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Effects of inosine 5′-monophosphate supplementation in high fishmeal and high soybean diets on growth, immune-related gene expression in gibel carp (*Carassius auratus gibelio* var. CAS III), and its challenge against *Aeromonas hydrophila* infection

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ABSTRACT

The present study was conducted to evaluate dietary inosine 5′-monophosphate (5′-IMP) on growth, immune genes expression and disease resistance against *Aeromonas hydrophila* in juvenile gibel carp (*Carassius auratus gibelio* var. CAS III) (initial body weight: 7.48 g). Six diets were formulated containing exogenous 5′-IMP at three gradient levels (0, 0.1% and 0.2%) in the high dietary fishmeal group (15% fishmeal: D1, D2, D3) and in the high dietary soybean meal group (33% soybean meal: D4, D5, D6). Each diet was randomly allotted to triplicate tanks in a recirculating system. After the feeding trial, fish were exposed to *Aeromonas hydrophila* challenge. Hematological and immunological responses were analyzed before and after challenge. The results indicated that feeding rate in all 5′-IMP supplemented treatments (D2, D3, D5 and D6) and daily growth coefficient in D5 and D6 were reduced compared with those of respective control treatments (D1 and D4) without 5′-IMP addition ($P < 0.05$). The cumulative survival rates were numerically improved by dietary 5′-IMP supplementation ($P > 0.05$). Compared with the respective control treatment, in the high fishmeal group, plasma SOD and MPO were significantly elevated in D3 at the end of feeding trial ($P < 0.05$), plasma SOD and lysozyme were significantly increased in D3 after bacterial challenge ($P < 0.05$); in high soybean meal group, plasma lysozyme activity was significantly elevated in D5 post bacterial challenge ($P < 0.05$). Most of the expression of immune related genes (intelectin, major histocompatibility complex class II β (MHC II β), Complement 3 (C3), Complement component C7-1 (ccC7), lysozyme C, Interleukin 1β (IL-1β), Tumor necrosis factor α1 (TNF-α1), Transforming growth factor-beta (TGF-β) and Interleukin 8 (IL-8)) in spleen, kidney and liver of the fish were significantly affected by supplementation of 5′-IMP at the end of feeding trial and post bacterial challenge. Additionally, adding 5′-IMP in high soybean meal diets exerted further effects of promoting immunity than counterparts in high fishmeal diets. Considering enhanced disease resistance, the immunopotentiation of 5′-IMP was manifested when the addition level was 0.1% in high soybean meal diets and 0.2% in high fishmeal diets.

1. Introduction

During the last decades, global aquaculture sector has been rapidly expanding due to huge demand of aquatic animal food, which results in intensification of aquaculture practice and ultimately leads to emergence of several pathogenic organisms. It is known that inappropriate use of antibiotics in aquaculture will bring about increment of resistant pathogen, antibiotics residue and eradication of normal microbiota in fish [1]. Recently, nucleotides and their related products have attracted increasing attention due to their potential immunomodulatory effects. Several studies have reported successful application of nucleotides as immunomodulator in different cultured fish species, such as sea cucumber (*Apostichopus japonicas*) [2], hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂) [3] and channel catfish (*Ictalurus*

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punctatus) [4].

Nucleotides are ubiquitous molecules, which are building block of tissue RNA, DNA and of ATP and play key roles in physiological and biochemical processes. A nucleotide consists of nitrogenous base linked to a sugar (ribose or deoxyribose) and one to three phosphate groups. Inosine monophosphate (IMP) is the main product in the de novo synthesis process of nucleotides [5]. Currently, a wide range of immunostimulatory effects of dietary IMP in fish such as elevated antioxidant status [6], enhanced innate immunity [7], improved disease resistance [8] have been described. Nevertheless, to our best of knowledge, very little information is available about potential effects of exogenous IMP on immune-related gene expression.

