaptation lasted for a week[52] and then fish fed with their respective treatment diets four times per d under the same feeding trial conditions for 10 d as described by Peres et al.[53]. At the end of the experiment, the faeces of each cage (all of the thirty fish) were respectively collected by applying slight pressure between the ventral fin and anus, following the method of Austreng[54]. Finally, the faecal samples were stored at –20°C for further analyses according to Omnes et al.[55]. The crude protein content was assayed using the Kjeldahl method (N-Kjeldahl × 6·25). The crude fat content was determined by using the Soxhlet exhaustive extraction technique. The Cr content was determined using atomic absorption spectrometry.

Immunisation test and sample collection

After the 70 d' feeding trial, an immunisation test was conducted via infecting fish with a widespread pathogenic bacterium to investigate the influence of dietary CT on the intestinal immune function of on-growing grass carp[56]. Aeromonas hydrophila, one of the most common pathogenic bacteria causing bacterial enteritis in freshwater fish[57], was friendly provided by College of Veterinary Medicine, Sichuan Agricultural University. From each treatment group, twenty-four fish with a similar body weight were injected intraperitoneally with 1·0 ml A. hydrophila at a concentration of $2·5 × 10^8$ colony-forming units/ml under ventral fin by an 1·0 ml syringe and 26-gauge needle. The injection angle was about 50°, with the injection depth being 1 cm approximately. At the same time, the other twenty-four fish from the control group were intraperitoneally injected with 1·0 ml physiological saline as a positive control (saline) (online Supplementary Figs. S1 and S2). The injected concentration of bacteria was a nonlethal dosage which was sufficient to activate the immune system and consequently enable the investigation of effluent on reactivity against a threatening disease according to our preliminary study (unpublished results). The immunisation test lasted for 14 d, according to our previous study[58]. During the test, the experimental conditions and feeding management were same as the feeding trial. At the end of the immunisation test, a scoring system was conducted to evaluate the severity

of intestine haemorrhage and red swelling morbidity with a semiquantitative method according to Song *et al.*[57]. According to the sample collection method of feeding trial, all fish were anaesthetised in a benzocaine (50 mg/l) bath, then their intestine was quickly removed, segmented (PI, MI and DI), frozen in liquid N_2 and stored at –80°C.

Immunological analysis

The intestinal samples were homogenised in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at 6000 *g* at 4°C for 20 min; then, the collected supernatant was stored for the analysis of related parameters as described by Chen *et al.*[59]. The LZ and ACP activities were assayed by using the commercial kit (Nanjing Jiancheng Bioengineering Institute) according to the method of Jiang *et al.*[60]. The contents of C3, C4 and IgM were measured using the immunoturbidimetric kit (Zhejiang Elikan Biological Technology Co. Ltd), according to the method of Wan *et al.*[61].

Histopathological analysis

According to the changes of growth performance results, only the 0, 30, 50 g/kg diet CT groups were used to observe histopathological damage. The intestines were fixed in 4 % paraformaldehyde, dehydrated in ethanol/methanol and embedded in paraffin. Then tissue was sectioned to 4 µm. The sections were stained using standard haematoxylin–eosin and examined by a light microscope (Nikon Eclipse TS100, Nikon Corporation) according to the method of Wu *et al.*[62]. Then a scoring system was used to evaluate the proportion of histology changes in different treatments according to Liu *et al.*[63]. The morphological changes are based on the evaluation of ten images randomly selected from each fish in each group (three fish in each group). The symptoms were scored as follows: 0 = not observed; 1 = low frequency (1–3 out of 10 images); 2 = moderate frequency (4–6 out of 10 images) and 3 = high frequency (≥7 out of 10 images).

Real-time PCR analysis

The procedures of RNA analysis were conducted according to the method of a study from our laboratory[64]. Total RNA samples were extracted from the PI, MI and DI using an RNAiso Plus Kit (Takara), and the RNA was dissolved in DEPC treated-water. The RNA quality and quantity were assessed by agarose gel (1 %) electrophoresis and spectrophotometric analysis (A260:280 nm ratio). The total RNA was used to synthesise cDNA by a PrimeScript™ RT Reagent Kit (TaKaRa), according to the manufacturer's instructions. The specific primers for the genes were designed according to the grass carp sequences (online Supplementary Table S1). And then, analysing melt curve was used to confirm the specificity and purity of all PCR products after amplification. According to the results of our preliminary experiment about the evaluation of internal control genes (data not shown), *β*-actin was used as a reference gene to normalise cDNA loading. Based on the specific gene, standard curves were generated from 10-fold serial dilutions to calculate the target and housekeeping gene amplification efficiency. The $2^{-\Delta\Delta CT}$ method was used to calculate the expression results after verifying that

the primers amplified with an efficiency of approximately 100 %, as described by Aditya *et al.*[65].

Western blot analysis

The method of Western blotting was based on our previous study[66]. Briefly, protein homogenates of PI, MI and DI were prepared and determined by a BCA assay kit (Beyotime Biotechnology Inc.). The protein samples were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane for the Western analysis. The membrane was blocked for 1·5 h with 0·5 % BSA at room temperature and then incubated with a primary antibody overnight at 4°C. The total TOR (T-TOR, AF6308, 1:1000 dilution), phosphorylation of TOR on residue Ser2448 (p-TOR Ser 2448) (AF3308, 1:1000 dilution), NF-κBp65 (DF7003, 1:1000 dilution), *β*-actin (AF7018, 1:3000 dilution) and Lamin B1 (AF5161, 1:1000 dilution) antibodies were same with our previous studies[64,67]. The *β*-actin and Lamin B1 were used as control proteins for total and nuclear protein, respectively. After being washed, the polyvinylidene difluoride membrane was incubated for 1·5 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in TRIS-buffered saline with Tween. The immune complexes were visualised using electrochemiluminescence reagents (Affinity Biosciences Inc.). The Western bands were quantified using NIH Image 1.63 software (National Institutes of Mental Health). The density of the protein was quantified and then normalised to the control CT group as relative density referring to Jiang *et al.*[68]. This experiment was repeated at least three times, and similar results were obtained each time.

Calculations and statistical analysis

The PWG, specific growth rate and FE were calculated by initial body weight (IBW), final body weight and FI, respectively. The values of growth performances are means of three replicate groups, with thirty fish in each group. The intestinal length and intestinal weight were used to calculate ILI and ISI, respectively. All above were according to Wu *et al.*[69]. The ADC of nutrient (one ADC per cage) were calculated, based on the ratio of the marker in the diets and faeces, according to the formula (NRC, 2011).

$$PWG = 100 \times (\text{final body weight (g/fish)} - \text{IBW (g/fish)})/\text{IBW (g/fish)};$$

$$\text{Specific growth rate} = 100 \times \ln(\text{final body weight (g/fish)}/\text{IBW (g/fish)})/d;$$

$$FI = \text{total feed consumption}_{(dry)} \text{ (g/fish)} - \text{total uneaten feed}_{(dry)} \text{ (g/fish)};$$

$$FE = (\text{final body weight (g/fish)} - \text{IBW (g/fish)})/\text{FI (g/fish)};$$

$$ILI = 100 \times (\text{intestinal length (cm)}/\text{total body length (cm)});$$

$$ISI = 100 \times (\text{intestinal weight (g)}/\text{wet body weight (g)});$$

$$ADC_{DM} = 100 \times (1 - (Cr_{diet}/Cr_{faeces}));$$

$$ADC_{nutrient} = 100 \times (1 - (Cr_{diet}/Cr_{faeces}) \times (Nutrient_{faeces}/Nutrient_{diet})).$$

where $Cr_{diet/faeces}$ is the Cr content in diet or faeces, respectively; $\text{nutrient}_{diet/faeces}$ is the crude protein and crude fat in diet or faeces.

Table 2. Growth performance and intestinal growth and bacterial counts of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of condensed tannins (CT) for 70 d
(Mean values and standard deviations)

CT level (g/kg diet)...	0		10·00		20·00		30·00		40·00		50·00	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IBW*	232·22[a]	0·38	232·22[a]	0·38	233·11[a]	1·02	233·33[a]	0·67	233·89[a]	0·38	232·22[a]	0·77
FBW*	890·11[d]	23·11	872·22[d]	20·41	862·22[d]	8·39	781·00[c]	18·18	693·59[b]	8·64	597·33[a]	27·38
FI*	1051·19[d]	7·37	1047·77[d]	8·94	1041·15[d]	3·34	992·22[c]	2·11	909·12[b]	1·91	833·57[a]	6·06
FE*	0·63[d]	0·02	0·61[d]	0·02	0·60[d]	0·01	0·55[c]	0·02	0·51[b]	0·01	0·44[a]	0·03
PWG*	283·29[d]	9·36	275·60[d]	9·01	269·88[d]	3·61	234·70[c]	6·97	197·83[b]	3·22	157·24[a]	12·33
SGR*	1·92[d]	0·03	1·89[d]	0·03	1·87[d]	0·01	1·73[c]	0·03	1·56[b]	0·02	1·35[a]	0·07
IL†	61·90[c]	3·07	60·34[c]	7·14	60·30[c]	5·76	54·70[b]	2·27	51·77[a,b]	3·64	49·64[a]	1·50
ILI†	150·06[b]	6·41	147·61[b]	15·23	146·02[b]	10·49	135·45[a]	4·22	133·57[a]	5·66	133·06[a]	4·03
IW†	15·27[c]	1·08	14·31[c]	1·56	13·97[c]	1·53	11·97[b]	1·23	10·70[b]	0·90	9·24[a]	0·96
ISI†	1·71[b]	0·09	1·71[b]	0·12	1·68[b]	0·05	1·58[a]	0·14	1·55[a]	0·05	1·54[a]	0·08
Protein content (%)‡§												
PI	5·25[c]	0·48	5·29[c]	0·32	5·04[b,c]	0·24	4·79[a,b]	0·33	4·49[a]	0·37	4·40[a]	0·25
MI	5·15[c]	0·47	5·06[c]	0·42	4·91[c]	0·21	4·47[b]	0·18	4·38[a,b]	0·25	4·06[a]	0·28
DI	5·27[b]	0·39	5·16[b]	0·29	4·96[b]	0·30	4·62[a]	0·16	4·36[a]	0·31	4·32[a]	0·23
Aeromonas spp.‡	7·23[d]	0·15	7·14[c]	0·12	6·93[b]	0·11	6·55[a]	0·16	6·47[a]	0·11	6·37[a]	0·24
Escherichia coli‡	7·29[d]	0·08	7·10[c]	0·07	6·94[b]	0·16	6·78[a]	0·09	6·69[a]	0·18	6·62[a]	0·17
Lactobacillus spp.‡	7·70[e]	0·05	7·65[e]	0·07	7·19[d]	0·19	7·01[c]	0·03	6·84[b]	0·20	6·64[a]	0·16
Bifidobacterium spp.‡	7·50[d]	0·06	7·44[d]	0·12	7·04[c]	0·23	6·78[b]	0·17	6·69[a,b]	0·16	6·49[a]	0·28

