Dietary choline deficiency and excess induced intestinal inflammation and alteration of intestinal tight junction protein transcription potentially by modulating NF-κB, STAT and p38 MAPK signaling molecules in juvenile Jian carp

Pei Wu ^{1,2,3}, Wei-Dan Jiang ^{1,2,3}, Jun Jiang ^{2,3}, Juan Zhao ¹, Yang Liu ^{1,2,3}, Yong-An Zhang ⁴, Xiao-Qiu Zhou ^{1,2,3 *}, Lin Feng ^{1,2,3 *}

¹*Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, 611130, China*

²*Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu, 611130, China*

³*Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Sichuan Agricultural University, Chengdu, 611130, China*

⁴*Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China*

Running title: Choline modulated intestinal immunity

* Corresponding authors. Tel.: +86-835-2885157; Fax: +86-835-2885968.

E-mail address: zhouxq@sicau.edu.cn (X.-Q. Zhou); fenglin@sicau.edu.cn (L. Feng).

Abstract

This study investigated the effects of choline on intestinal mucosal immune and the possible mechanisms in fish by feeding juvenile Jian carp (*Cyprinus carpio* var. Jian) with graded levels of dietary choline (165-1820 mg/kg diet) for 65 days. The results firstly showed that choline deficiency induced inflammatory infiltration in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of fish. Meanwhile, compared with the optimal choline group, choline deficiency decreased the activities of lysozyme and acid phosphatase, contents of complement 3 and IgM in the intestine, downregulated the mRNA levels of antimicrobial peptides (liver-expressed antimicrobial peptide (LEAP) 2A and defensin-3 in the PI and MI, LEAP-2B and hepcidin in the PI, MI and DI), anti-inflammatory cytokines (interleukin (IL) 10 and transforming growth factor β 2 in the PI, MI and DI), and signaling molecule I κ B in the PI, MI and DI; while upregulated the mRNA levels of pro-inflammatory cytokines (IL-6a and tumor necrosis factor α in the MI and DI, interferon γ 2b in the PI and MI, IL-1 β and IL-6b in the PI, MI and DI), and signaling molecules (Toll-like receptor 4 in the MI, myeloid differentiation primary response 88 in the PI and MI, Janus kinase 3 and tyrosine kinase 2 in the MI and DI, nuclear factor kappa B (NF- κ B), signal transducers and activators of transcription (STAT) 4 and STAT5 in the PI, MI and DI) of juvenile Jian carp, further indicating that choline deficiency caused inflammation and immunity depression in the intestine of fish. But choline deficiency decreased the PI IL-6a mRNA level, and increased the DI LEAP-2A and defensin-3 mRNA levels with unknown reasons. Furthermore, dietary choline deficiency downregulated

44 mRNA levels of tight junction (TJ) proteins (claudin 3c in the PI and MI, claudin 7,
45 claudin 11 and occludin in the PI, MI and DI) and signaling molecule
46 **mitogen-activated protein kinases p38** in the PI, MI and DI of juvenile Jian carp,
47 whereas upregulated the mRNA levels of claudin 3b in the MI and DI, and claudin 3c
48 in the DI. Moreover, the excessive choline exhibited negative effects on intestinal
49 immunity and TJ proteins that were similar to the choline deficiency. In summary,
50 dietary choline deficiency or excess caused the depression of intestinal mucosal
51 immune by inducing inflammation and dysfunction of the intestinal physical barrier,
52 and regulating related signaling molecules of fish.

53 **Keywords:** Choline, Cytokine, Intestinal mucosal immunity, NF- κ B, STAT, Tight
54 junction protein

55

1. Introduction

The intestine is an important site for nutrient digestion and absorption in fish, especially in stomachless species, and an important barrier for preventing entrance of antigens and pathogens in fish [1]. It was reported that the impaired intestinal barrier function resulted in increasing of disease susceptibility in Atlantic salmon (*Salmo salar* L.) [2]. Therefore, the intestinal barrier function is of outmost importance to maintain health of fish. Recently, the dietary nutrition stimulus is considered as one of the major strategies to increase intestinal barrier function in fish. Study from our laboratory firstly found that several vitamins such as thiamin [3], riboflavin [4], niacin [5] and folic acid [6] improved the intestinal barrier in grass carp (*Ctenopharyngodon idella*). Choline is considered to be an essential vitamin for fish [7]. In our previous study, we found that choline improved the intestinal digestive function [8], and enhanced the diseases resistance and systemic immunity of juvenile Jian carp (*Cyprinus carpio* var. Jian) [9]. However, whether choline could improve the intestinal barrier function of fish is unclear.

Fish intestinal immune barrier is an important part of the intestine barrier line, and consists of antibacterial compounds including lysozymes, complement factors and antimicrobial peptides (AMPs) [10]. Meanwhile, study from our laboratory found that intestinal immune barrier was impaired by the intestinal inflammatory disorders which were accompanied with upregulated pro-inflammatory cytokines and decreased anti-inflammatory cytokines in fish [11]. Nuclear factor kappa B (NF- κ B), and signal transducers and activators of transcription (STAT) are important transcriptional

activators that regulate cytokines transcription [12]. NF- κ B can be regulated by the inhibitory protein I κ B [13], and the upstream signaling molecules Toll-like receptor 4 (TLR4) and myeloid differentiation primary response 88 (MyD88) in mammal [14]. STAT can be activated by the upstream signaling molecule Janus kinases (JAKs) [15]. To date, no information concerned about the effect of choline on the intestinal immune barrier and the possible underlying mechanisms in fish. Our previous study showed that choline enhanced serum lysozyme activity and complement 3 (C3) content, decreased pro-inflammatory cytokines interleukin (IL) 1 β and tumor necrosis factor (TNF) α mRNA levels while increased anti-inflammatory cytokines IL-10 mRNA levels in spleen and head kidney of juvenile Jian carp [9]. Furthermore, choline is the precursor for biosynthesis of acetylcholine (ACh) which is the cholinergic neurotransmitter [16]. In rat, a cholinergic agonist induced the secretion of intestinal lysozyme and defensin [17]. Stimulation of the vagus nerve decreased the IL-6 levels and activated STAT3 in mouse intestinal macrophages [18]. Choline also acts as an agonist of α 7 nicotinic acetylcholine receptor (α 7nAChR) in mice [19]. Activating α 7nAChR attenuated the TNF- α level in lamina propria mononuclear cells of colitis mice [20], inhibited the NF- κ B activation and increased the I κ B levels in human microvascular endothelial cells [21]. Accordingly, choline may affect the intestinal immune barrier potentially by NF- κ B and STAT signaling pathways in fish, however, this warrants further study.

Fish intestinal physical barrier is another important part of intestinal barrier line, and composes of intestinal epithelial cells and the tight junction (TJ) [1]. Occludins

and claudins are two main protein families found in TJs and form the backbone of the TJ [22]. Moreover, mitogen-activated protein kinases (MAPKs) have emerged as important regulators of the TJ in mammal [23]. In human gastric epithelial cells, inhibition of p38^{MAPK} prevented the decrease of occludin expression and disruption of TJ [24]. Yet no literature data are available concerning the effect of choline on intestinal TJs and the related signaling pathway. As mentioned above, the important metabolite of choline, acetylcholine is the principle vagal neurotransmitter. In rabbit, vagal stimulation increased the intestinal epithelial permeability [25]. Moreover, our previous study showed that choline downregulated the TNF- α expressions in spleen of Jian carp [9]. In human colon carcinoma cells, TNF- α decreased the occludin expression [26]. In rat, choline inhibited the activation of p38^{MAPK} in ventricular myocytes [27]. Thus, there might be a relationship between choline and the intestinal TJs and signaling molecule p38^{MAPK}, which warrants investigation.

This study was a part of a larger study that involved in exploring the effects of choline on fish growth and disease resistance, and used the same growth trial as we previously reported [8]. Our previous study showed that dietary choline improved the intestinal digestive function [8], the systemic immunity and disease resistance of fish [9]. Here, the present study was performed to investigate the effect of dietary choline on the mucosal immune components, cytokines, tight junction proteins and related signaling molecules in the intestine of juvenile Jian carp aiming to provide partial theoretical evidence for the mechanisms by which choline improved health of fish.

2. Material and methods

2.1. Diets and feeding trail

The present study used the same animal trial as our previous study [8]. Formulation of the basal diet is presented in Table 1. The basal diet was formulated to contain 315.3g crude protein/kg diet and 44.0g lipid/kg diet. Choline chloride (Sigma, St Louis, MO, USA) was added to the basal diet to form six experimental diets with graded levels of choline, i.e. 165 (choline deficiency), 310, 607, 896, 1167 and 1820 mg/kg diet, which was measured according to the method of Venugopal [28]. The diets were fan-dried at room temperature and stored at -20 °C until use as our previous study [8].