Many researches have proved that the application of immunomodulators in aquaculture are affected by a variety of factors including fish species, dose and frequency, mode of administration, and so forth. It is reported that the appropriate dose of dietary inosine in juvenile red sea bream (*Pagrus major*) with initial body weight of 8 g is 4 g/kg feed among five graded level (0, 2, 4, 6, 8 g/kg) [9], whereas the recommended usage of this immunomodulator in juvenile amberjack (*Seriola dumerili*: 26 g) are 5.4 and 6.7 g/kg in diet based on quadratic regression analyses of weight gain and lysozyme activity [10]. Sajeevan et al. [11] proved that the maximum survival of post larvae of Indian white shrimp (*Fenneropenaeus indicus*) was observed in 0.2% glucan incorporated group post-oral challenge with white spot syndrome virus when shrimp were fed with six increasing doses (0, 0.05, 0.1, 0.2, 0.3, 0.4 g glucan/100 g feed) of yeast glucan for 21 days. Subsequently, the authors selected this dose of glucan to feed fish at different feeding intervals (daily, once every two/five/seven/ten days), the fish fed glucan once every seven days showed highest survival rate post viral challenge. Three routes of administration of immunomodulators are reported in aquaculture, including immersion, oral addition and intraperitoneal injection. The injection method is stressful to aquatic animal, whereas oral addition and immersion methods are less stressful and more applicable [12]. In addition, it has been demonstrated that inosine supplementation at 0.6% in diets in which 50% and 75% of fishmeal protein were replaced by soybean meal exerted a positive impact on immune responses of amberjack (*Seriola dumerili*) rather than in the 25% fishmeal replacement group, it is partly implied that diet formulation also might affect appropriate dosage of the immunostimulants in some fish species.

Gibel carp (*Carassius auratus gibelio*) is a very popular cultivated species in China and the annual production of this species was approximate three million tons in 2016 [13]. It is also encountering the problem of reduced immunity because of intensive farming, diseases and plant-derived anti-nutritional factors. Taking all these considerations, the present study was designed to investigate the effects of exogenous 5′-IMP on immune-related genes and disease resistance in gibel carp and compare differences of immune gene expression between high fishmeal group and high soybean meal group.

2. Materials and methods

All experimental animal care protocols were approved by the ethic committee of the Institute of Hydrobiology of Chinese Academy of Sciences.

2.1. Experimental design and diet preparation

The diet formulation and chemical composition are shown in Table 1. Six practical diets (termed as D1, D2, D3, D4, D5 or D6, respectively) were designed to be isonitrogenous (39% crude protein) and isoenergetic (19.0 MJ kg^{-1}), using fishmeal, soybean meal and rapeseed meal as blended protein sources, wheat middling and cornstarch as mixed carbohydrate sources and soybean oil as lipid source. D1, D2 and D3 had a higher fishmeal content with 15% of fishmeal and 25% soybean meal. Compared with the formula of high fishmeal diets, 40% of fishmeal was replaced by soybean meal in D4, D5 and D6, with 9% of fishmeal and 33% soybean meal, while contents of other major ingredients were kept constant. Exogenous 5′-IMP was incorporated at three gradient levels (0, 0.1% and 0.2%) at the expense of monocalcium phosphate.

Table 1
Formulation and chemical composition of six practical diets (% dry matter).

Content (%)	D1	D2	D3	D4	D5	D6
Fishmeal[a]	15.00	15.00	15.00	9.00	9.00	9.00
Soybean meal[b]	25.00	25.00	25.00	33.00	33.00	33.00
Rapeseed meal[b]	25.00	25.00	25.00	25.00	25.00	25.00
Wheat middling	15.00	15.00	15.00	15.00	15.00	15.00
Corn starch	3.60	3.60	3.60	0.00	0.00	0.00
Soy oil	5.00	5.00	5.00	5.50	5.50	5.50
Mineral premix[c]	5.00	5.00	5.00	5.00	5.00	5.00
Vitamin premix[d]	0.39	0.39	0.39	0.39	0.39	0.39
Choline chloride[e]	0.11	0.11	0.11	0.11	0.11	0.11
Carboxymethyl cellulose	3.00	3.00	3.00	3.00	3.00	3.00
Monocalcium phosphate	2.00	1.90	1.80	2.00	1.90	1.80
5′-IMP[f]	0.00	0.10	0.20	0.00	0.10	0.20
Cellulose	0.90	0.90	0.90	2.00	2.00	2.00
Proximate composition						
Crude protein	39.30	39.82	38.99	39.00	38.93	39.5
Crude lipid	5.42	5.21	5.65	5.62	5.57	5.60
Moisture	10.56	9.94	10.09	9.57	10.72	9.63
Crude ash	11.14	10.99	10.96	10.87	10.64	10.78
Gross energy (MJ/kg)	18.49	19.21	19.35	19.33	19.29	19.23
5′-IMP (%)	0.06	0.11	0.15	0.02	0.08	0.12

[a] White fish meal was purchased from American Seafood Company, Seattle, Washington, USA.