IBW, initial body weight (g/fish); FBW, final body weight (g/fish); FI, feed intake (g/fish); FE, feed efficiency; PWG, percentage weight gain (%); SGR, specific growth rate (%/d); IL, intestine length (cm); ILI, intestine length index; IW, intestine weight (g/fish); ISI, intestinal somatic index; PI, proximal intestine; MI, mid intestine; DI, distal intestine.
[a,b,c,d,e] Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$; ANOVA and Duncan's multiple range tests).
* Mean values and standard deviations for three replicate groups, with thirty fish in each group.
† Mean values and standard deviations (n 7).
‡ Mean values and standard deviations (n 6).
§ Protein content (%) = protein content (g)/intestine weight (g) $\times$ 100.

The results are represented with mean values and standard deviation. Data were subjected to one-way ANOVA followed by Duncan's multiple range tests to determine significant differences among six treatment groups at $P < 0.05$ with SPSS 18.0 (SPSS Inc.). Pearson correlation coefficient analysis was conducted using the Bivariate Correlation program in SPSS 18.0. On the basis of the means and standard deviations of growth and intestinal immune-related parameters, the minimum effect size was calculated to be 0·68 according to the method of Searcy-Bernal *et al.*[70]. With the effect size of 0·68, a significance level of 0·05 and the six replicates in each treatment, the statistical power was calculated to be 0·84 using the R pwr package according to Grey *et al.*[71]. Broken-line analysis was used to evaluate the maximum allowable level of CT in the diets of on-growing grass carp, according to Wang *et al.*[9].

Results

Growth performance and intestinal function related indices of on-growing grass carp

Effects of CT on growth performance, intestinal growth and intestinal bacterial counts of on-growing grass carp and feed ADC are presented in Table 2 and Fig. 1. Compared with the control group, the growth performance (FI, FE and PWG) of on-growing grass carp had no significant differences with increasing CT to 10 and 20 g/kg, but were significantly decreased when CT levels were up to 30 g/kg and above ($P < 0.05$). Compared with the control group, the intestinal growth parameters (intestinal length, ILI, intestinal weight, ISI and protein content) of on-growing grass carp had no significant differences with increasing CT to 10 and 20 g/kg, but were strikingly decreased when CT levels were up to 30 g/kg and above ($P < 0.05$). Meanwhile, in comparison with control treatment, the ADC of DM and crude lipid were significantly decreased when adding 20 g/kg CT and 50 g/kg ($P < 0.05$). The ADC of crude protein had no significant difference when adding 20 g/kg CT but significantly decreased with CT levels up to 50 g/kg ($P < 0.05$). Additionally, compared with the control diet, adding 10 g/kg CT to the diet has significantly decreased the *Aeromonas* spp. and *E. coli* amounts ($P < 0.05$). The *Lactobacillus* spp. and *Bifidobacterium* spp. amounts had no significant differences when the CT levels were up to 10 g/kg but strikingly lowered when the CT levels were up to 20 g/kg and above ($P < 0.05$).

The histopathological results are shown in Fig. 2. Compared with the control group, goblet cell hyperplasia and blood capillary hyperaemia were observed in the PI, MI and DI of on-growing grass carp with the CT levels up to 30 g/kg. Increasing the dietary levels of CT to 50 g/kg resulted in goblet cell heavily hyperplasia, thickening of lamina propria and leucocytes infiltration in the lamina propria in three intestinal segments of fish. The summary scoring results of morphological changes are presented in Table 3. Compared with the control group, the morphological change was not significant with CT level up to 30 g/kg but significantly aggravated with CT level up to 50 g/kg ($P < 0.05$) in the PI of on-growing grass carp. In the MI and DI, the intestinal morphological changes were significantly aggravated with the CT levels up to 30 and 50 g/kg CT ($P < 0.05$) in fish. The survival rate of all of the groups after

infection with *A. hydrophila* was 100 % (unpublished results). Based on the noticeable hypertrophy and hyperaemia of intestine (shown in Fig. 3), the enteritis severity of twenty-four fish from each treatment was evaluated as described by Huang *et al.*[66] and Song *et al.*[57]. As shown in Fig. 4, the enteritis morbidity of on-growing grass carp had no significant differences with increasing CT to 10 and 20 g/kg, but were significantly enhanced with CT levels up to 30 g/kg and above (*P* < 0·05).
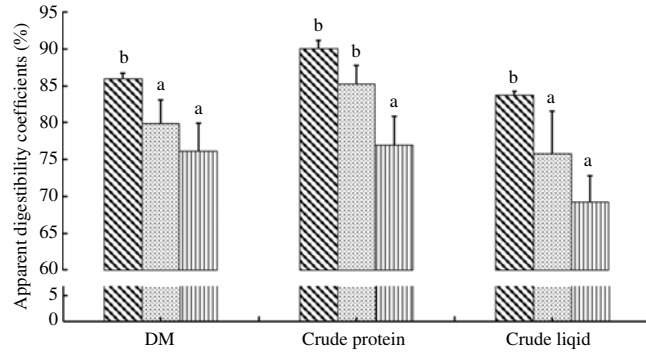

Fig. 1. Apparent digestibility coefficients of DM, crude protein and crude lipid of experimental diets. Values are means and standard deviations for three replicate groups, with thirty fish in each group. [a,b] Mean values with unlike letters in the same row are significantly different (*P* < 0·05; ANOVA and Duncan's multiple range tests). ▨, 0 g/kg diet condensed tannins (CT); ▧, 20 g/kg CT; ▥, 50 g/kg CT.

Table 3. Intestinal morphological changes in on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing different levels of condensed tannins (CT) for 70 d*
(Mean values)

Morphology	Dietary CT level (g/kg)		
	Control (0)	30·00	50·00
PI			
Blood capillary hyperaemia	0	0·33	0·33
Goblet cell hyperplasia	0	0·33	0
Thickening of lamina propria	0	0·33	1·33
Leucocyte infiltration	0	0·33	1·00
Column totals	0[a]	1·33[a,b]	2·67[b]
MI			
Blood capillary hyperaemia	0	0·67	0·33
Goblet cell hyperplasia	0	1·33	1·67
Thickening of lamina propria	0	0·67	2·00
Leucocyte infiltration	0	0·67	1·67
Column totals	0[a]	3·33[b]	5·67[c]
DI			
Blood capillary hyperaemia	0	1·33	0·67
Goblet cell hyperplasia	0·33	1·33	2·00
Thickening of lamina propria	0·67	0·33	2·00
Leucocyte infiltration	0	0·67	1·67
Column totals	1·00[a]	3·67[b]	6·33[c]

PI, proximal intestine; MI, mid intestine; DI, distal intestine.
[a,b,c] Mean values with unlike superscript letters among groups are significantly different (*P* < 0·05).
* The morphological changes are based on the microscopy evaluation of ten micrographs from each fish in each treatment group. Values are means, *n* 3 fish in each treatment. Tissue changes were assessed as follows: 0 = not observed; 1 = low frequency (1–3 out of 10 images); 2 = moderate frequency (4–6 out of 10 images) and 3 = high frequency (≥7 out of 10 images).
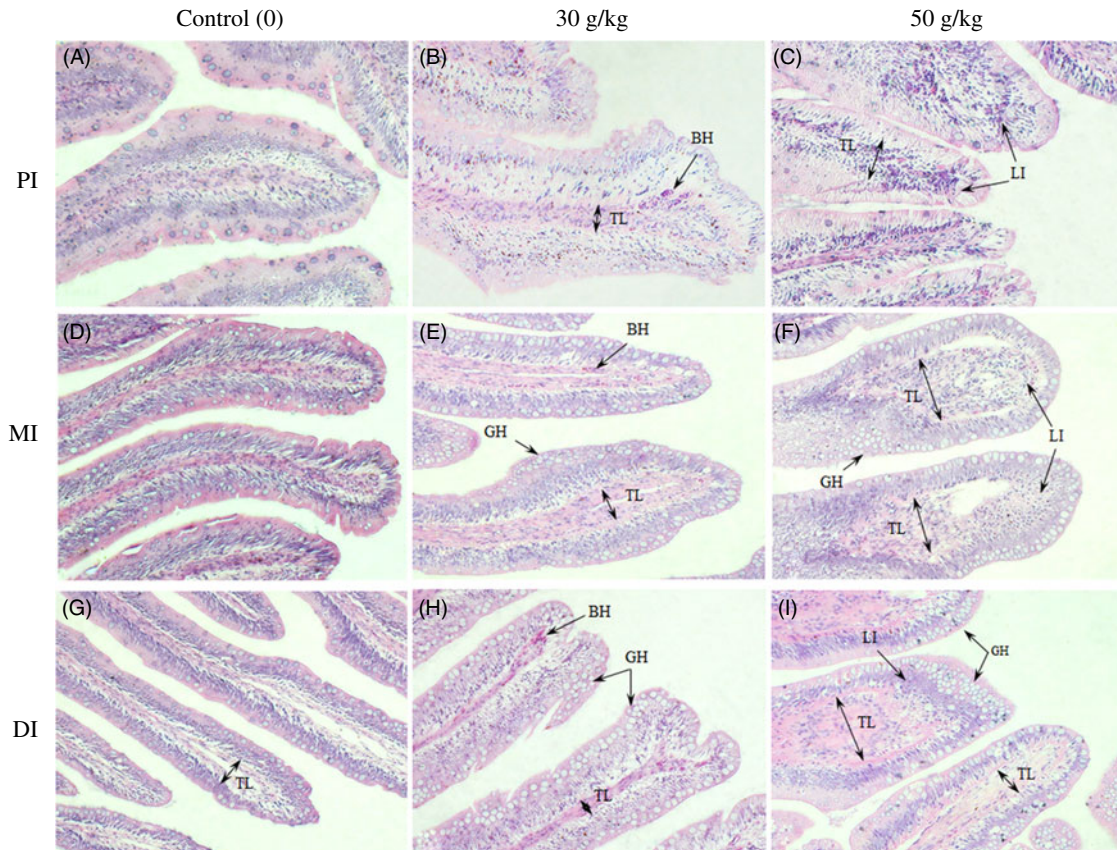