The Guidelines for the Care and Use of Laboratory Animals of Animal Nutrition Institute, Sichuan Agricultural University were strictly followed during the feeding trail. After obtained from Tong Wei Hatchery (Sichuan, China), juvenile Jian carp were adapted to the experimental environment for one month. A total of 1200 fish with average initial weight 7.94 ± 0.03 g were randomly assigned to 24 aquaria (90 L×30 W×40 H cm). The aquaria system, water quality, and operation of the culture system were as previously described [8]. In brief, fish were reared in a closed recirculating water system with dissolved oxygen higher than 5 mg/L. Water temperature and pH were controlled to be 25 ± 1 °C and 7.0 ± 0.3 , respectively. Quadruplicate of fish were fed with each of the six experimental diets to satiation for 65 days as previously described [8]. The uneaten feed was removed by siphoning after each meal.

2.2. Tissue collection and immune parameters assay

After anaesthetizing the fish by benzocaine bath (50 mg/L), the whole intestine of five fish from each aquarium were removed, weighed and frozen in liquid nitrogen, then stored at -70 °C until analyzed. Intestines of another five fish from each aquarium were collected as three intestinal segments (proximal intestine, mid intestine and distal intestine) according to Syakuri et al. [29]. Parts of the proximal intestine, mid intestine and distal intestine were preserved in 10% neutral buffered formalin for histological examination. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University.

The whole intestine samples were homogenized in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at 6000 g at 4 °C for 20 min, and the supernatant was conserved for immune parameters analysis. Lysozyme activity was determined according to El-Boshy et al. [30]. **Acid phosphatase (ACP)** activity was assayed according to the method described by Barka and Anderson [31]. C3 and IgM contents were determined using the corresponding kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocols. Intestine protein content was determined by the method of Bradford [32].

2.3. Histological examination

Preserved tissue segments were embedded in paraffin wax after dehydration and clear. Tissue was sectioned to 4 mm. Sections were stained using standard hematoxylin and eosin (H & E) and examined by a Nikon TS100 light microscope.

2.4. Quantitative real-time PCR (RT-qPCR) analysis

Total RNA was extracted from the proximal intestine (PI), mid intestine (MI) and

distal intestine (DI) using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's procedure followed by DNase I treatment. Quantity and quality of RNA were determined by electrophoresis on 1% agarose gels and by spectrophotometry at 260 and 280 nm. The RNA was used to synthesize cDNA using the PrimeScript^{TM} RT reagent Kit (TaKaRa, Dalian, China) following the manufacturer's protocol. RT-qPCR analysis was performed with CFX96^{TM} Real-Time PCR Detection System (Bio-Rad) using the fluorescent agent SYBR Green (Takara, Dalian, China) as previously described [33]. The specific primers for target and housekeeping genes and the annealing temperature for each gene are presented in Table 2. Melting curve analysis of amplification products was performed at the end of each amplification to confirm that only a single PCR product was amplified and detected. Gene transcription level was normalized to the β -actin content in each sample using the $2^{-\Delta\Delta C_t}$ method [34].

2.5. Statistical analysis

Results were presented as means $\pm$ SE. All data were subjected to one-way analysis of variance (ANOVA) followed by the Duncan's method to determine significant differences among groups at the level of $P < 0.05$ through SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The parameters with significant differences were subjected to a linear regression or quadratic regression model.

3. Results

3.1. Histopathological examination

The histological results showed that dietary choline deficiency and excess induced

inflammatory infiltration of the intestine in juvenile Jian carp. In the proximal intestine, the edema and leucocyte infiltration in the lamina propria were observed in the choline-deficient group (Fig. 1A), the blood capillary hyperemia was found in the group fed with choline of 310 mg/kg diet (Fig. 1B), and the increased leucocytes in the lamina propria was observed in the group fed with choline of 1820 mg/kg diet (Fig. 1F). In the mid intestine, the choline-deficient diet induced the leucocyte infiltration and blood capillary hyperemia in the lamina propria (Fig. 2A). Meanwhile, the blood capillary hyperemia was observed in the mid intestine of fish fed with 310, 1167 and 1820 mg choline/kg diet (Fig. 2B, 2E and 2F). Besides, the increased leucocyte in the lamina propria of mid intestine was observed in the group fed with 1820 mg choline/kg diet (Fig. 2F). In the distal intestine, the choline-deficient diet induced the same histological changes as those in the mid intestine (Fig. 3A). Meanwhile, the blood capillary hyperemia in the distal intestine was appeared in the group fed with choline of 310 mg/kg diet (Fig. 3B). The leucocyte infiltration in the lamina propria of the distal intestine was found in the group fed with choline of 1820 mg/kg diet (Fig. 3F).

3.2. Intestinal immune parameters

The results of lysozyme, ACP, C3 and IgM are shown in Table 3. Lysozyme activity was improved with increase of dietary choline levels up to 896 mg/kg diet, and then decreased significantly with further increase of choline ($P<0.05$). ACP activity was the highest in groups fed with choline of 310 and 607 mg/kg diet ($P<0.05$). Intestinal C3 contents in groups fed with choline equal to or higher than 310

mg/kg diet were significantly higher than that in the choline-deficient group ($P<0.05$). IgM content was the highest in groups fed with 607 and 896 mg choline/kg diet. Regression analysis showed that the intestinal lysozyme activity was quadratic responses to dietary choline levels (Table 3).

3.3. Changes of antimicrobial peptide mRNA levels in the intestine

As shown in Fig. 4A, transcription levels of liver-expressed antimicrobial peptide (LEAP) 2A, LEAP-2B, hepcidin and defensin-3 genes in the proximal intestine (PI) of juvenile Jian carp were significantly affected by dietary choline. The relative mRNA level of LEAP-2A in the PI was significantly higher in groups fed the diets supplemented with 310 and 607 mg choline/kg diet than those in other groups ($P<0.05$). The mRNA level of LEAP-2B in the PI was upregulated by dietary choline and the lowest in the choline-deficient group ($P<0.05$). The relative mRNA level of hepcidin in the PI was the highest in the group of 310 mg choline/kg diet ($P<0.05$). The mRNA level of defensin-3 in the PI was the highest in the group fed with 607 mg choline/kg diet ($P<0.05$). The mRNA levels of LEAP-2A, LEAP-2B, hepcidin and defensin-3 in the mid intestine (MI) of juvenile Jian carp are presented in Fig. 4B. The transcription level of LEAP-2A gene in the MI was the highest in the group fed with choline of 310 mg/kg diet ($P<0.05$). The transcription levels of LEAP-2B and hepcidin gene in the MI were upregulated by dietary choline and the lowest in the choline-deficient group ($P<0.05$). The mRNA level of defensin-3 in the MI was the highest in the group fed with 310 mg choline/kg diet ($P<0.05$). As presented in Fig. 4C, the relative mRNA levels of LEAP-2B and hepcidin in the DI were the highest in

the group fed with 310 mg choline/kg diet ($P<0.05$), while the relative mRNA levels of LEAP-2A and defensin-3 in the DI were the highest in the choline-deficient group ($P<0.05$). Regression analysis showed that the relative mRNA levels of LEAP-2A and defensin-3 in the DI quadratically responded to the increased dietary choline levels (Table 4).