[b] Soybean and rapeseed meal were purchased from Coland Feed Co. Ltd., Wuhan, Hubei, China.

[c] Mineral premix (mg kg^{-1} diet): NaCl, 500.0; $MgSO_4{\cdot}7H_2O$, 8155.6; $NaH_2PO_4{\cdot}2H_2O$, 12500.0; KH_2PO_4, 16000.0; $Ca(H_2PO_4)_2{\cdot}H_2O$, 7650.6; $FeSO_4{\cdot}7H_2O$, 2286.2; $C_6H_{10}CaO_6{\cdot}5H_2O$, 1750.0; $ZnSO_4{\cdot}7H_2O$, 178.0; $MnSO_4{\cdot}4H_2O$, 61.4; $CuSO_4{\cdot}5H_2O$, 15.5; $CoSO_4{\cdot}6H_2O$, 0.91; KI, 1.5; Na_2SeO_3, 0.60; Corn starch, 899.7.

[d] Vitamin premix (mg kg^{-1} diet): thiamin, 20; riboflavin, 20; pyridoxine, 20; cyanocobalamine, 0.02; folic acid, 5; calcium patotheniate, 50; inositol, 100; niacin, 100; biotin, 5; Vitamin A, 11; Vitamin D_3, 2; Vitamin E, 100; Vitamin K_3, 10; starch, 3522.

[e] Choline chloride was composed of 50% choline chloride and 50% silicon dioxide.

[f] 5′-IMP was supplied by CJ Cheiljedang, 330, Dongho-ro, Jung-gu, Seoul 04560, Korea.

All ingredients were passed through a 375 μm sieve before mixing thoroughly and then pellets were made (2 mm in diameter) using laboratory pellet machine (SLP-45; Fishery Mechanical Facility Research Institute, Shanghai, China). The pellets were dried in an oven (CT-C, Tonghao Drying equipment Co. Ltd, Nanjing, Jiangsu, China) at 50 °C and stored at −20 °C until used.

2.2. Experimental fish and feeding regime

Gibel carp were obtained from Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China. Fish were reared in fiberglass tanks (300 L). Before the feeding trial, fish were acclimated to laboratory conditions for one month and fed with the control diet twice daily (09:00 and 15:00). At the beginning of the experiment, fish were fasted for 24 h. 20 fish of similar size were selected (initial weight: 7.48 ± 0.09 g ind^{-1}, mean ± SEM), batch-weighed and stocked into 18 tanks. Three tanks were randomly selected for each treatment. During the trial, ~~the~~ fish were fed to apparent satiation twice daily (09:00 and 15:00). The feeding trial lasted for 88 days from 16th Oct 2016 to 12th Jan 2017.

Table 2
Sequences of primers applied for quantitative real-time PCR analysis in gibel carp.

gene	Acronym	prime sequence	Amplicon size (bp)	Annealing temp. (°C)	PCR efficiency A/B/C	Accession No.
β-actin	β-actin	F: TTGAGCAGGAGATGGGAACCG R: AGAGCCTCAGGGCAACGGAAA	115	60.0	2.01/1.99/2.03	AB039726.2
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	F: ATCCAACCAGATGGGAGAACG R: ACCGTGTATGTGACCTGATGG	103	60.0	1.97/1.98/1.99	MH820410
Intelectin	Intelectin	F: GGAAGTGCTCTCTTGGTG R: AACTGGCTGTCTGTATGG	250	57.0	2.02/2.01/1.99	KF954510.1
Major histocompatibility complex IIβ	MHC II β	F: CCGTGATAAAACAGTTAAA R: CATCTCCATAGTGGAGGTC	175	57.0	1.95/1.96/1.98	AJ556943.1
Complement 3	C3	F: GAGATGAAGTGCGTTTAG R: GAGGTCAGAGATGCCGAG	374	59.0	1.99/2.00/1.99	KF110786.1
Complement component C7-1	ccC7	F: AGAAAGAAGGCGAAGCAGA R: GACGCACAGAGCCACAAAT	207	59.0	1.96/2.00/1.99	KC969197.1
Lysozyme C	Lyz C	F: CTCATTGTGAAAACCGAAG R: TAGCTAATTAAAGCTCCTG	139	57.0	1.96/1.99/1.97	KF417503.1
Interleukin 1β	IL-1β	F: TTTGTGAAGATGCGCTGCTC R: CCAATCTCGACCTTCCTGGTG	133	54.0	2.01/1.98/1.97	AB757758.1
Tumor necrosis factor α1	TNF-α1	F: CGCTACTCTGATTCCTATGGC R: GCTTTCGCTGTTGCCTTTCT	199	54.0	2.00/1.97/1.98	KF500408.1
Transforming growth factor-beta	TGF-β	F: ATGAGGGTGGAGAGTTTAT R: AGTCGTAGTTTGCTGAGAA	155	53.0	1.95/1.96/1.98	EU086521.1
Interleukin 8	IL-8	F: TGAAGGAATGAGTCTTAGA R: AGCTCCACACTCTCTATGTG	99	52.0	1.85/1.90/1.88	KC184490.1