Fig. 2. Histology of the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) in on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing different levels of condensed tannins (CT) (g/kg diet) for 70 d. (A) Control (0), (B) 30 g/kg diet and (C) 50 g/kg diet group in PI; (D) control (0), (E) 30 g/kg diet and (F) 50 g/kg diet in MI; (G) control (0), (H) 30 g/kg diet and (I) 50 μg/kg diet in DI. The sections were stained with haematoxylin–eosin and observed at 200 × original magnification. In each panel, BH, blood capillary hyperaemia; GH, goblet cell hyperplasia; TL, thickening of lamina propria; LI, leucocyte infiltration.
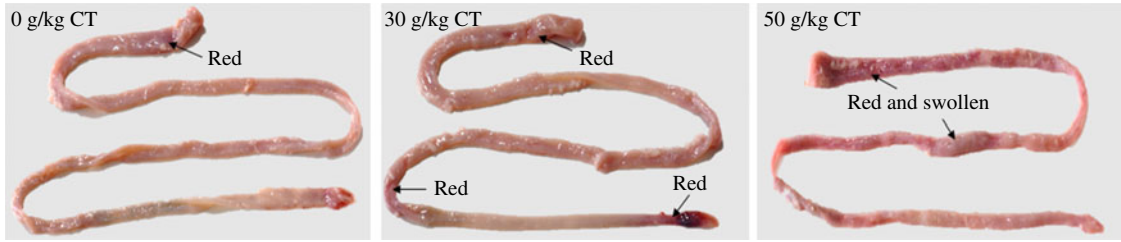
Fig. 3. Enteritis symptoms of on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing different levels of condensed tannins (CT) after challenge with *Aeromonas hydrophila* for 14 d.
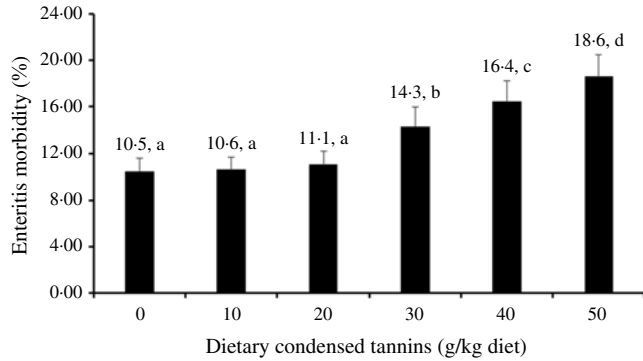

Fig. 4. Effects of graded levels of condensed tannins (CT) on enteritis morbidity of on-growing grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. Values are means (*n* 24 individuals in each group), with standard deviations represented by vertical bars. [a,b,c,d] Mean values with unlike letters are significantly different (*P* < 0·05; ANOVA and Duncan's multiple range tests).

Immune parameters in the intestine of on-growing grass carp

As shown in Table 4, the activities of LZ and ACP as well as C3, C4 and IgM contents in the intestine of on-growing grass carp were significantly down-regulated when the CT was added to appropriate levels, and then sustainably decreased or gradually plateaued. In the PI, compared with the control group, the activities of LZ and ACP as well as C3 and IgM contents were all obviously lower with increasing CT levels to 30 g/kg diet (*P* < 0·05), while the C4 contents were obviously decreased as the CT levels increased to 20 g/kg diet (*P* < 0·05). In the MI, compared with the control group, the LZ and ACP activities as well as C4 and IgM contents were significantly decreased as the CT levels increased to 20 g/kg diet (*P* < 0·05), while the C3 contents were obviously decreased as the CT levels increased to 30 g/kg diet (*P* < 0·05). In comparison with the control group, with CT levels up to 20, 20, 30, 20 and 30 g/kg diet (*P* < 0·05), the LZ and ACP activities as well as C3, C4 and IgM contents in the DI were remarkably decreased, respectively.

Gene expression in the intestine of on-growing grass carp

Relative mRNA levels of antimicrobial peptides and anti-inflammatory cytokines in the intestine of on-growing grass carp. As shown in Fig. 5, the relative mRNA levels of *Hepcidin*, liver-expressed antimicrobial peptide (*LEAP*)-*2A*, -*2B*, *β-defensin-1*, *Mucin-2*, *TGF-β1*, *IL-4/13A*, *IL-10* and *IL-11*

in the intestine of on-growing grass carp were significantly down-regulated when the CT was added to appropriate levels, and then sustainably decreased or gradually plateaued. In the PI, compared with the control group, the relative mRNA levels of *Hepcidin*, *LEAP-2A*, -*2B*, *β-defensin-1*, *Mucin-2*, *TGF-β1*, *TGF-β2*, *IL-4/13A*, *IL-10* and *IL-11* were significantly down-regulated when the CT levels were up to 40, 30, 30, 20, 30, 30, 30, 40, 40 and 30 g/kg diet (*P* < 0·05), respectively. Compared with the control group, the mRNA levels of *Hepcidin*, *LEAP-2A*, -*2B*, *β-defensin-1*, *Mucin-2*, *TGF-β1*, *IL-4/13A*, *IL-10* as well as *IL-11* in the MI of on-growing grass crap were significantly down-regulated when the CT levels reached 30, 30, 30, 30, 30, 40, 30, 30 and 30 g/kg diet (*P* < 0·05), respectively. In the DI, compared with the control group, the mRNA levels of *Hepcidin*, *LEAP-2A*, -*2B*, *β-defensin-1*, *Mucin-2*, *TGF-β1*, *IL-4/13A*, *IL-10* as well as *IL-11* obviously descended when the CT increased to 20, 10, 30, 20, 30, 30, 50, 40 and 20 g/kg diet (*P* < 0·05), respectively. Interestingly, CT had no effect on the mRNA expression of *TGF-β2* in the MI and DI and *IL-4/13B* in the three intestinal segments of on-growing grass crap (*P* > 0·05).

Relative mRNA levels of pro-inflammatory cytokines in the intestine of on-growing grass carp. As shown in Fig. 6, the relative mRNA levels of *IFN-γ2*, *IL-1β*, -*6*, -*12p35*, -*12p40*, -*15* and -*17D* in the intestine of on-growing grass carp were significantly up-regulated when the CT was added to appropriate levels, and then sustainably increased or gradually plateaued. In the PI of on-growing grass crap, compared with the control group, the relative mRNA levels of *IFN-γ2*, *IL-1β*, -*6*, -*12p35*, -*12p40*, -*15* and -*17D* were significantly up-regulated with the CT levels up to 30, 30, 40, 40, 30, 30 and 30 g/kg diet (*P* < 0·05), respectively. In the MI, compared with the control group, the relative mRNA levels of *IFN-γ2*, *IL-1β*, -*6*, -*8*, -*12p35*, -*12p40*, -*15* and -*17D* significantly increased when the CT levels reached 40, 30, 30, 30, 30, 30, 40 and 20 g/kg diet (*P* < 0·05), respectively. Similarly, when the CT levels were up to 30, 30, 20, 30, 30, 30, 30 and 30 g/kg diet (*P* < 0·05) in the DI, the relative mRNA levels of *IFN-γ2*, *IL-1β*, -*6*, -*8*, -*12p35*, -*12p40*, -*15* and -*17D* were significantly increased, respectively. Specifically, the CT level had no significant impact on the relative mRNA level of *IL-8* in the PI and *TNF-α* in the three intestinal segments of on-growing grass carp (*P* > 0·05).