3.4. Changes of cytokines and related signaling molecules mRNA levels in the intestine

The relative mRNA levels of IL-1 β , IL-6a, IL-6b, TNF- α , interferon γ 2b (IFN- γ 2b), IL-10 and transforming growth factor β 2 (TGF- β 2) in three intestinal segments are presented in Fig. 5. In the PI, the IL-1 β mRNA levels in groups of choline deficiency, 1167 and 1820 mg choline/kg diet were significantly higher than those in groups fed with 310, 607 and 896 mg choline/kg diet ($P<0.05$) (Fig. 5A). The expression levels of IL-6b and IFN- γ 2b gene in the PI were decreased by choline, and the highest in the choline-deficient group ($P<0.05$) (Fig. 5A). However, the mRNA level of IL-6a in the PI was significantly higher in groups fed with 310, 607 and 896 mg choline/kg diet ($P<0.05$), while there was no significant difference in PI TNF- α mRNA level among dietary groups ($P>0.05$) (Fig. 5A). The relative mRNA level of IL-10 in the PI was upregulated significantly in response to the increasing levels of choline up to 607 mg/kg diet ($P<0.05$), and plateaued thereafter (Fig. 5A). The mRNA level of TGF- β 2 in the PI was significantly upregulated with increasing dietary choline, and the highest in the group fed with choline of 310 mg/kg diet ($P<0.05$) (Fig. 5A). The relative mRNA levels of IL-1 β , IL-6a, IL-6b and IFN- γ 2b in the MI were significantly

downregulated by dietary choline and the highest in the choline-deficient group ($P<0.05$), meanwhile, the relative mRNA level of TNF- α was the highest in groups fed with the choline-deficient diet and 1820 mg choline/kg diet ($P<0.05$) (Fig. 5B). The relative mRNA level of IL-10 in the MI was upregulated significantly by supplementing choline of 607, 896, 1167 and 1820 mg/kg diet ($P<0.05$) (Fig. 5B). The relative mRNA level of TGF- β 2 in the MI was upregulated significantly by choline, and the highest in groups fed with choline of 310 and 607 mg/kg diet ($P<0.05$) (Fig. 5B). In the DI, the relative mRNA levels of IL-1 β , IL-6a, IL-6b and TNF- α were significantly decreased by dietary choline and the highest in the choline-deficient group ($P<0.05$) (Fig. 5C). The relative mRNA level of IL-10 in the DI was increased significantly with the increasing levels of choline up to 896 mg/kg diet ($P<0.05$), and then plateaued (Fig. 5C). The relative mRNA level of TGF- β 2 in the DI was significantly upregulated with increasing dietary choline levels up to 310 mg/kg diet, and downregulated with further increase of dietary choline ($P<0.05$) (Fig. 5C). Regression analysis showed that mRNA levels of IL-1 β , IL-6b and IL-10 in the PI, IL-6b, IL-10, TNF- α and IFN- γ 2b in the MI, and IL-10, TNF- α in the DI quadratically responded to increased dietary choline levels (Table 4).

The relative mRNA levels of TLR4, MyD88, NF- κ B and I κ B in the PI, MI and DI are presented in Fig. 6. The relative mRNA level of PI TLR4 was not significantly affected by dietary choline ($P>0.05$), while the relative mRNA levels of PI MyD88 in groups of choline deficiency and 310 mg choline/kg diet were significantly higher than those in groups of 607 and 1820 mg choline/kg diet ($P<0.05$) (Fig. 6A). The

relative mRNA level of PI NF- κ B was significantly decreased by choline and the highest in the choline-deficient group ($P<0.05$), however, the relative mRNA level of PI I κ B was significantly increased by choline and the highest in groups supplemented with choline of 310 and 607 mg/kg diet ($P<0.05$) (Fig. 6A). The relative mRNA levels of MI TLR4, MyD88 and NF- κ B were significantly downregulated by choline and the highest in the choline-deficient group ($P<0.05$), while the relative mRNA level of MI I κ B was the highest in the 310 mg choline/kg diet group ($P<0.05$) (Fig. 6B). The relative mRNA levels of TLR4 and MyD88 in the DI showed no significant difference among groups ($P>0.05$) (Fig. 6C). The relative mRNA level of DI NF- κ B was the highest in groups of choline-deficient and 310 mg choline/kg diet ($P<0.05$), while the relative mRNA level of DI I κ B was the highest in the 310 mg choline/kg diet group ($P<0.05$) (Fig. 6C). Regression analysis showed that NF- κ B mRNA levels in the PI, MI and DI quadratically responded to the increase of dietary choline levels (Table 4).

There were significant correlations between dietary choline level and relative mRNA levels of JAKs and STATs in three intestinal segments as shown in Fig. 7. The mRNA level of PI JAK3 was significantly higher in fish fed the diet supplemented with choline of 607 mg/kg diet than that fed with choline of 1820 mg/kg diet ($P<0.05$); whereas, no significant effect was found about another member of the Janus kinases, i.e. TYK2 mRNA levels among dietary groups in PI ($P>0.05$) (Fig. 7A). The PI STAT4 and STAT5 mRNA levels were significantly lower in fish fed diets supplemented with choline of 896, 1167 and 1820 mg/kg diet than those fed

the choline-deficient diet and diets supplemented with choline of 310 and 607 mg/kg diet ($P<0.05$) (Fig. 7A). In the MI, the mRNA level of JAK3 was significantly downregulated by choline levels of 896, 1167 and 1820 mg/kg diet ($P<0.05$), meanwhile, the mRNA level of TYK2 was downregulated by dietary choline and the highest in the choline-deficient group ($P<0.05$) (Fig. 7B). The MI STAT4 mRNA level in the choline-deficient group was significant lower than that in groups fed with 310 and 607 mg choline/kg diet, but significant higher than that in groups fed with 1167 and 1820 mg choline/kg diet ($P<0.05$) (Fig. 7B). The MI STAT5 mRNA level in the choline-deficient group was significant lower than that in the group fed with 310 mg choline/kg diet, but significant higher than that in other groups ($P<0.05$) (Fig. 7B). In the DI, the relative mRNA level of JAK3 in the choline-deficient group was significantly higher than that in groups fed with choline of 607 and 896 mg/kg diet ($P<0.05$) (Fig. 7C). The highest mRNA levels of DI TYK2 and STAT4 were observed in the choline-deficient group, and the highest mRNA level of DI STAT5 was observed in groups of choline-deficient and 310 mg choline/kg diet ($P<0.05$) (Fig. 7C).

3.5. Changes of tight junction protein and $p38^{MAPK}$ mRNA levels in the intestine

The mRNA levels of claudin 3b, claudin 3c, claudin7, claudin 11, occludin and $p38^{MAPK}$ in three intestinal segments are given in Fig. 8. The mRNA levels of claudin 3c and occludin in the PI were significantly upregulated with increase of dietary choline levels up to 1167 and 896 mg/kg diet respectively ($P<0.05$) (Fig. 8A). Meanwhile, the relative mRNA level of PI claudin 7 was upregulated by choline and

the lowest in the choline-deficient group ($P<0.05$) (Fig. 8A). The PI claudin 11 mRNA levels in groups fed with 896, 1167 and 1820 mg choline/kg diet were significantly higher than those in other groups ($P<0.05$) (Fig. 8A). The mRNA level of PI p38^{MAPK} in the choline-deficient group was significantly lower than those in groups fed with 310 and 607 mg choline/kg diet ($P<0.05$) (Fig. 8A). However, the relative mRNA level of MI claudin 3b was significantly downregulated with increment of dietary choline levels up to 310 mg/kg diet ($P<0.05$), and then plateaued (Fig. 8B). The relative mRNA levels of MI claudin 3c, claudin 7 and occludin were significantly increased with the increase of choline levels up to 896, 607 and 896 mg/kg diet ($P<0.05$) respectively (Fig. 8B). The transcription level of claudin 11 gene in the MI was the highest in the group fed with 310 mg choline/kg diet ($P<0.05$), and showed no significant difference among other groups (Fig. 8B). The mRNA level of MI p38^{MAPK} in the choline-deficient group was significantly lower than those in the group fed with 310 mg choline/kg diet ($P<0.05$) (Fig. 8B). The mRNA level of claudin 3b in the DI was the lowest in fish fed the diet supplemented with 896 mg choline/kg diet ($P<0.05$), while the mRNA level of claudin 3c in the DI was the highest in the choline-deficient group ($P<0.05$) (Fig. 8C). The DI claudin 7 mRNA levels in groups fed with 310 and 607 mg choline/kg diet were significantly higher than those in other groups ($P<0.05$) (Fig. 8C). The DI claudin 11 mRNA level in the group fed with choline of 310 mg/kg diet was significantly higher than that in the choline-deficient group ($P<0.05$) (Fig. 8C). The DI occludin mRNA level was increased by increasing dietary choline levels up to 310 mg/kg diet ($P<0.05$), and then

plateaued (Fig. 8C). The mRNA level of DI p38^{MAPK} in the choline-deficient group was significantly lower than those in groups fed with 310, 607 and 896 mg choline/kg diet ($P<0.05$) (Fig. 8C). Regression analysis suggested that the mRNA levels of claudin 3c, claudin 11 and occludin in the PI, occludin in the MI, as well as p38^{MAPK} in the DI quadratically responded to the increase of dietary choline levels (Table 4).

4. Discussion

The present study was a part of a larger research that involved in determination of the effects of choline on fish growth and disease resistance, and used one growth trial as our previous study [8]. The intestinal barrier function plays an important role in maintaining fish health. Therefore, the present study explores the effects of choline on the intestinal barrier function and the related signaling pathways.