Note: [A]liver, [B]spleen, [C]kidney. β-actin and GAPDH were used as internal reference in spleen, in liver and kidney, respectively.

During the experiment, water temperature was maintained at 26.07 ± 2.05 °C (mean ± SEM) using automatic heater (Guang Dong RiSheng group Co., Ltd, Guang Dong, China) and pH range was 7.37 ± 0.03. Each tank was provided with continuous aeration through an air stone. The dissolved oxygen content was kept above 7.4 mg/L, and ammonia nitrogen content was less than 0.5 mg/L. The photoperiod was 12 h light: 12 h dark with light period from 08:00 to 20:00 and light intensity was 30 ± 5 lux.

2.3. Sample collection

At the end of the experiment, fish were fasted for 24 h, then all fish were rapidly netted and anesthetized by MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA, USA) at a density of 50 mg/L and batch-weighed. Three of them were used for blood sample and remaining fish were returned to original tank. Blood samples were withdrawn with heparinized syringe from caudal vein. After centrifugation (3000*g*, 15min, 4 °C), plasma samples were separated into 200 μl PCR tube and stored at −80 °C for further analyses of lysozyme, myeloperoxidase (MPO) and superoxide dismutase (SOD). The phlebotomized fish were gutted to isolate liver, spleen and kidney and quickly frozen in liquid nitrogen and stored at −80 °C for further analyses of immune-related gene expression profile.

2.4. Bacterial challenge test

The single colony of *A. hydrophila* used in the challenge test was kindly supplied by Prof. LI Aihua, State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, Hubei, China). It was originally isolated from gibel carp. The pathogen was cultured on brain heart infusion (BHI) agar plate, a single colony of bacteria was picked out by sterile inoculating loop and incubated in liquid BHI at 30 °C for 6 h, then centrifuged at 3500 g for 10 min to obtain the bacteria. Using sterile PBS to wash the sediment for three times and suspend the bacteria. Four concentrations of bacteria (1.0×10^6 CFU ml^{-1}, 5×10^6 CFU ml^{-1}, 1.0×10^7 CFU ml^{-1} and 1.0×10^8 CFU ml^{-1}) were applied in the pre-challenge test to find out the median lethal dose. Two fish per tank were randomly chosen and mixed in one barrel. Then the fish were divided in four equal aliquots and injected with above four doses of bacteria on the weight basis (1 ml/100 g fish). The mortality was recorded every two hours, and the bacterial density of 5×10^6 CFU ml^{-1} resulted in around 50% of fish mortality.

Right after sampling of feeding trial, 15 fish per tank were injected intraperitoneally with median lethal dose of bacteria. At 7 h after bacterial challenge, plasma samples and three internal organs (liver, spleen and kidney) of two fish from each tank were obtained as described in previous method. Fish mortality was observed continuously for four days, the two samples of fish were excluded in the following equation. The mortalities (%) = (final number of fish death)/(initial number of injected fish) × 100.

2.5. Biochemical assays

Proximate composition analyses of the diets were performed in duplicate using the AOAC methods [14]. Dry matter, crude protein, crude lipid and crude ash were measured according to previous lab work [15].

The plasma superoxide dismutase (SOD) and myeloperoxidase (MPO) activities were tested using the commercial kits according to the instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The plasma lysozyme level was measured by turbidimetric assay with slight modification as previously established [16,17].