Relative mRNA levels of inflammation-related signalling molecules in the intestine of on-growing grass carp. As shown in Fig. 7, the relative mRNA levels of *TOR*, *S6K1* and *IκBα* in the

Table 4. Effect of condensed tannin (CT) levels on immune parameters in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of on-growing grass carp (*Ctenopharyngodon idella*) with fed diets containing graded levels of CT for 70 d after injection infected with *Aeromonas hydrophila* for 14 d (*n* 6) (Mean values and standard deviations)

CT level (g/kg)...	0		10·00		20·00		30·00		40·00		50·00	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PI												
LZ	109·14[b]	5·40	107·99[b]	7·76	103·06[b]	3·95	87·44[a]	5·83	86·34[a]	7·64	81·59[a]	4·16
ACP	228·91[c]	18·14	221·72[c]	9·37	218·53[c]	12·97	170·76[b]	7·70	164·88[b]	13·43	143·10[a]	9·84
C3	62·97[c]	5·28	61·02[c]	2·28	59·93[c]	4·25	55·07[b]	3·12	51·66[a,b]	4·16	49·90[a]	3·13
C4	10·37[d]	0·76	9·83[c,d]	0·96	9·30[c]	0·67	7·12[b]	0·17	6·12[a]	0·47	5·86[a]	0·31
IgM	61·93[c]	3·70	61·08[c]	4·56	59·20[c]	2·66	50·01[b]	4·27	49·33[b]	1·75	44·17[a]	3·40
MI												
LZ	107·74[d]	7·66	102·38[c,d]	3·83	97·66[c]	4·96	88·69[b]	6·01	83·30[b]	6·24	72·37[a]	3·73
ACP	230·65[d]	10·69	215·68[c,d]	18·52	207·66[c]	14·22	173·00[b]	11·65	158·76[a,b]	14·69	155·01[a]	5·74
C3	60·86[c]	3·10	60·88[c]	4·51	57·06[b,c]	3·08	54·87[a,b]	4·08	53·91[a,b]	4·37	52·02[a]	1·49
C4	15·32[d]	0·65	14·72[c,d]	1·14	14·31[c]	0·48	10·12[b]	0·27	9·65[b]	0·35	8·27[a]	0·39
IgM	58·66[d]	2·50	56·76[c,d]	3·21	54·81[c]	3·98	49·37[b]	1·57	46·12[a,b]	3·78	42·61[a]	2·54
DI												
LZ	103·28[d]	7·34	96·85[c,d]	3·12	94·84[c]	5·75	87·58[b]	3·46	82·67[a,b]	5·94	77·13[a]	6·79
ACP	209·20[c]	4·66	201·31[c]	13·58	171·90[b]	9·55	178·68[b]	5·45	145·96[a]	13·59	144·38[a]	10·64
C3	59·04[c]	5·11	57·37[c]	1·77	56·89[c]	2·70	52·73[b]	2·43	51·08[a,b]	3·05	47·87[a]	3·26
C4	13·95[d]	0·91	13·28[c,d]	0·68	13·13[c]	0·36	11·88[b]	0·45	11·41[b]	0·68	10·29[a]	0·53
IgM	64·27[c]	1·85	63·94[c]	3·98	62·23[b,c]	3·14	59·44[a,b]	3·00	59·01[a,b]	4·62	57·98[a]	1·33

LZ, lysozyme activity (U/mg protein); ACP, acid phosphatase (U/mg protein); C3, complement 3 (mg/g protein); C4, complement 4 (mg/g protein).
a,b,c,d Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$; ANOVA and Duncan's multiple range tests).

intestine of on-growing grass carp were significantly down-regulated as the CT added to appropriate levels, and then sustainably decreased or gradually plateaued, while the relative mRNA levels of *4E-BP2*, *NF-κBp65*, *c-Rel*, *IKKα*, *β* and *γ* were significantly advanced as the CT added to appropriate levels, and then sustainably increased or gradually plateaued. In the PI, compared with the control group, the relative mRNA levels of *TOR*, *S6K1* and *IκBα* were all significantly decreased with the CT levels reached to 20 g/kg diet ($P < 0.05$), while the relative mRNA levels of *4 E-BP2*, *NF-κBp65*, *c-Rel*, *IKKα*, *β* and *γ* were significantly advanced with the CT levels reached to 20, 20, 30, 20, 30 and 20 g/kg diet ($P < 0.05$), respectively. In the MI, compared with the control group, the relative mRNA levels of *TOR*, *S6K1* and *IκBα* were all significantly decreased with the CT levels reached to 30 g/kg diet ($P < 0.05$), while the relative mRNA levels of *4E-BP2*, *NF-κBp65*, *c-Rel*, *IKKα*, *β* and *γ* were significantly advanced with the CT levels reached to 30, 30, 20, 30, 30 and 20 g/kg diet ($P < 0.05$), respectively. In the DI, compared with the control group, the relative mRNA levels of *TOR*, *S6K1* and *IκBα* were significantly decreased with the CT levels reached to 40, 30 and 30 g/kg diet ($P < 0.05$), while the relative mRNA levels of *4E-BP2*, *NF-κBp65*, *c-Rel*, *IKKα*, *β* and *γ* were significantly advanced with the CT levels reached to 30, 20, 40, 30, 30 and 30 g/kg diet ($P < 0.05$), respectively. Interestingly, CT had no effect on the mRNA expression of *4 E-BP1* and *NF-κBp52* in the three intestinal segments of on-growing grass crap ($P > 0.05$).

Protein levels of NF-κB p65, phosphorylated target of rapamycin Ser2448 and total target of rapamycin in the intestine of on-growing grass carp

As shown in Figs. 8 and 9, in comparison with the control group, the protein levels of T-TOR and p-TOR Ser2448 protein levels in the PI, MI and DI were both significantly decreased when CT levels were up to 30, 20 and 30 g/kg ($P < 0.05$), respectively. Compared with the control group, the protein levels of NF-κBp65 in the PI, MI and DI were significantly increased when CT levels were up to 20, 20 and 10 g/kg ($P < 0.05$), respectively.

Discussion

Condensed tannins reduced the growth performance and enteritis resistance ability of fish

The results in the present study illustrated that CT reduced the growth performance (FI, FE and PWG) and decreased the feed-stuff utilisation (DM, crude protein and crude lipid) of on-growing grass carp. As we know, the fish growth is closely associated with intestinal function[72]. The present study observed that CT also decreased the intestinal growth (intestinal length, ILI, intestinal weight, ISI and protein content), lessened all of the studied intestinal bacteria amounts (*Aeromonas* spp., *E. coli*, *Lactobacillus* spp. and *Bifidobacterium* spp.), induced the fish intestinal histopathological lesions (goblet cell hyperplasia, thickening of lamina propria, blood capillary hyperaemia and leucocytes infiltration) and diminished enteritis resistance ability of on-growing grass carp (the enteritis morbidity of fish in 50 g/kg CT treatment was 18·6 %, which increases by 77·1 % when compared with that of control group (10·5 %) after infection with *A. hydrophila*). These observations indicated that CT weakens the intestinal function of on-growing grass carp. Interestingly, not only beneficial bacteria amounts but also harmful bacteria amounts have been reduced by CT. Among these, the decrease of *Lactobacillus* amount caused by CT is similar to the report in weaned piglets[73]. And, bacteria amount of *E. coli* depressed by CT might be partly associated with the cytoplasmic membrane
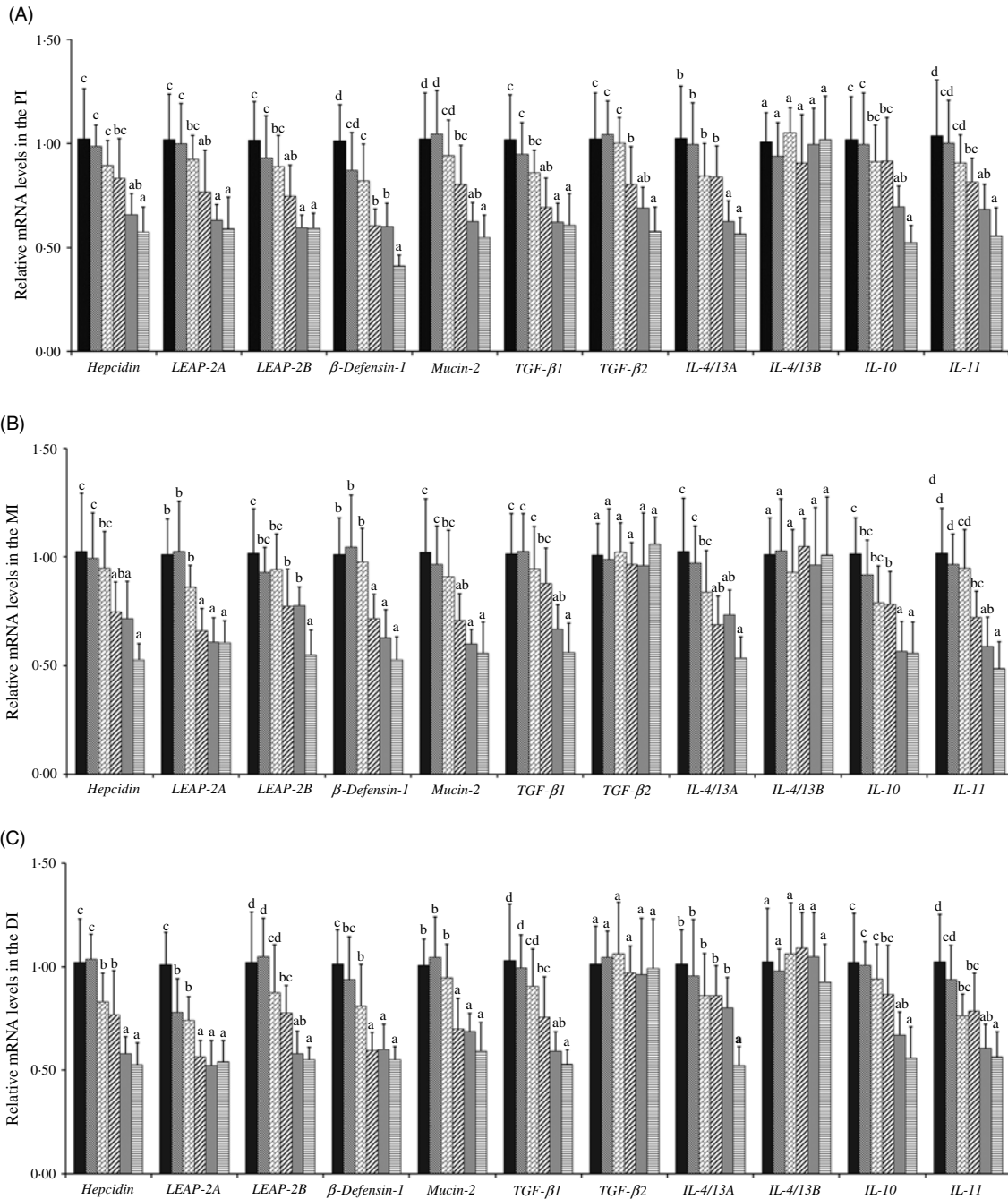
Fig. 5. Effects of condensed tannins (CT) on relative mRNA levels of antibacterial and anti-inflammatory cytokines in the proximal intestine (PI) (A), mid intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. Values are means of six fish in each group, with standard deviations represented by vertical bars. [a,b,c,d] Mean values with unlike letters are significantly different ($P < 0.05$; ANOVA and Duncan's multiple range tests). LEAP, liver-expressed antimicrobial peptide; TGF, transforming growth factor. ■, 0 g/kg diet CT; ◨, 10 g/kg CT; ▨, 20 g/kg CT; ◪, 30 g/kg CT; ▦, 40 g/kg CT; ▤, 50 g/kg CT.

integrity. Hoshino *et al.*[74] have discovered that CT exposure killed *E. coli* cells by damaging the cytoplasmic membrane.