4.1. Choline deficiency impaired the intestinal immune barrier of the fish

4.1.1. Choline deficiency decreased the intestinal antimicrobial defense of the fish

Lysozyme, complement factors and AMPs are the first line of the intestinal mucosal immune and play important roles against microbial invasion in fish [35]. The present results indicated that compared with the optimal choline group, choline deficiency decreased the activities of lysozyme and ACP, contents of C3 and IgM in the intestine, and downregulated the mRNA levels of AMPs (LEAP-2A and defensin-3 in the PI and MI, LEAP-2B and hepcidin in three intestinal segments) of juvenile Jian carp, suggesting that choline deficiency impaired the intestinal antimicrobial defense of fish. However, there is little information regarding the effects of choline on the intestinal antimicrobial defense in fish. As one of the main choline metabolites, ACh is the

cholinergic neurotransmitter [16]. Plasma choline depletion is associated with decreased peripheral blood leukocyte acetylcholine in children with cystic fibrosis [36]. Besides, choline also acts as an endogenous cholinergic agonist in mice [19]. In rat, a cholinergic agonist induced the secretion of lysozyme and defensin in the intestine [17]. Therefore, the impaired intestinal antimicrobial defense caused by the choline deficiency might be partly related to the decreased cholinergic agonist levels.

Interestingly, the present results showed that the relative mRNA levels of LEAP-2A and defensin-3 in the DI were the highest in the choline-deficient group. The reasons for these interesting results are unknown but might be partially explained by the observation that LEAP-2A and defensin-3 mRNA levels in the hindgut were higher than that in the foregut, and induced by infection in common carp (*Cyprinus carpio* L.) [37, 38]. Therefore, the choline deficiency might be a stress to fish and induced the mRNA expressions of LEAP-2A and defensin-3 in the DI. However, the detail mechanisms need further characterization.

4.1.2. Choline deficiency induced inflammation and regulated NF- κ B and STAT signaling molecules in the intestine of the fish

Intestinal inflammatory disorders injured the intestinal immune barrier in fish [11]. Thus, we next investigated the effects of choline on intestinal inflammation and the associated signaling molecules in juvenile Jian carp. The histological results showed that the choline deficiency induced the inflammatory infiltration in three intestinal segments. Furthermore, IL-1, IL-6, TNF- α and IFN- γ are central pro-inflammatory cytokines, while IL-10 and TGF- β are important anti-inflammatory cytokines in fish

[39]. In the present study, compared with the optimal choline groups, dietary choline deficiency upregulated the mRNA levels of pro-inflammatory cytokines (IL-1 β and IL-6 in three intestinal segments, IL-6 α and TNF- α in the MI and DI, and IFN- γ 2b in the PI and MI); whereas reduced the mRNA levels of anti-inflammatory cytokines (IL-10 and TGF- β 2 in three intestinal segments), further demonstrating that choline deficiency induced the inflammatory response in the intestine and optimal choline supplementation inhibited the occurrence of intestinal inflammation. To date, no reports have shown the relationship between choline and intestinal inflammation in fish. Choline deficiency-induced intestinal inflammation might be partly ascribed to the important role of choline in cholinergic nerve and choline metabolism. In rat with intestinal manipulation-induced inflammation, stimulation of the vagus nerve reduced the expression of TNF- α and IL-6 in peritoneal lavage fluid [18]. Activating the α 7nAChR attenuated the TNF- α content in colitis mice [20]. As mentioned above, choline and the metabolite ACh play important roles in vagus nerve system and α 7nAChR activation. Moreover, higher homocysteine induced mRNA and protein expressions of inflammatory cytokines TNF- α , IL-1 and IL-6 in human monocytes [40]. Choline-deficient increased the production of homocysteine in terrestrial animal [41], while higher dietary intake of choline reduced homocysteine concentrations in human [42]. However, the exact underlying mechanisms need further investigation.

The expressions of cytokines are regulated by several signaling pathways. NF- κ B is one of the critical signaling molecules for regulating transcription of cytokines in mammals [12]. Over-activation of NF- κ B signaling pathway would aggravate

inflammatory reaction in rat [43]. In the present study, the choline deficiency upregulated the mRNA levels of NF- κ B in the PI, MI and DI, whereas, downregulated I κ B mRNA levels in the PI, MI and DI. Correlation analysis showed that TNF- α mRNA levels were positively correlated with NF- κ B mRNA levels in the DI ($r=+0.887$, $P<0.05$) and negatively correlated with I κ B mRNA levels in the PI ($r=-0.901$, $P<0.05$), while IL-10 mRNA levels were negatively correlated with NF- κ B mRNA levels in the MI ($r=-0.851$, $P<0.05$), TGF- β 2 mRNA levels were positively correlated with I κ B mRNA levels in the MI and DI ($r_{MI}=+0.888$, $P<0.05$; $r_{DI}=+0.888$, $P<0.05$). These results indicated that the upregulated pro-inflammatory cytokine TNF- α mRNA level and the downregulated anti-inflammatory cytokines IL-10 and TGF- β 2 mRNA levels might be related to the changes of signaling molecules NF- κ B and I κ B in the intestine caused by the choline deficiency. However, no information concerned about the effect of choline on NF- κ B signaling pathway in fish. In mammal, TLR4 and MyD88 are the upstream signaling molecules of NF- κ B and can activate it [14]. The present study showed that choline deficiency upregulated the mRNA levels of TLR4 in the MI and MyD88 in the PI and MI. To date, scanty data could be found in the literature on the effect of choline on TLR4 and MyD88. The underlying mechanism might be partly related to the α 7nAChR. In human, activation of α 7nAChR inhibited the expression of MyD88 in airway epithelial cell line [44]. As mentioned above, choline is an agonist of α 7nAChR in mice [19]. However, the exact mechanisms await further elucidation.

Furthermore, the signaling molecules JAKs and STATs also play critical roles in

regulation of cytokines transcription in mammal. In mice, STAT4 is required for IFN- γ production by the natural killer cells [45]. Meanwhile, STAT5 is also involved in the regulation of IFN- γ production in the T helper 1 cells of mice [46]. In the present study, compared with the group of 896 mg choline/kg diet, choline deficiency upregulated the mRNA levels of STAT4 and STAT5 in three intestinal segments, as well as the mRNA levels of the upstream signaling molecules JAK3 and TYK2 in the MI and DI. The IFN- γ 2b mRNA levels in the PI and MI showed the same trends as those of the STAT4 and STAT5. Thus, the changes of IFN- γ 2b mRNA levels in the PI and MI caused by the choline deficiency might be partly attributed to the changes of signaling molecules JAKs and STATs in the group. However, this is the first report investigated the relationship between choline and the signaling molecules JAKs and STATs in fish. The mechanisms underlying the increase of JAKs and STATs expression under choline deficiency are unknown but might be partially related to the increased reactive oxygen species (ROS). In human retinoblastoma cells, H₂O₂, one important ROS induced the expression of JAK3 and STAT4 genes [47]. Our unpublished data showed that choline deficiency increased the ROS levels in the intestine of Jian carp, which supported the hypothesis.

4.2. Choline deficiency impaired the intestinal physical barrier and regulated signaling molecule p38^{MAPK} gene expression of the fish

In fish, function of the intestinal immunological barrier could be affected by the physical intestinal barrier [2], which formed by TJ that consist of occludins and claudins [22]. Claudin 3 and claudin 11 were recognized as builders of the barrier,

while claudin 7 was reported to function both as barrier builder and anion channel [29]. Occludin plays an important role in the formation and stability of tight junction strands [48]. The present results indicated that dietary choline deficiency downregulated mRNA levels of claudin 7, claudin 11 and occludin in three intestinal segments, as well as claudin 3c in the PI and MI, suggesting that choline deficiency impaired the intestinal physical barrier. However, choline deficiency upregulated the mRNA levels of claudin 3b in the MI and DI, as well as claudin 3c in the DI. The reasons for the different effects of choline on claudin 3b and claudin 3c in different intestinal segments are unclear. There may be two reasons for explaining the downregulated TJs expressions by the choline deficiency. First, it might partly be related to the intestinal flora. In mice, the probiotic containing *Lactobacillus* increased occludin expression in ilea [49]. Our previous study showed that the choline deficiency decreased the *Lactobacillus* count in the intestine of Jian carp [9]. Second, it might partly be attributed to the cytokines expressions in intestine. In human, TNF- α decreased the occludin expression in the colon carcinoma cells [26] and IL-1 β increased the TJ permeability in intestinal epithelial cells [50]. The present study showed that choline deficiency upregulated the TNF- α and IL-1 β expressions in the MI and DI of Jian carp. Furthermore, inhibition of p38^{MAPK} increased occludin expression and prevented the disruption of TJ in human gastric epithelial cells [24]. However, the present result showed that the mRNA level of p38^{MAPK} was downregulated by the choline deficiency. The mechanism responsible for this change is unknown. We speculated that it might partly be due to an adaptive mechanism

responding to the choline deficiency. In human intestinal epithelial cells, IL-1 β increased the phosphorylated and activity of p38^{MAPK} kinase [51]. Our present study showed that the choline-deficient diet upregulated the IL-1 β mRNA level in intestine. Thus, the choline deficiency might result in the increase of phosphorylated p38^{MAPK}, which might induce a feedback signal that contributed to the downregulated p38^{MAPK} mRNA levels; however, the involved mechanism needs further investigation.