2.6. qPCR analysis

D1 and D3 in high fishmeal group, D4 and D5 in high soybean meal group were selected to measure immune genes expression profile according to the results of plasma immunity and survival rate post infection. Total RNAs of liver, spleen and kidney were extracted by the trizol method. The reverse transcription of mRNA to complementary DNA (cDNA), design of PCR primers, PCR amplification protocols, identification of PCR product and quantitative real time PCR procedure were conducted based on previous lab work [18]. Primers sequence, product size, annealing temperature and gene bank number were listed in Table 2. GAPDH and actin genes were used as the internal reference in liver and kidney, in spleen, respectively. Transcriptional levels were calculated according to Ref. [19]. Six samples were used for each treatment and each sample was measured in duplicate.

Table 3
Growth performance of gibel carp fed diets with different 5′-IMP levels.

	D1 (control)	D2	D3
IBW (g/fish)[1]	7.50 ± 0.04	7.48 ± 0.02	7.46 ± 0.04
FBW (g/fish)[2]	14.40 ± 0.46	13.88 ± 0.49	14.38 ± 0.43
FR (%BW/d)[3]	2.26 ± 0.05[b]	2.13 ± 0.06[a]	2.05 ± 0.06[a]
DGC (%)[4]	0.65 ± 0.03	0.62 ± 0.03	0.63 ± 0.01
FE (%)[5]	33.54 ± 1.11	33.93 ± 2.51	37.25 ± 2.45
SR (%)[6]	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	D4 (control)	**D5**	**D6**
IBW (g/fish)[1]	7.43 ± 0.03	7.51 ± 0.01	7.45 ± 0.02
FBW (g/fish)[2]	13.94 ± 0.49	12.51 ± 0.27	12.08 ± 0.09
FR (%BW/d)[3]	2.15 ± 0.04[b]	2.01 ± 0.01[a]	1.95 ± 0.01[a]
DGC (%)[4]	0.58 ± 0.01[b]	0.52 ± 0.02[a]	0.51 ± 0.01[a]
FE (%)[5]	34.15 ± 2.45	29.85 ± 1.25	29.26 ± 0.42
SR (%)[6]	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Values are presented as means ± SE (n = 3). Values in the same row with different lowercases differ significantly ($P < 0.05$).

[1] IBW (g): Average initial body weight.

[2] FBW (g): Average final body weight.

[3] FR (Feeding rate, %BW d^{-1}) = 100total feed intake/days/[(IBW + FBW)/2].

[4] Daily growth coefficient (DGC, %) = 100 × (final body weight$^{1/3}$ - initial body weight$^{1/3}$)/Σ (days).

[5] Feed efficiency (FE, %) = (100 × wet body weigh gain)/dry feed intake.

[6] Survival rates (SR, %) = (final fish number/initial fish number) × 100.

2.7. Statistical analysis

All data were examined for normal distribution by Shapiro-Wilk test and for homogeneity of variances by Levene's test. Results of growth, gene expression, plasma SOD, MPO and lysozyme activities were analyzed in both meal groups separately using one-way analysis of variance (ANOVA). Plasma SOD, MPO and lysozyme activities at the end of growth trial and after challenge within each meal group, while gene expression results between D1 and D3, D4 and D5 were compared using independent *t*-test. Mean Values were considered significantly different at $P < 0.05$. SPSS statistical package version 20.0 (SPSS Inc., Chicago, IL, USA) were used for analyses. The data are presented as means ± standard error (S.E.) in Tables and Figures.

3. Results

Table 3 described growth performance of gibel carp fed with high fishmeal and high soybean diets containing different 5′-IMP levels for 88 days. Feeding rate in all 5′-IMP supplemented treatments was significantly depressed compared with that of control ($P < 0.05$) irrespective of diet formulas. Among high fishmeal group, there were no significant differences of daily growth coefficient (DGC) and feed efficiency in fish fed D2 and D3 compared with control treatment. Among high soybean group, significant lower DGC were observed in fish fed 5′-IMP incorporated diets compared with control treatment ($P < 0.05$).