As shown in Fig. 10, according to the broken-line analysis of the PWG, the maximum allowable level of CT for on-growing grass carp was estimated to be 18·6 g/kg diet, which was a little higher than that based on the enteritis resistance ability (17·4 g/kg). This phenomenon was in agreement with the study

of gossypol in on-growing grass carp[9]. The result indicated that infection with intestinal pathogenic bacteria could decrease the tolerance of on-growing grass carp on CT. In addition, research has confirmed that the enteritis resistance ability is closely associated with the intestinal immune function in fish[75]. Therefore, we next investigated the influence of CT on the intestinal function of on-growing grass carp.
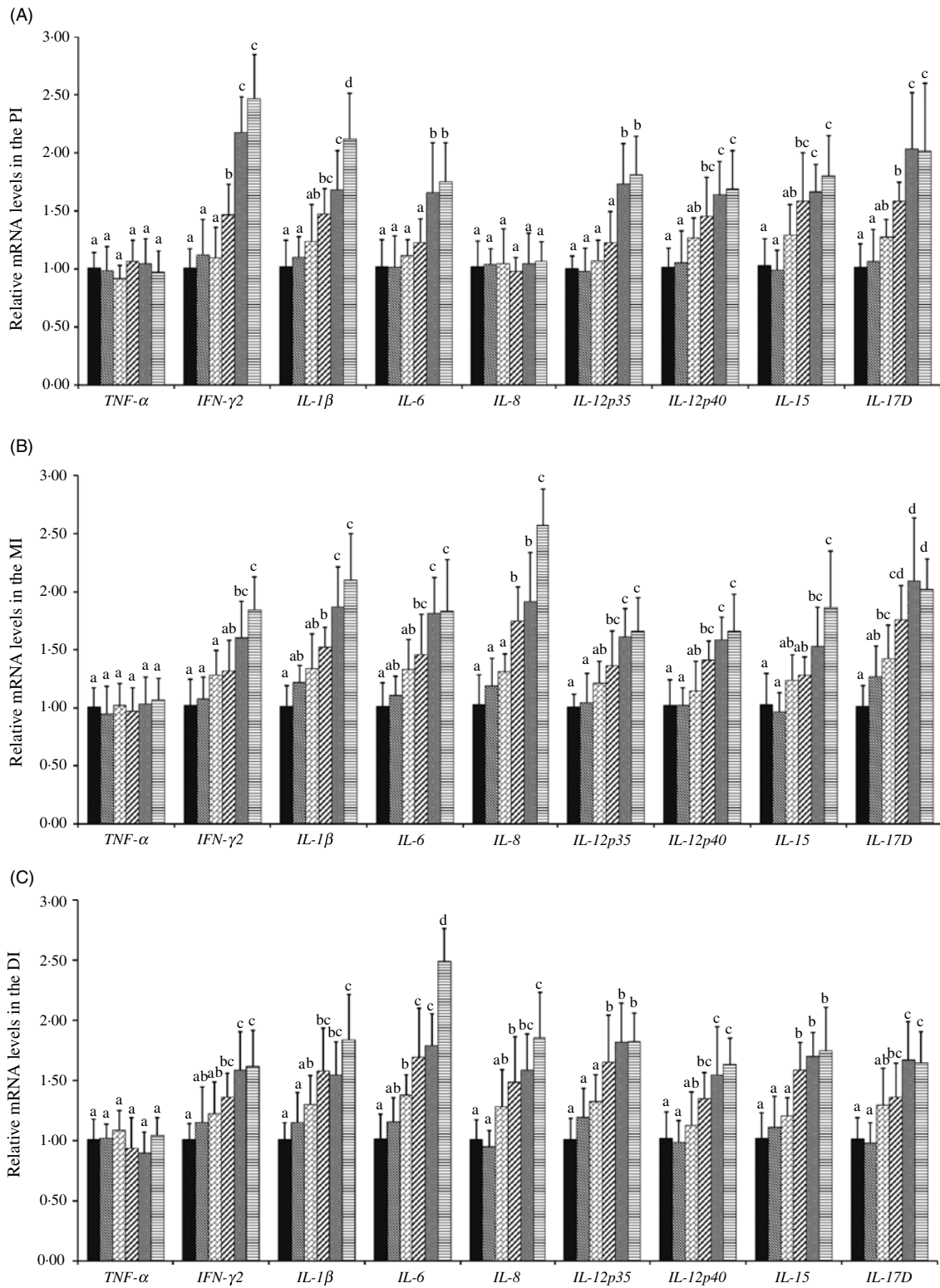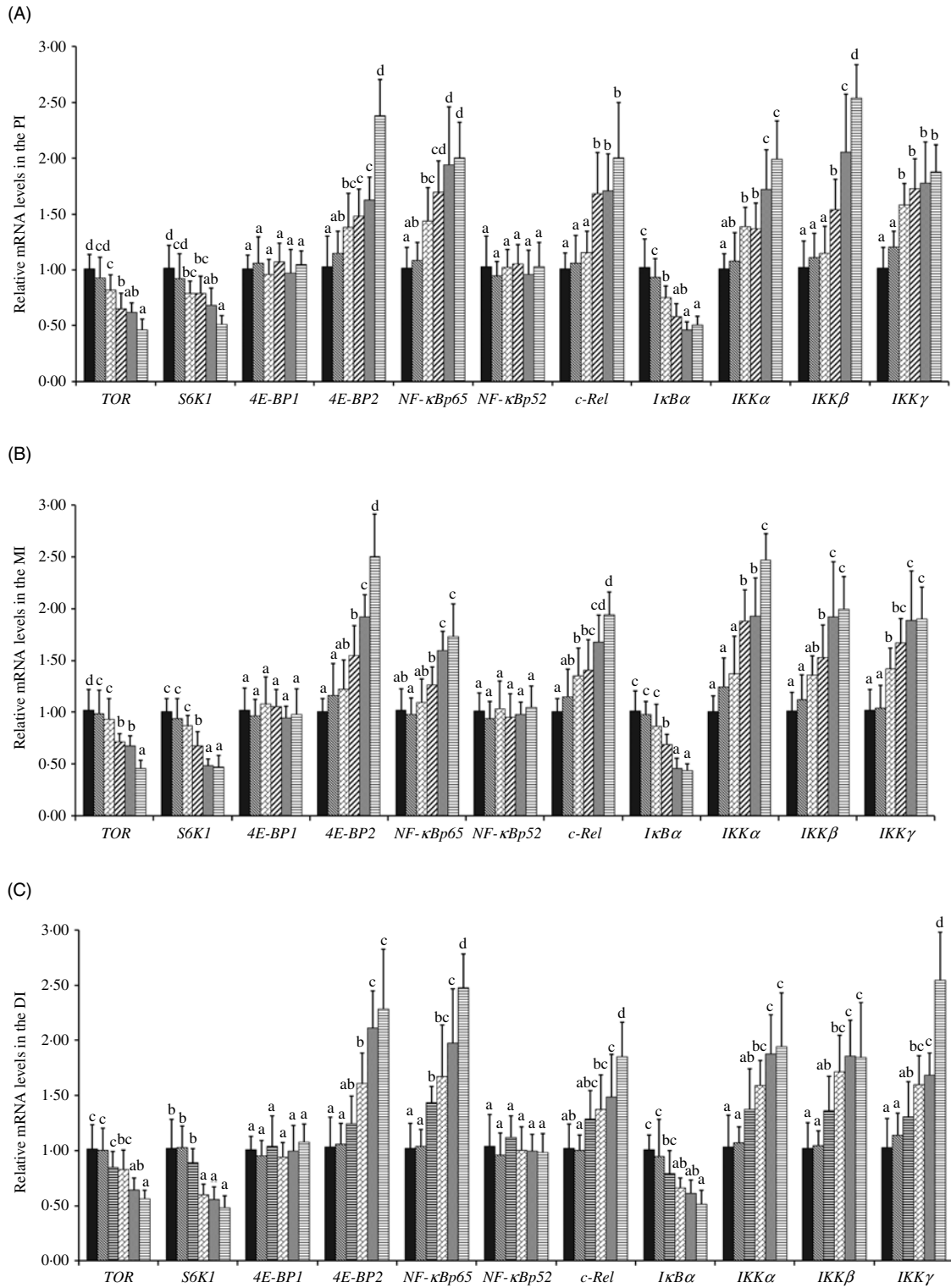
Fig. 6. Effects of condensed tannins (CT) on relative mRNA levels of pro-inflammatory cytokines in the proximal intestine (PI) (A), mid intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. Values are means of six fish in each group, with standard deviations represented by vertical bars. [a,b,c,d] Mean values with unlike letters are significantly different ($P < 0.05$; ANOVA and Duncan's multiple range tests). IFN-γ2, interferon-γ2. ■, 0 g/kg diet CT; ▨, 10 g/kg CT; ▨, 20 g/kg CT; ▨, 30 g/kg CT; ■, 40 g/kg CT; ▤, 50 g/kg CT.