4.3. Choline excess impaired the intestinal barrier of the fish

Although no negative effects of high level of choline (1820 mg/kg diet) on the growth performances were observed in our previous study on juvenile Jian carp [8], excessive choline (1820 mg/kg diet) induced negative effects on the intestinal barrier as compared with the optimal choline group in the present study. We found that excessive choline: (1) depressed intestinal antimicrobial defense by decreasing intestinal lysozyme and ACP activities, IgM content, and mRNA levels of AMPs (LEAP-2A and defensin in the PI and MI, hepcidin in the PI and DI) of juvenile Jian carp, (2) induced leucocytes infiltration and inflammation in the intestine associated with the upregulated mRNA levels of pro-inflammatory cytokines (IL-1 β in the PI, TNF- α and IFN- γ 2b in the MI), and **the downregulated** mRNA levels of anti-inflammatory cytokines (TGF- β 2 in the MI and DI) and signaling molecule (I κ B in the PI, MI and DI) of juvenile Jian carp, (3) impaired intestinal physical barrier by decreasing the mRNA levels of TJs (claudin 3c and occludin in the MI, claudin 7 and claudin 11 in the MI and DI) and signaling molecule p38^{MAPK} in the MI and DI of juvenile Jian carp.

These negative effects of excessive choline on intestinal immunity and physical barrier might be partially explained by the finding that excessive choline increased the harmful bacteria *Aeromonas hydrophila* in the intestine of fish. In grass carp, *Aeromonas hydrophila* induced the inflammatory responses that was associated with the expression of pro-inflammatory cytokines (IL-1 β and TNF- α) in the intestine [52], and downregulated the transcription levels of claudin b, claudin 2 and claudin 4 in the spleen [53]. Our previous study found that excessive choline increased the *Aeromonas hydrophila* counts in the intestine of juvenile Jian carp compared with the optimal choline group [9], which supported the hypothesis.

5. Conclusions

The present study provided evidences that dietary choline deficiency impaired the intestinal mucosal immunity of fish, and provided partial theoretical evidence for the underlying molecular mechanisms. Firstly, the results showed that choline deficiency depressed intestinal antimicrobial defense by decreasing antimicrobial component levels, and induced intestinal inflammation via upregulating pro-inflammatory cytokines expressions and downregulating anti-inflammatory cytokines expressions, which might partly be ascribed to the changed transcription levels of signaling molecules related to NF- κ B and STAT signaling pathways in fish. Secondly, the present study firstly indicated that choline deficiency impaired the intestinal physical barrier through downregulating the mRNA levels of TJ proteins (mainly claudin 7, claudin 11 and occludin) in the intestine of fish. Meanwhile, the results firstly showed a possible relationship between choline-regulated p38^{MAPK} gene expression and the

changed TJ proteins transcript abundance in fish. Furthermore, the negative effects of excessive choline on intestinal immunity and physical barrier of fish were observed.

Acknowledgments

This research was financially supported by the National Basic Research Program of China (973 Program) (2014CB138600), National Science Foundation of China (31502184), the National Department Public Benefit Research Foundation (Agriculture) of China (201003020), the Specialized Research Fund for the Doctoral Program of Higher Education of China (20135103110001), Science and Technology Support Program of Sichuan Province of China (2014NZ0003), Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China (2012NC0007; 2013NC0045), the Demonstration of Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China (2015CC0011) and Natural Science Foundation for Young Scientists of Sichuan Province (2014JQ0007). The authors would like to thank these teams for their financial support, and are grateful to Yang He for her kind help with histological analysis.

References

- [1] L. Niklasson, H. Sundh, F. Fridell, G.L. Taranger, K. Sundell, Disturbance of the intestinal mucosal immune system of farmed Atlantic salmon (*Salmo salar*), in response to long-term hypoxic conditions, Fish Shellfish Immun. 31 (2011) 1072-80.
- [2] H. Sundh, Chronic stress and intestinal barrier function: implications for infection and inflammation in intensive salmon aquaculture, Doctoral thesis, University of Gothenburg, Göteborg, Sweden, 2009.
- [3] L. Wen, W. Jiang, Y. Liu, P. Wu, J. Zhao, J. Jiang, et al., Evaluation the effect of thiamin deficiency on intestinal immunity of young grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immun. 46 (2015) 501-15.
- [4] L. Chen, L. Feng, W. Jiang, J. Jiang, P. Wu, J. Zhao, et al., Intestinal immune function, antioxidant status and tight junction proteins mRNA expression in young grass carp (*Ctenopharyngodon idella*) fed riboflavin deficient diet, Fish Shellfish Immun. 47 (2015) 470-84.
- [5] L. Feng, S. Li, W. Jiang, Y. Liu, J. Jiang, P. Wu, et al., Deficiency of dietary niacin impaired intestinal mucosal immune function via regulating intestinal NF- κ B, Nrf2 and MLCK signaling pathways in young grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immun. 49 (2016) 177-93.
- [6] L. Shi, L. Feng, W. Jiang, Y. Liu, J. Jiang, P. Wu, et al., Immunity decreases, antioxidant system damages and tight junction changes in the intestine of grass carp (*Ctenopharyngodon idella*) during folic acid deficiency: Regulation of

558 NF- κ B, Nrf2 and MLCK mRNA levels, Fish Shellfish Immun. 51 (2016) 405-19.

559 [7] NRC, Nutrient requirements of fish and shrimp, The National Academies Press,
 560 Washington, DC, 2011.

561 [8] P. Wu, L. Feng, S.Y. Kuang, Y. Liu, J. Jiang, K. Hu, et al., Effect of dietary
 562 choline on growth, intestinal enzyme activities and relative expressions of target
 563 of rapamycin and eIF4E-binding protein2 gene in muscle, hepatopancreas and
 564 intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian), Aquaculture 317 (2011)
 565 107-16.

566 [9] P. Wu, J. Jiang, Y. Liu, K. Hu, W.D. Jiang, S.H. Li, et al., Dietary choline
 567 modulates immune responses, and gene expressions of TOR and eIF4E-binding
 568 protein2 in immune organs of juvenile Jian carp (*Cyprinus carpio* var. Jian), Fish
 569 Shellfish Immun. 35 (2013) 697-706.

570 [10] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: The
 571 evolution of tolerating commensals while fighting pathogens, Fish Shellfish
 572 Immun. 35 (2013) 1729-39.

573 [11] J.X. Zhang, L.Y. Guo, L. Feng, W.D. Jiang, S.Y. Kuang, Y. Liu, et al., Soybean
 574 beta-conglycinin induces inflammation and oxidation and causes dysfunction of
 575 intestinal digestion and absorption in fish, PLoS ONE 8 (2013) e58115.

576 [12] W.J. de Jonge, L. Ulloa, The $\alpha 7$ nicotinic acetylcholine receptor as a
 577 pharmacological target for inflammation, Br. J. Pharmacol. 151 (2007) 915-29.

578 [13] H. Wang, Y. Zhu, X. Xu, X. Wang, Q. Hou, Q. Xu, et al., *Ctenopharyngodon*
 579 *idella* NF- κ B subunit p65 modulates the transcription of I κ B α in CIK cells, Fish

580 Shellfish Immun. 54 (2016) 564-72.

581 [14] L. Zhu, L. Nie, G. Zhu, L. Xiang, J. Shao, Advances in research of fish
582 immune-relevant genes: A comparative overview of innate and adaptive
583 immunity in teleosts, Dev. Comp. Immunol. 39 (2013) 39-62.