In high fishmeal group, plasma SOD and MPO activities were significantly elevated in fish fed D3 compared with those of fish fed D1 at the end of feeding trial ($P < 0.05$), whereas after bacterial challenge plasma SOD and lysozyme activities were significantly increased in fish fed D3 compared with those of fish fed D1 ($P < 0.05$). In high soybean meal group, there were no significant variations of plasma SOD, MPO and lysozyme in gibel carp among three treatments prior to bacterial challenge, whereas plasma lysozyme was significantly improved in fish fed D5 compared with those of fish fed D4 or D6 diets after infection ($P < 0.05$). After 7 h of the bacterial challenge, plasma SOD and lysozyme activities significantly dropped in both meal groups compared with counterparts before challenge ($P < 0.05$), but plasma MPO activity significantly increased in high fishmeal group compared with

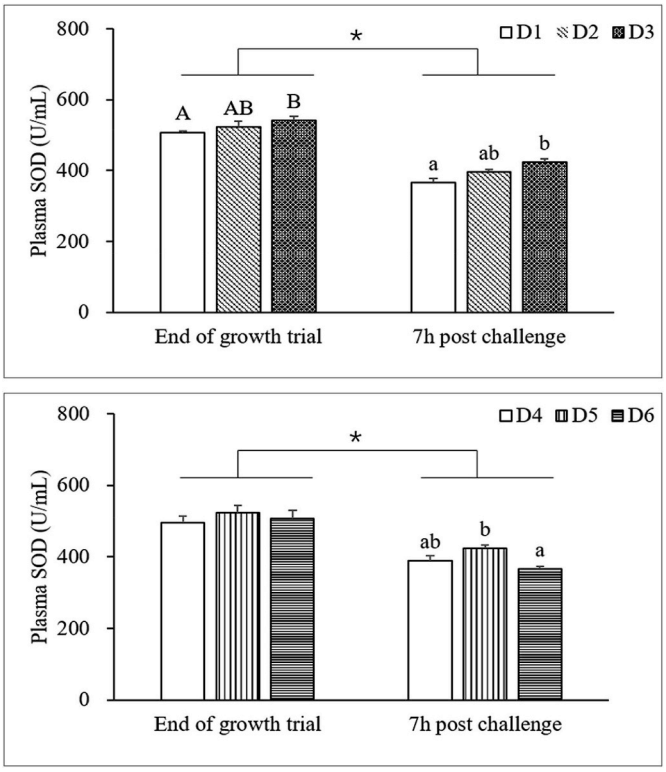


Fig. 1. Effects of dietary 5′-IMP inclusions on plasma SOD activity in gibel carp of high fishmeal group and high soybean meal group at the end of feeding trial (pre-challenge) or 7 h post challenge with *A. hydrophila*. Bars with different capital letters mean significant changes among treatments at the end of growth trial ($P < 0.05$). Bars with different lower cases mean significant changes among treatments 7 h post bacterial challenge ($P < 0.05$). Bars with * mean significant changes within meal groups before challenge and after challenge ($P < 0.05$).

counterparts before challenge ($P < 0.05$) (Figs. 1–3).

After bacterial challenge, most of immune related gene expression (intelectin, C3, ccC7, IL-1β, TNF-α1, IL-8) were significantly up-regulated in liver, spleen and kidney of infected fish compared with the non-infected counterparts irrespective of diet formula and 5′-IMP supplementations ($P < 0.05$) (Fig. 4-a, 4-b and 4-c). In the liver of fish fed D3, gene expression of MHC II β, IL-1β, TGF-β and IL-8 at the end of growth trial were significantly up-regulated compared with those of fish fed D1 ($P < 0.05$). After challenge in the liver of fish fed D3, gene expression of ccC7, TNF-α1 and TGF-β were significantly up-regulated compared with those of fish fed D1 ($P < 0.05$). Among high soybean group, TNF-α1 gene expression was significantly increased in the liver of fish fed D5 before and after the challenge ($P < 0.05$) (Fig. 4-a).

In the spleen of fish among high fishmeal group, intelectin gene was significantly down-regulated by dietary supplementation of 5′-IMP at the end of growth trial ($P < 0.05$). While for high soybean group, 5′-IMP supplementation significantly improved mRNA abundance of genes (MHC II β, lysozyme, IL-1β, TNF-α1 and IL-8) at the end of growth trial compared with the control treatment ($P < 0.05$). Relative expression of lysozyme, IL-1β, TNF-α1 and IL-8 were significantly up-regulated in fish fed D5 after challenge compared with counterparts in D4 ($P < 0.05$). While genes of intelectin and ccC7 were significantly depressed by dietary 5′-IMP at the end of growth trial and after bacterial infection compared with control fish ($P < 0.05$) (Fig. 4-b).