Fig. 7. Effects of condensed tannins (CT) on relative mRNA levels of inflammatory related signalling molecules in the proximal intestine (PI) (A), mid intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. Values are means of six fish in each group, with standard deviations represented by vertical bars. [a,b,c,d] Mean values with unlike letters are significantly different (*P* < 0·05; ANOVA and Duncan's multiple range tests). TOR, target of rapamycin. ☐, 0 g/kg diet CT; ◪, 10 g/kg CT; ☐, 20 g/kg CT; ◪, 30 g/kg CT; ☐, 40 g/kg CT; ◼, 50 g/kg CT.
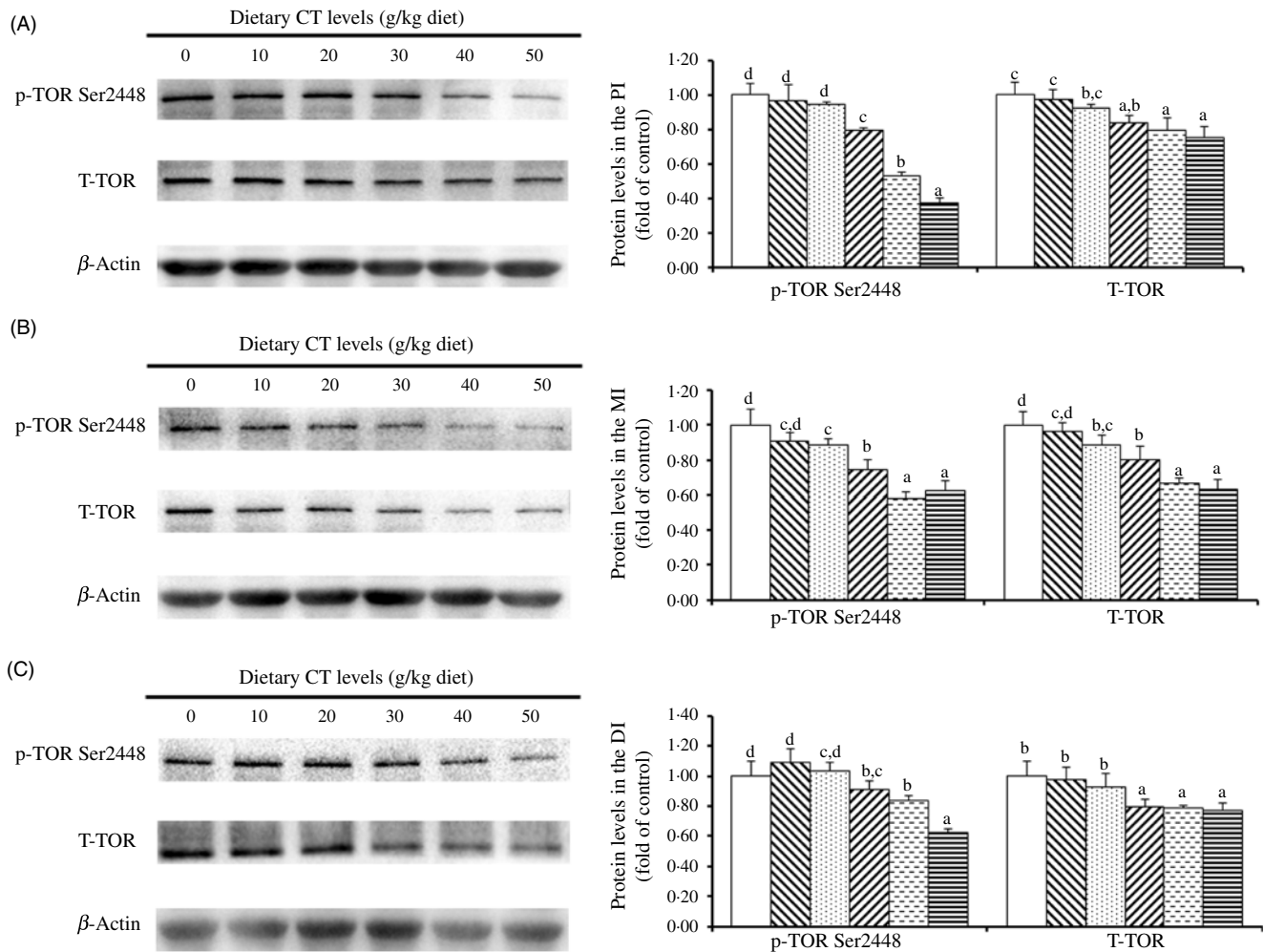
Fig. 8. Western blot analysis of target of rapamycin (TOR) phosphorylation levels at Ser2448 and total TOR (T-TOR) protein levels in the proximal intestine (PI) (A), mid intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila* (β-actin is added as a loading control). Values are means of three fish in each group, with standard deviations represented by vertical bars. [a,b,c,d] Mean values with unlike letters are significantly different ($P < 0.05$; ANOVA and Duncan's multiple range tests). □, 0 g/kg diet condensed tannins (CT); ◨, 10 g/kg CT; ▨, 20 g/kg CT; ◪, 30 g/kg CT; ▨, 40 g/kg CT; ▨, 50 g/kg CT.

Condensed tannins decreased innate and adaptive immunity in the intestine of fish

The immune function of fish relies on the immune response, which is closely related to innate and adaptive immune components such as LZ, ACP, complements, antibacterial peptides and Ig[76]. From our research, CT ($\geq$30 g/kg) decreased the LZ and ACP activities, C3, C4 and IgM contents and down-regulated the *Hepcidin*, *LEAP-2A*, *-2B*, *β-defensin-1* and *Mucin2* mRNA levels in the three intestinal segments of on-growing grass carp. These results demonstrated that CT decreased intestinal immune function of fish.

In addition, another dietary ANF, gossypol, also determined above antibacterial peptides in the intestine of on-growing grass carp[9], but interestingly, partial results were unalike the present study. Firstly, CT decreased the *Hepcidin* mRNA levels in the whole of three intestinal segments. However, gossypol caused down-regulation of the *Hepcidin* mRNA levels only in the PI and MI (not in DI) of fish. The reason for causing such results might be in part associated with the TGF-β1. It was reported that

the inhibition of TGF-β1 could down-regulate the *Hepcidin* mRNA level in mice hepatic cells[77]. In the present study, CT down-regulated *TGF-β1* mRNA levels in all the three intestinal segments, while gossypol down-regulated it only in the PI and MI (not in DI) of fish, which, to some extent, might explain the differences of *Hepcidin* mRNA level induced by CT and gossypol. Secondly, in the three intestinal segments of fish, CT down-regulated the mRNA levels of *LEAP-2A*, whereas this change was unobserved in the study of gossypol, which might be partially associated with IKKα. A study from mice found that IKKα could increase the mRNA level of *IL-22*[78], which could up-regulate the *LEAP-2A* gene expression in splenocytes of rainbow trout (*Oncorbynchus mykiss*)[79]. However, the comparison found that CT increased the *IKKα* gene expression but gossypol had no distinct effect on it in the three intestinal segments of fish. Therefore, we speculated that the different influences of CT and gossypol on the *LEAP-2A* mRNA level might be partially correlated with their different impacts in IKKα. Certainly, these diversities induced by CT and gossypol occurred in intestinal immune
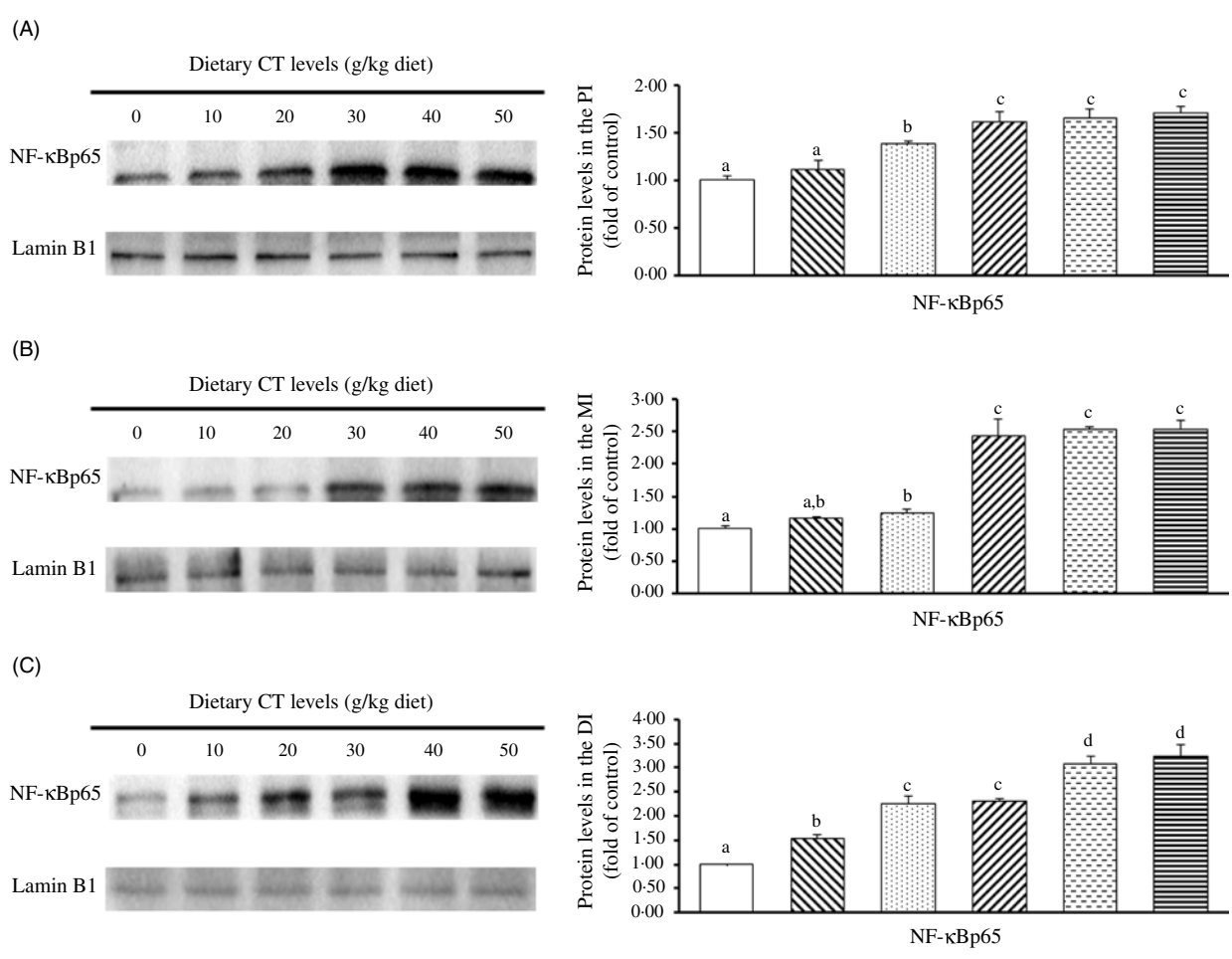
Fig. 9. Western blot analysis of NF-κBp65 protein levels in the proximal intestine (PI) (A), mid intestine (MI) (B) and distal intestine (DI) (C) of on-growing grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila* (Lamin B1 is added as a loading control). Values are means of three fish in each group, with standard deviations represented by vertical bars. [a,b,c,d] Mean values with unlike letters are significantly different ($P < 0.05$; ANOVA and Duncan's multiple range tests). ☐, 0 g/kg diet condensed tannins (CT); ◣, 10 g/kg CT; ▦, 20 g/kg CT; ◪, 30 g/kg CT; ▨, 40 g/kg CT; ▤, 50 g/kg CT.