584 [15] B. Robertsen, The interferon system of teleost fish, Fish Shellfish Immun. 20
585 (2006) 172-91.

586 [16] S.H. Zeisel, K.D. Costa, P.D. Franklin, E.A. Alexander, J.T. Lamont, N.F.
587 Sheard, et al., Choline, an essential nutrient for humans, FASEB J. 5 (1991)
588 2093-8.

589 [17] X. Qu, K.C.K. Lloyd, J.H. Walsh, R.I. Lehrer, Secretion of type II phospholipase
590 A2 and cryptdin by rat small intestinal paneth cells, Infect. Immun. 64 (1996)
591 5161-5.

592 [18] W.J. de Jonge, E.P. van der Zanden, F.O. The, M.F. Bijlsma, D.J. van Westerloo,
593 R.J. Bennink, et al., Stimulation of the vagus nerve attenuates macrophage
594 activation by activating the Jak2-STAT3 signaling pathway, Nat. Immunol. 6
595 (2005) 844-51.

596 [19] W.R. Parrish, M. Rosas-Ballina, M. Gallowitsch-Puerta, M. Ochani, K. Ochani,
597 L. Yang, et al., Modulation of TNF release by choline requires $\alpha 7$ subunit
598 nicotinic acetylcholine receptor-mediated signaling, Mol. Med. 14 (2008) 567-74.

599 [20] A. Bai, Y. Guo, N. Lu, The effect of the cholinergic anti-inflammatory pathway
600 on experimental colitis, Scand. J. Immunol. 66 (2007) 538-45.

601 [21] R.W. Saeed, S. Varma, T. Peng-Nemeroff, B. Sherry, D. Balakhaneh, J. Huston,

et al., Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation, *J. Exp. Med.* 201 (2005) 1113-23.

[22] K.E. Pijls, D.M. Jonkers, E.E. Elamin, A.A. Masclee, G.H. Koek, Intestinal epithelial barrier function in liver cirrhosis: an extensive review of the literature, *Liver Int.* 33 (2013) 1457-69.

[23] L. González-Mariscal, R. Tapia, D. Chamorro, Crosstalk of tight junction components with signaling pathways, *BBA-Biomembranes* 1778 (2008) 729-56.

[24] H. Wu, X. Gao, Z. Jiang, Z. Duan, S. Wang, B. He, et al., Attenuated expression of the tight junction proteins is involved in clopidogrel-induced gastric injury through p38 MAPK activation, *Toxicology* 304 (2013) 41-8.

[25] B. Greenwood, M. Mantle, Mucin and protein release in the rabbit jejunum: Effects of bethanechol and vagal nerve stimulation, *Gastroenterology* 103 (1992) 496-505.

[26] L.J. John, M. Fromm, J. Schulzke, Epithelial barriers in intestinal inflammation, *Antioxid. Redox Sign.* 15 (2011) 1255-70.

[27] S. Wang, H. Han, Z. Pan, P. Hang, L. Sun, Y. Jiang, et al., Choline inhibits angiotensin II-induced cardiac hypertrophy by intracellular calcium signal and p38 MAPK pathway, *N.-S. Arch. Pharmacol.* 385 (2012) 823-31.

[28] P.B. Venugopal, Choline, in: J Augustin, BP Klein, D Becker, PB Venugopal, editors, *Methods of vitamin assay*, John Wiley and Sons, New York, 1985, pp. 555-73.

[29] H. Syakuri, M. Adamek, G. Brogden, K.L. Rakus, M. Matras, I. Irnazarow, et al.,

Intestinal barrier of carp (*Cyprinus carpio* L.) during a cyprinid herpesvirus
 3-infection: molecular identification and regulation of the mRNA expression of
 claudin encoding genes, Fish Shellfish Immun. 34 (2013) 305-14.

[30] M.E. El-Boshy, A.M. El-Ashram, F.M. Abdelhamid, H.A. Gadalla,
 Immunomodulatory effect of dietary *Saccharomyces cerevisiae*, beta-glucan and
 laminaran in mercuric chloride treated Nile tilapia (*Oreochromis niloticus*) and
 experimentally infected with *Aeromonas hydrophila*, Fish Shellfish Immun. 28
 (2010) 802-8.

[31] T. Barka, J.P. Anderson, Histochemical methods for acid phosphatase using
 hexazonium pararosanalin as coupler, J. Histochem. Cytochem. 10 (1962) 741-53.

[32] M. Bradford, A rapid and sensitive method for the quantification of microgram
 quantities of protein utilizing the principle of protein dye-binding, Anal. Biochem.
 72 (1976) 248.

[33] P. Wu, W.D. Jiang, Y. Liu, G.F. Chen, J. Jiang, S.H. Li, et al., Effect of choline
 on antioxidant defenses and gene expressions of Nrf2 signaling molecule in the
 spleen and head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian), Fish
 Shellfish Immun. 38 (2014) 374-82.

[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using
 real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402-8.

[35] J.H. Rombout, L. Abelli, S. Picchietti, G. Scapigliati, V. Kiron, Teleost intestinal
 immunology, Fish Shellfish Immun. 31 (2011) 616-26.

[36] S.M. Innis, A.G.F. Davidson, B.N. Bay, P.J. Slack, D. Hasman, Plasma choline

depletion is associated with decreased peripheral blood leukocyte acetylcholine in children with cystic fibrosis, *Am. J. Clin. Nutr.* 93 (2011) 564-8.

[37] G. Yang, H. Guo, H. Li, S. Shan, X. Zhang, J.H.W.M. Rombout, et al., Molecular characterization of LEAP-2 cDNA in common carp (*Cyprinus carpio* L.) and the differential expression upon a *Vibrio anguillarum* stimulus; indications for a significant immune role in skin, *Fish Shellfish Immun.* 37 (2014) 22-9.

[38] H. Li, H. Guo, S. Shan, C. Qi, L. An, G. Yang, Characterization and expression pattern of a novel β -defensin in common carp (*Cyprinus carpio* L.): implications for its role in mucosal immunity, *Biosci. Biotech. Bioch.* 78 (2014) 430-7.

[39] H. Cordero, M. Mauro, A. Cuesta, M. Cammarata, M.A. Esteban, In vitro cytokine profile revealed differences from dorsal and ventral skin susceptibility to pathogen-probiotic interaction in gilthead seabream, *Fish Shellfish Immun.* 56 (2016) 188-91.

[40] S. Su, L. Huang, L. Pai, H. Liu, K. Chan, Homocysteine at pathophysiologic concentrations activates human monocyte and induces cytokine expression and inhibits macrophage migration inhibitory factor expression, *Nutrition* 21 (2005) 994-1002.

[41] S.H. Zeisel, Choline, homocysteine, and pregnancy, *Am. J. Clin. Nutr.* 82 (2005) 719-20.

[42] E. Cho, S.H. Zeisel, P. Jacques, J. Selhub, L. Dougherty, G.A. Colditz, et al., Dietary choline and betaine assessed by food-frequency questionnaire in relation

668 to plasma total homocysteine concentration in the Framingham Offspring Study,
669 Am. J. Clin. Nutr. 83 (2006) 905-11.

670 [43] B. Hoesel, J.A. Schmid, The complexity of NF- κ B signaling in inflammation and
671 cancer, Mol. Cancer 2013, 12:86 12 (2013) 86.

672 [44] Q. Li, X. Zhou, V.P. Kolosov, J.M. Perelman, Nicotine reduces TNF- α
673 expression through a α 7nAChR/MyD88/NF- κ B pathway in HBE16 airway
674 epithelial cells, Cell. Physiol. Biochem. 27 (2011) 605-12.

675 [45] T. Miyagi, M.P. Gil, X. Wang, J. Louten, W. Chu, C.A. Biron, High basal
676 STAT4 balanced by STAT1 induction to control type 1 interferon effects in
677 natural killer cells, J. Exp. Med. 204 (2007) 2383-96.

678 [46] W. Liao, J. Lin, L. Wang, P. Li, W.J. Leonard, Modulation of cytokine receptors
679 by IL-2 broadly regulates differentiation into helper T cell lineages, Nat. Immunol.
680 12 (2011) 551-9.

681 [47] S. Vandhana, T.S.R. Lakshmi, D. Indra, P.R. Deepa, S. Krishnakumar,
682 Microarray analysis and biochemical correlations of oxidative stress responsive
683 genes in retinoblastoma, Curr. Eye Res. 37 (2012) 830-41.