In the kidney of gibel carp of high fishmeal group, dietary 5′-IMP supplementation caused a significant increase in the expression of intelectin, ccC7, IL-1β and IL-8 genes post challenge ($P < 0.05$). In high soybean group, relative expression of intelectin, MHC II β, IL-1β, TNF-α1, TGF-β and IL-8 at the end of feeding trial were significantly

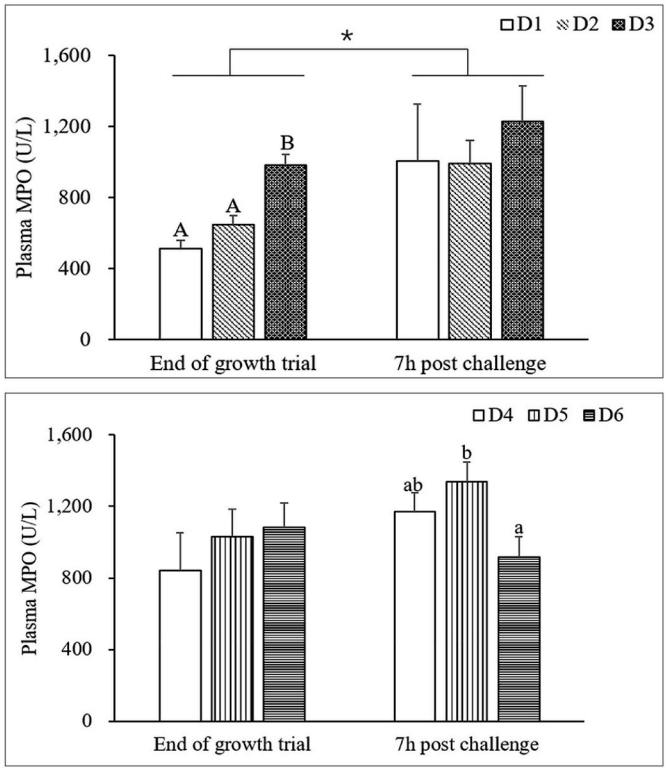


Fig. 2. Effects of dietary 5′-IMP inclusions on plasma MPO activity in gibel carp of high fishmeal group and high soybean meal group at the end of feeding trial (pre-challenge) or 7 h post challenge with *A. hydrophila*. Bars with different capital letters mean significant changes among treatments at the end of growth trial ($P < 0.05$). Bars with different lower cases mean significant changes among treatments 7 h post bacterial challenge ($P < 0.05$). Bars with * mean significant changes within meal groups before challenge and after challenge ($P < 0.05$).

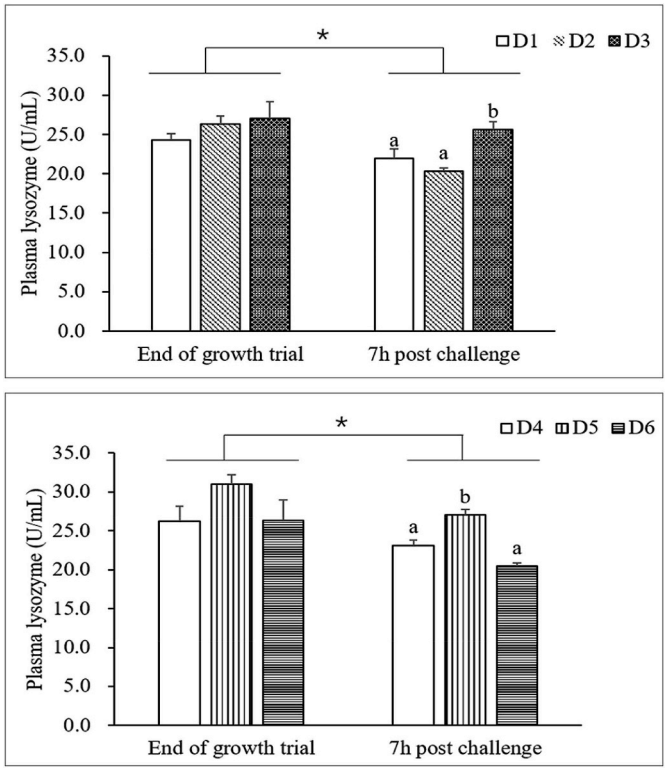


Fig. 3. Effects of dietary 5′-IMP inclusions on plasma lysozyme activity in gibel carp of high fishmeal group and high soybean meal group at the end of feeding trial (pre-challenge) or 7 h post challenge with *A. hydrophila*. Bars with different capital letters mean significant changes among treatments at the end of growth trial ($P < 0.05$). Bars with different lower cases mean significant changes among treatments 7 h post bacterial challenge ($P < 0.05$). Bars with * mean significant changes within meal groups before challenge and after challenge ($P < 0.05$).