function of on-growing grass carp need more researches to explicate. In addition, the fish intestinal immune function is also correlated with its inflammatory responses, which are mainly mediated by cytokines[80] and related signalling molecules such as TOR and NF-κB[81]. Thus, we next examined the effects of CT on inflammatory cytokines and researched the potential regulation mechanism in the intestine of fish.

Condensed tannins aggravated inflammatory responses by regulating inflammatory cytokines partly related to the target of rapamycin and NF-κB signalling pathways in the intestine of fish

Condensed tannins aggravated intestinal inflammation by regulating inflammatory cytokines in fish. Research indicated that down-regulating anti-inflammatory cytokines (like IL-10) and up-regulating pro-inflammatory cytokines (like IL-1β) mRNA levels could aggravate inflammatory responses in fish[82]. The present results showed that compared with the control group, CT (≥30 g/kg) down-regulated most of the studied anti-inflammatory cytokines (such as *TGF-β1, IL-4/13A, IL-10*

and *IL-11*) and up-regulated the majority of studied pro-inflammatory cytokines (such as *IFN-γ2, IL-1β, -6, -12p35, -12p40, -15* and *-17D*) mRNA levels in the three intestinal segments of on-growing grass carp. These results illustrated that CT could aggravate the intestinal inflammatory responses of fish.

Surprisingly, we discovered four interesting phenomena on the changes of intestinal inflammatory cytokines mRNA levels in fish caused by CT. These might be analysed as follows. Firstly, CT had no effect on the *TNF-α* mRNA levels in the three intestinal segments of on-growing grass carp, which might be partially relevant to the growth hormone. Research has found that growth hormone deficiency caused plasma *TNF-α* level to increase in children[83]. In ewes, CT had no impact upon plasma concentrations of growth hormone[84]. Hence, we speculated that CT had no influence on the level of growth hormone, leading to unaffected *TNF-α* mRNA levels in the three intestinal segments of fish, but the concrete mechanism needs further study. Secondly, CT down-regulated *IL-4/13A* mRNA levels (rather than *IL-4/13B*) in the three intestinal segments of on-growing grass carp, which might be partially associated with TOR and GATA-3. A previous study reported that the absence of
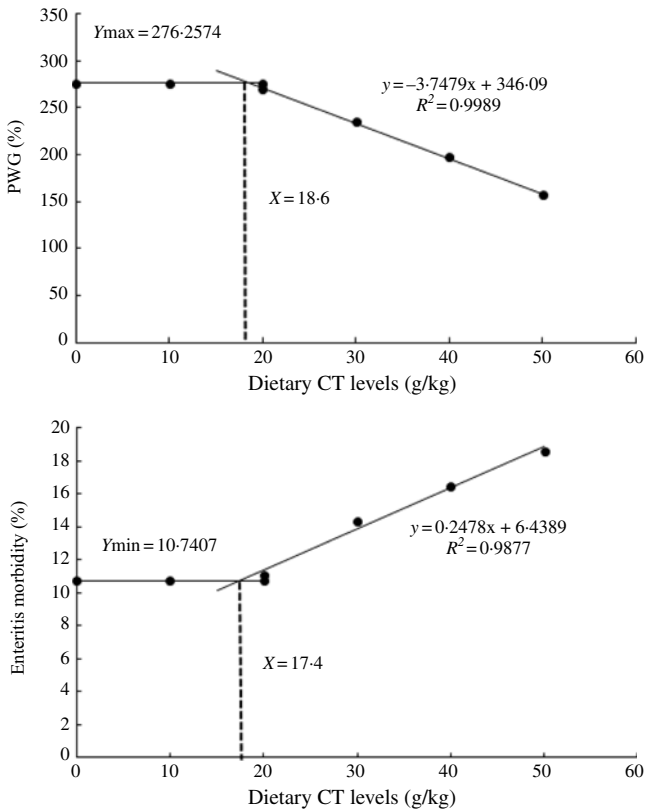
Fig. 10. Broken-line analysis of percentage weight gain (PWG) and enteritis morbidity of on-growing grass carp (*Ctenopharyngodon idella*) containing graded levels of condensed tannins (CT).

mammalian TOR decreased the protein expression of GATA-3 in mice Th2 cells[85]. Ohtani *et al.* indicated that GATA-3 could directly enhance the gene transcription of IL-4/13A rather than IL-4/13B in most teleost[86]. Our data found that CT down-regulated the p-TOR Ser2448 protein levels in three intestinal segments of fish, supporting our hypothesis. Thirdly, CT up-regulated *IL-8* mRNA levels in the MI and DI (rather than PI) of on-growing grass carp, which might be partially associated with cholesterol and glucocorticoid. It was reported that CT decreased the cholesterol level in an *in vitro* experiment[87]. Cholesterol serves as a precursor of glucocorticoid[88], and lessened glucocorticoid could raise the *IL-8* gene expression in human fibroblasts[89]. Moreover, the mRNA abundance of glucocorticoid receptor in the MI and DI was higher than that in the PI of tilapia (*Oreochromis mossambicus*)[90]. Therefore, we supposed that CT up-regulated *IL-8* mRNA levels in the MI and DI (rather than PI) which is partially associated with the higher mRNA levels of glucocorticoid receptor in the MI and DI than PI of fish. However, this supposition needs deeper investigation. Fourthly, CT down-regulated *TGF-β2* mRNA level in the PI (rather than MI and DI) of fish, which might be partially associated with butyric acid, cholecystokinin (CCK) and protein kinase C. It was reported that CT lessened the butyric acid concentrations in the caecum of rat[32]. In neonatal piglets, supplementation with Na-butyrate increased the CCK concentrations in plasma[91]. A literature discovered that CCK could

activate protein kinase C in pancreatic acinar cells[92], whereas inhibited protein kinase C down-regulated proteins of TGFβ-2 in the guinea pig myopic eye[93]. However, Yuan *et al.* found that the mRNA expression of *CCK* in the PI was much higher than that in the MI and DI of *Schizothorax prenanti*[94]. Consequently, we hypothesised that CT down-regulated butyric acid level resulting in decreasing the expression of CCK only in the PI (rather than MI and DI) of fish, which depressed the activity of protein kinase C and then leaded to down-regulate the *TGF-β2* mRNA level only in the PI (rather than MI and DI) of fish. However, further investigation should be conducted to verify this assumption.

The identical inflammatory cytokines were also investigated in the study of gossypol[9]; however, these detrimental effects showed that CT and gossypol differed in ways whereby regulating cytokines to aggravate intestinal inflammatory responses in on-growing grass carp. Examples of CT down-regulated anti-inflammatory cytokines *TGF-β1* and *IL-10* mRNA levels and enhanced pro-inflammatory cytokine *IL-6* mRNA levels in all the three intestinal segments, whereas gossypol decreased *TGF-β1* only in the PI and MI and *IL-10* only in the PI and increased *IL-6* just in the PI of fish. These observations indicated that CT-induced inflammatory responses might be relative equilibrium in the three intestinal segments, whereas gossypol impaired the PI more severely than MI and DI of fish. However, the reasons for these different results are largely unknown, but the difference of IL-6 could be speculated, which might be partly associated with c-Rel. A study found that c-Rel could increase the *IL-6* gene expression in the primary B cells[95]. In the present study, in the whole of three intestinal segments of fish, the mRNA level of *c-Rel* was up-regulated by CT, while that was up-regulated by gossypol just in the PI (rather than MI and DI). Hence, this indicated that CT increased *IL-6* mRNA levels in the three intestinal segments, while gossypol up-regulated it only in PI (rather than MI and DI) of fish, which might be related to their diverse influences on c-Rel. For other differences on inflammatory cytokines between CT and gossypol in the intestine of on-growing grass carp, further studies are needed.