684 [48] I.E. Blasig, C. Bellmann, J. Cording, V.G. Del, D. Zwanziger, O. Huber, et al.,
685 Occludin protein family: oxidative stress and reducing conditions, Antioxid.
686 Redox Sign. 15 (2011) 1195-219.

687 [49] D. Corridoni, L. Pastorelli, B. Mattioli, S. Locovei, D. Ishikawa, K.O. Arseneau,
688 et al., Probiotic bacteria regulate intestinal epithelial permeability in experimental
689 ileitis by a TNF-dependent mechanism, PLoS ONE 7 (2012) e42067.

690 [50] R. Al-Sadi, D. Ye, H.M. Said, T.Y. Ma, IL-1 β -induced increase in intestinal
691 epithelial tight junction permeability is mediated by MEKK-1 activation of
692 canonical NF- κ B pathway, Am. J. Pathol. 177 (2010) 2310-22.

693 [51] R. Al-Sadi, S. Guo, D. Ye, K. Dokladny, T. Alhmoud, L. Ereifej, et al.,
694 Mechanism of IL-1beta modulation of intestinal epithelial barrier involves p38
695 kinase and activating transcription factor-2 activation, J. Immunol. 190 (2013)
696 6596-606.

697 [52] X. Song, J. Zhao, Y. Bo, Z. Liu, K. Wu, C. Gong, *Aeromonas hydrophila* induces
698 intestinal inflammation in grass carp (*Ctenopharyngodon idella*): An
699 experimental model, Aquaculture 434 (2014) 171-8.

700 [53] Y. Yang, H. Yu, H. Li, A. Wang, Transcriptome profiling of grass carp
701 (*Ctenopharyngodon idellus*) infected with *Aeromonas hydrophila*, Fish Shellfish
702 Immun. 51 (2016) 329-36.

703

704

705 **Table 1** Composition and nutrients content of the basal diet.

Ingredients	g/kg diet	Nutrients content ^a	g/kg diet
Fish meal	20.0	Crude protein	315.3
Soybean protein concentrate	170.3	Crude lipid	44.0
Casein	180.3	Crude ash	48.1
Rice protein meal	33.5	Available phosphorus	6.6
Gelatin	38.2	n-3	10.0
DL-methionine (99%)	4.2	n-6	10.0
Thr (98.5%)	3.6	Methionine	11.3
Fish oil	26.8		
Soy bean oil	16.7		
α-starch	140.0		
Corn starch	276.8		
Ca(H ₂ PO ₄) ₂	19.1		
Choline-free vitamin premix ^b	10.0		
Trace mineral premix ^c	10.0		
Choline chloride premix ^d	30.0		
Ethoxyquin (30%)	0.5		
cellulose	20.0		

706 ^a Crude protein, crude fat, crude ash, methionine and available phosphorus were
707 measured value. n-3 and n-6 contents were calculated according to NRC (1993) and
708 Bell (1984).

^b Per kilogram of choline-free vitamin premix(g/kg): retinyl acetate (500000 IU/g) 0.800g, cholecalciferol (500000 IU/g) 0.480g, DL- α -tocopherol acetate (500 g/kg) 20.000g, menadione (500 g/kg) 0.200g, cyanocobalamin (100 g/kg) 0.010g, D-biotin (200 g/kg) 0.500g, folic acid (960 g/kg) 0.521g, thiamin nitrate (980 g/kg) 0.104g, ascorhyl acetate (920 g/kg) 7.247g, niacin (980 g/kg) 2.857g, inositol (980 g/kg) 52.857g, calcium-D-pantothenate (980 g/kg) 2.511g, riboflavine (800 g/kg) 0.625g, pyridoxine hydrochloride (980 g/kg) 0.755g. All ingredients were diluted with corn starch to 1 kg.

^c Per kilogram of trace mineral premix (g/kg): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (250.0 g/kg Cu) 1.201g, KI (38.0 g/kg I) 2.895g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (318.0 g/kg Mn) 4.089g, NaSeO_3 (10.0 g/kg Se) 2.500g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (197.0 g/kg Fe) 69.695g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (225.0 g/kg Zn) 21.640g. All ingredients were diluted with CaCO_3 to 1 kg.

^d Per kilogram of choline chloride premix (g/kg): each treatment group containing choline chloride 0g, 5.5170g, 17.2769g, 29.0368g, 40.7967g and 64.3166g, respectively. Each choline chloride mixture was diluted with corn starch to 1 kg.

Table 2 Primer sequences and annealing temperature for target and the housekeeping genes.

Target gene	Forward (5'→3')	Reverse (5'→3')	Annealing temperature	Accession number
LEAP-2A	GCAATCCTGTGCCAAAGTC	TAGTGCCCATGATCCTCCA	55°C	KC551971
LEAP-2B	GCGTCTGCATCACTTTAACC	CGAACAATGACCGTTCCTG	63.3°C	KC551972
Defensin-3	ACGGGAACCTCTGGGACTG	AATCGCAAAGCACAGCAC	55.7°C	KF321773
Hepcidin	ACATGCGTCTGCTTCCTCC	CTGGTTCTCCTGTGGTGCTT	55.7°C	JX855261
TNF- α	AAGTCTCAGAACAATCAGGAA	TGCCTTGGAAGTGACATT	61.5°C	AJ311800
IL-1 β	AACTTCACACTTGAGGAT	GACAGAACATAACAACAAC	61.5°C	AB010701
IL-6a	CAGATAGCGGACGGAGGGGC	GCGGGTCTCTTCGTGTCTT	59°C	KC858890
IL-6b	GGCGTATGAAGGAGCGAAGA	ATCTGACCGATAGAGGAGCG	59°C	KC858889
IFN- γ 2b	TGTCTTCCTACGGTTCCTT	TCTTCAGTTGAGTTGCTTCT	63.3 °C	AM168524
IL-10	GCCAGCATAAAGAACTCG	CCAAATACTGCTCGATGT	59.4°C	AB110780
TGF- β 2	GGGACATCATCGCCATCT	TGACATTCTCGGCAGGGT	57.5°C	CCU66874
TLR4	TGTCGCTTTGAGTTTGAAT	TCCAGAATGATGATGATGATC	55°C	HM564033
Myd88	AAGAGGATGGTGGTAGTCA	GAGTGCGAACTTGGTCTG	55.5°C	GU321987
NF- κ B p65	TATTCAGTGCGTGAAGAAG	TATTAAGGGGTGTTCCTGT	58°C	LN590704
I κ B	CATCTTGCCATTATTCACGAGG	GTTTTGTATGTTGAGGAAGGGGT	62.7°C	LN594784
JAK3	GGATGGACTACAAGGACTG	CGTGCTGTGCTGATAGAA	60°C	AF148993
TYK2	TGTTGGAGGAAGGCATCA	GAAGTCGCAGAAGGTGTT	60°C	KJ782031
STAT4	CATCTGTAACCTGCCTGAGG	CGCTGCTTCTTATGATTGC	61°C	KJ782030
STAT5	GCAGAATCGTCAGCAGAT	TGAGATGATGTCCGTGATG	61°C	KJ782032

Claudin 3b	ACTCCATGCTGGCTCTGA	CATAACTTTGGCTTTGACG	61.5°C	JQ767156
Claudin 3c	GCACCAACTGTATCGAGGATG	GGTTGTAGAAGTCCCGAATGG	61.5°C	JQ767157
Claudin 7	CTTCTATAACCCCTTCACACCAG	ACATGCCTCCACCCATTATG	60.4°C	JQ767155
Claudin 11	TCGGAAGTGAACCAGAAAGC	GAAGCCAAAGGACATCAAGC	59.2°C	JQ767158
Occludin	ATCGGTTTCAGTACAATCAGG	GACAATGAAGCCCATAACAA	54°C	KF975606
p38 ^{MAPK}	ACCTCAATAATATCGTCAA	TAAGTTCACAGTCTTCATT	56°C	AB023481
β-actin	CGTGATGGACTCTGGTGATG	TCGGCTGTGGTGGTGAAG	60°C	M24113

727 LEAP, liver-expressed antimicrobial peptide; IFN- γ 2b, interferon γ 2b; TNF- α , tumor
 728 necrosis factor α ; IL-1 β , interleukin 1 β ; IL-6a, interleukin 6a; IL-6b, interleukin 6b;
 729 IL-10, interleukin 10; TGF- β 2, transforming growth factor β 2; TLR4, Toll-like
 730 receptor 4; MyD88, myeloid differentiation primary response 88; NF- κ B, nuclear
 731 factor kappa B, I κ B, inhibitor of NF- κ B; JAK3, Janus kinase 3; TYK2, tyrosine
 732 kinase 2; STAT, signal transducers and activators of transcription; p38^{MAPK},
 733 mitogen-activated protein kinase p38.