Condensed tannin-induced intestinal inflammation partially associated with target of rapamycin and NF-κB signalling in fish. The study proved that mammalian TOR signalling partly regulated anti-inflammatory cytokines by activating S6K1 and inhibiting 4E-BP associated with eIF4E in humans[96]. The phosphorylation of TOR on residue Ser2448 can be used to monitor the activation of TOR signalling in rainbow trout[97]. In our study, CT (≥30 g/kg) down-regulated *TOR* and *S6K1* mRNA levels, up-regulated the mRNA levels of *4E-BP2* and depressed p-TOR Ser2448 protein levels in the three intestinal segments of on-growing grass carp. Furthermore, correlation analysis indicated that anti-inflammatory cytokines (except IL-4/13B and TGF-β2 in MI and DI) were positively correlated with the p-TOR Ser2448 protein levels (online Supplementary Table S2). Hence, these results implied that CT down-regulated most of the studied anti-inflammatory cytokines gene expressions
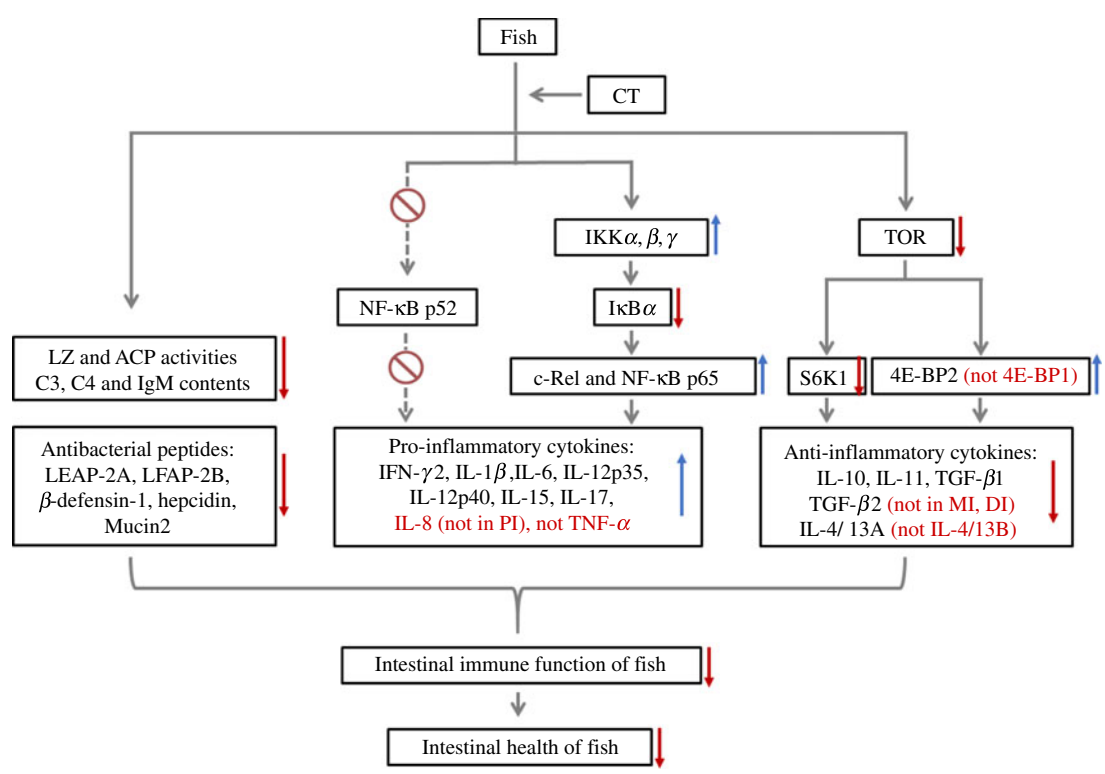
Fig. 11. Potential action pathways of condensed tannins (CT)-disrupted intestinal immune function in fish. TOR, target of rapamycin; LZ, lysozyme; ACP, acid phosphatase; IFN-γ2, interferon-γ2; PI, proximal intestine; TGF, transforming growth factor; MI, mid intestine; DI, distal intestine.

partly associated with (TOR/(S6K1, 4 E-BP2)) signalling pathways in the intestine of fish. Interestingly, CT up-regulated *4E-BP2* mRNA levels rather than *4E-BP1* in the three intestinal segments of fish, which might be partly related to the low protein level. In the present study, our data found that CT reduced the protein contents in the three intestinal segments of fish. A previous study from our laboratory observed that a low level of protein led to the increase of *4E-BP2* mRNA levels, but had no influence on the *4E-BP1* mRNA levels in the three intestinal segments of on-growing grass carp[98]. Hence, we supposed that CT enhanced *4E-BP2* mRNA levels rather than *4E-BP1* in the three intestinal segments of fish partly due to the decrease of protein level in the intestine. However, further investigation should be conducted to verify this assumption.

Additionally, one study reported that the IKK complex (including IKKα, β and γ) promoted the phosphorylation and proteasome degradation of IκBα and then induced the activation of NF-κB (including p65, p52 and c-Rel), which regulated the expression of pro-inflammatory cytokines in mammalian cells[99]. The NF-κB protein levels could be considered hallmarks for the activation of the NF-κB signalling pathway in humans[100]. In the present study, our results showed that CT ($\geq$30 g/kg) induced the up-regulation of *NF-κBp65, c-Rel, IKKα, β, γ* and the NF-κBp65 protein levels and the down-regulation of *IκBα* mRNA levels in the three intestinal segments of on-growing grass carp. Furthermore, the correlation analysis showed that pro-inflammatory cytokines (except *TNF-α* and *IL-8* in PI) mRNA levels were positively correlated with the NF-κBp65 protein levels and *c-Rel* mRNA levels (online Supplementary Table S2). The above data indicated that CT up-regulated the mRNA levels of most of the studied pro-inflammatory cytokines which might be partly associated with ((IKKα, β, γ)/IκBα/(NF-κBp65, c-Rel)) signalling pathways in the intestine of fish. However, it is interesting that CT have up-regulated the mRNA levels of *NF-κBp65*, but unaffected *NF-κBp52* mRNA levels in the three intestinal segments of on-growing grass carp, which might be relevant to the different activated NF-κB signalling pathways. In mammals, NF-kBp65 and NF-kBp52 could be activated by classical NF-kB signalling pathways and noncanonical NF-kB signalling pathways, respectively[101]. Classical NF-kB activity could suppress noncanonical NF-κB signalling in peripheral blood mononuclear cells[102]. In the present study, we found that CT increased *NF-κB p65* rather than *NF-κB p52* mRNA levels. Hence, we speculated that CT might regulate inflammatory responses in the intestine of fish through classical NF-κB signalling pathways rather than alternative NF-κB signalling pathways. Nevertheless, the supposition still remains to be elucidated.

Interestingly, about TOR and NF-κB signalling pathways, discrepancies still presented between CT and gossypol in the intestine of on-growing grass carp. CT might regulate inflammatory cytokines which are partially associated with (IKKα, β, γ/IκBα/ NF-κBp65, c-Rel) and (TOR/S6K1,4E-BP2 (not 4E-BP1)) signalling pathways, but gossypol activated (IKKβ, γ (not IKKα)/IκBα/ NF-κBp65, c-Rel (not in MI and DI)) and (TOR/S6K1, 4E-BP1 (not 4E-BP2)) signalling pathways in the intestine of fish[9]. The reasons of these discrepant results have been still unclear. Besides, whether the different signalling pathways activated by CT and gossypol were the accounts for the discrepancies

on inflammatory cytokines in the intestine of on-growing grass carp needs to be found.

Conclusion

In summary (Fig. 11), we found that dietary CT decreased the growth performance, feed utilisation, intestinal growth and bacterial counts of on-growing grass carp. Meanwhile, we for the first time found that dietary CT impaired intestinal immune function of fish as displayed in the following aspects. Compared with the control group, dietary CT (1) induced intestinal histopathological lesions and aggravated enteritis of on-growing grass carp; (2) reduced the innate and adaptive immune immunity partly related to decrease the immune components including LZ, ACP, C3, C4, IgM and antimicrobial peptides; (3) aggravated intestinal inflammatory responses partially by down-regulating most of the studied anti-inflammatory cytokines (except *IL-4/13B* and *TGF-β2* in the MI and DI) mRNA levels and up-regulating the majority of studied pro-inflammatory cytokines (except *TNF-α* and *IL-8* in the PI) in the intestine of fish, which were partially associated with (TOR/(S6K1 and 4E-BP2)) and (IKKα, β, γ/IκBα/NF-κB (p65 and c-Rel)) signalling pathways, respectively, but not TOR/4E-BP1 and NF-κBp52 signalling pathways; (4) induced inflammatory responses might be relative equilibrium in the three intestinal segments, whereas gossypol, our previous study by Wang *et al.*[9], impaired the PI more severely than MI and DI of fish. In addition, based on the PWG, enteritis morbidity, the maximum allowable levels of CT for on-growing grass carp (232·22–890·11 g), were estimated to be 18·6 and 17·4 g/kg diet, respectively. Furthermore, our experiment has discussed the on-growing grass carp, so what the effect of CT on adult grass carp will be much interesting. Moreover, on the basis of our result that infection with pathogenic bacteria decreased the tolerance of on-growing grass carp on CT, it is betokening that the maximum allowable level of fish like carps even other animals on CT might be relatively lower in the wild or commercial farms, which needs more exploration and research.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114519003295

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