734

Table 3 Effects of dietary choline (mg/kg diet) on intestinal activities of lysozyme (U/mg protein) and acid phosphatase (ACP, U/mg protein), contents of complement 3 (C3, g/g protein) and IgM (g/g protein) in juvenile Jian carp.

Choline	165	310	607	896	1167	1820
Lysozyme	15.69±1.15 ^a	15.09±2.09 ^a	30.06±3.66 ^{bc}	34.62±1.49 ^c	25.42±0.83 ^b	16.49±1.83 ^a
ACP	71.13±1.88 ^a	84.61±5.80 ^c	85.82±2.85 ^c	83.31±3.72 ^{bc}	73.24±1.86 ^{ab}	67.29±4.15 ^a
C3	0.23±0.02 ^a	0.43±0.05 ^b	0.62±0.05 ^d	0.63±0.02 ^d	0.50±0.02 ^{bc}	0.56±0.02 ^{cd}
IgM	0.14±0.01 ^a	0.14±0.01 ^a	0.27±0.03 ^b	0.27±0.04 ^b	0.16±0.02 ^a	0.12±0.01 ^a
Regression						
$Y_{\text{lysozyme}} = -0.00002x^2 + 0.0447x + 6.8894$				$R^2 = 0.797$	$P = 0.092$	

Values are means ± SE of four replicate groups, with 5 fish in each group. Mean values with the different superscripts in the same row are significantly different ($P < 0.05$).

742 **Table 4** Regression analysis of genes transcription levels responded to dietary choline.

Genes	Regressions	R ²	P
Antimicrobial peptides			
DI LEAP-2A	$Y = 0.0000003x^2 - 0.0008x + 0.9972$	0.803	0.088
DI defensin-3	$Y = 0.0000002x^2 - 0.0008x + 0.9393$	0.718	0.145
Cytokines			
MI TNF- α	$Y = 0.0000005x^2 - 0.0011x + 1.052$	0.744	0.129
DI TNF- α	$Y = 0.0000004x^2 - 0.0011x + 1.0446$	0.769	0.110
MI IFN- γ 2b	$Y = 0.0000007x^2 - 0.0015x + 1.0761$	0.798	0.088
PI IL-1 β	$Y = 0.000002x^2 - 0.0022x + 1.1271$	0.962	< 0.01
PI IL-6b	$Y = 0.0000004x^2 - 0.0014x + 1.262$	0.937	< 0.05
MI IL-6b	$Y = 0.0000005x^2 - 0.0013x + 1.125$	0.919	< 0.05
PI IL-10	$Y = -0.0000006x^2 + 0.0015x + 0.8043$	0.792	0.094
MI IL-10	$Y = -0.000002x^2 + 0.0054x + 0.2234$	0.716	0.151
DI IL-10	$Y = -0.000001x^2 + 0.0036x + 0.2495$	0.970	< 0.01
Tight junction proteins			
PI claudin 3c	$Y = -0.00001x^2 + 0.0254x - 4.0671$	0.795	0.093
PI claudin 11	$Y = -0.000002x^2 + 0.0052x + 0.0585$	0.899	< 0.05
PI occludin	$Y = -0.00001x^2 + 0.025x - 3.6077$	0.758	0.119
MI occludin	$Y = -0.000002x^2 + 0.0042x + 0.3305$	0.718	0.149
Signaling molecules			
DI MAPK p38	$Y = -0.0000007x^2 + 0.0011x + 0.9976$	0.913	< 0.05

PI NF-κB	$Y = 0.0000004x^2 - 0.001x + 1.1288$	0.928	< 0.05
MI NF-κB	$Y = 0.0000006x^2 - 0.0015x + 1.21$	0.913	< 0.05
DI NF-κB	$Y = 0.0000004x^2 - 0.0009x + 1.1039$	0.775	0.106

743 PI, proximal intestine; MI, mid intestine; DI, distal intestine; LEAP-2A,
744 liver-expressed antimicrobial peptide 2A; TNF-α, tumor necrosis factor α; IFN-γ2b,
745 interferon γ2b; IL-1β, interleukin 1β; IL-6a, interleukin 6a; IL-6b, interleukin 6b;
746 IL-10, interleukin 10; MAPK, mitogen-activated protein kinase; NF-κB, nuclear
747 factor kappa B.

748

Fig. 1 The histology of proximal intestine in juvenile Jian carp fed diets containing graded levels of choline (mg/kg diet). A) The choline-deficient group. Arrowhead showed the edema and leucocyte infiltration in the lamina propria. B) The group of 310 mg choline/kg diet. Arrowhead showed the blood capillary hyperemia. C) The group of 607 mg choline/kg diet. D) The group of 896 mg choline/kg diet. E) The group of 1167 mg choline/kg diet. F) The group of 1820 mg choline/kg diet. Arrowhead showed the increased leucocytes in the lamina propria. The sections were H&E staining and observed at 400x original magnification.

Fig. 2 The histology of mid intestine in juvenile Jian carp fed diets containing graded levels of choline (mg/kg diet). A) The choline-deficient group. Arrowhead showed the blood capillary hyperemia. Triangle showed the leucocyte infiltration in the lamina propria. B) The group of 310 mg choline/kg diet. Arrowhead showed the blood capillary hyperemia. C) The group of 607 mg choline/kg diet. D) The group of 896 mg choline/kg diet. E) The group of 1167 mg choline/kg diet. Arrowhead showed the blood capillary hyperemia. F) The group of 1820 mg choline/kg diet. Arrowhead showed the blood capillary hyperemia. Triangle showed the increased leucocyte in the lamina propria. The sections were H&E staining and observed at 400x original magnification.

Fig. 3 The histology of distal intestine in juvenile Jian carp fed diets containing graded levels of choline (mg/kg diet). A) The choline-deficient group. Arrowhead

showed the blood capillary hyperemia. Triangle showed the leucocyte infiltration in the lamina propria. B) The group of 310 mg choline/kg diet. Arrowhead showed the blood capillary hyperemia. C) The group of 607 mg choline/kg diet. D) The group of 896 mg choline/kg diet. E) The group of 1167 mg choline/kg diet. F) The group of 1820 mg choline/kg diet. Arrowhead showed the leucocyte infiltration in the lamina propria. The sections were H&E staining and observed at 400x original magnification.

Fig. 4 Effect of choline (mg/kg diet) on mRNA levels of antimicrobial peptides in the proximal intestine (PI-A), mid intestine (MI-B) and distal intestine (DI-C) of juvenile Jian carp. Values are means $\pm$ SE of four replicates, with five fish in each replicate, and different letters denotes significant difference ($P < 0.05$).

Fig. 5 Effect of choline (mg/kg diet) on mRNA levels of cytokines in the proximal intestine (PI-A), mid intestine (MI-B) and distal intestine (DI-C) of juvenile Jian carp. Values are means $\pm$ SE of four replicates, with five fish in each replicate, and different letters denotes significant difference ($P < 0.05$).

Fig. 6 Effect of choline (mg/kg diet) on mRNA levels of TLR4, MyD88, NF- κ B and I κ B in the proximal intestine (PI-A), mid intestine (MI-B) and distal intestine (DI-C) of juvenile Jian carp. Values are means $\pm$ SE of four replicates, with five fish in each replicate, and different letters denotes significant difference ($P < 0.05$).

793

794 **Fig. 7 Effect of choline (mg/kg diet) on mRNA levels of JAK3, TYK2, STAT4 and**
795 **STAT5 in the proximal intestine (PI-A), mid intestine (MI-B) and distal intestine**
796 **(DI-C) of juvenile Jian carp.** Values are means $\pm$ SE of four replicates, with five fish
797 in each replicate, and different letters denotes significant difference ($P < 0.05$).

798

799 **Fig. 8 Effect of choline (mg/kg diet) on mRNA levels of tight junction proteins**
800 **and p38^{MAPK} in the proximal intestine (PI-A), mid intestine (MI-B) and distal**
801 **intestine (DI-C) of juvenile Jian carp.** Values are means $\pm$ SE of four replicates,
802 with five fish in each replicate, and different letters denotes significant difference ($P <$
803 0.05).

804

805

Fig. 1

Fig. 2

Fig. 3

Fig. 4

A

822 **B**

823

824

Fig. 5

A

832 **B**

833

834

Fig. 6

A

B

C

Fig. 7

A

B

C

Fig. 8

A

866

867

868