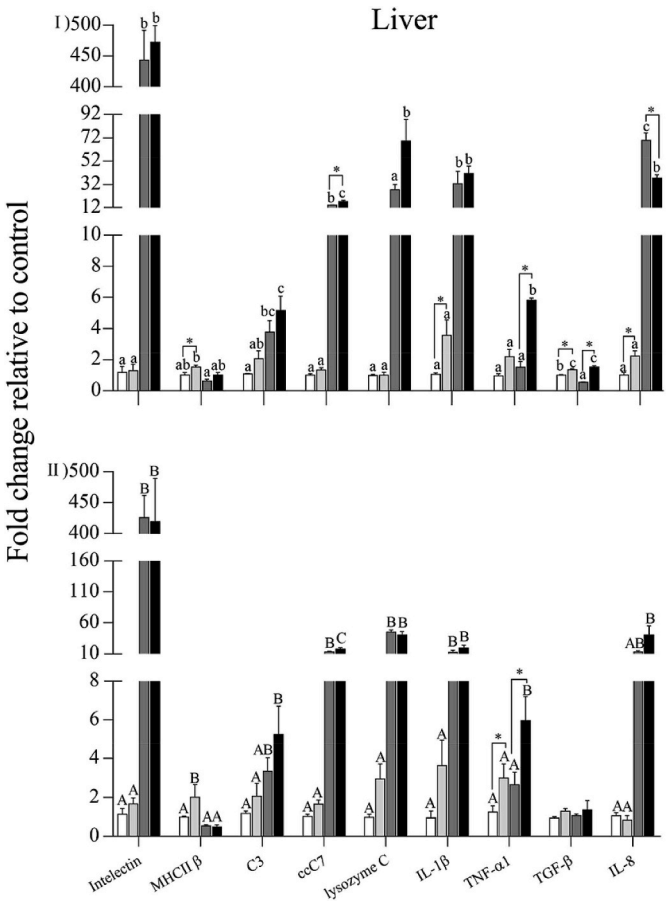
elevated in fish fed D5 compared with those of fish fed D4 ($P < 0.05$), and relative expression of C3, ccC7 IL-1β, TNF-α1 and IL-8 post infection were significantly up-regulated by dietary administration of 5′-IMP compared with those of control fish ($P < 0.05$) (Fig. 4-c).

The bacterial resistance of gibel carp was improved when fed with 5′-IMP supplemented diet without significant difference ($P > 0.05$) (Fig. 5). After challenged with *A. hydrophila*, the survival rate from D1 to D3 in high fishmeal group increased from 28.20 ± 12.82% to 46.15 ± 17.76%, while the survival rate from D4 to D5 in high soybean group increased from 12.80 ± 9.25% to 41.03 ± 6.79%.

4. Discussion

The results of the present study showed that dietary supplementation of 5′-IMP in gibel carp resulted in significantly reduced feeding rate regardless of diet formula. Moreover, among high soybean meal group, DGC in 5′-IMP supplemented treatments were markedly depressed compared to that of control group. It is assumed that this significantly depressed DGC in the present study possibly resulted from lower feeding rate. On the contrary, dietary inosine has been used as a chemical attractant in largemouth bass (*Micropterus salmoides*) and turbot (*Scophthalmus maximus*) [20,21]. It is also demonstrated in previous study that inosine supplementation at a concentration of 4 g/kg in juvenile red sea bream (*Pagrus major*) diet could improve feed intake [9]. Nevertheless, in shrimp (*Penaeus chinensis*), 5′-IMP did not exhibit attractive property in experimental ethology [22]. Similarly, addition of monosodium glutamate in combination with IMP-5 to a high-protein diet, either added to the food or in capsules, decreased overall desire to eat without affecting hunger and satiety in human experiment [23]. Under these situations, we assume that feeding-stimulating effect of 5′-IMP might vary due to species difference.

In the current study, the results demonstrated that an appropriate supplementation of 5′-IMP in gibel carp diets (both high fishmeal-based diets and high soybean meal-based diets) could, to some extent, improve immune response and disease resistance of fish to *A. hydrophila* infections. In high fishmeal group, significantly elevated MPO activities at the end of growth trial, higher lysozyme activities after challenge, higher SOD activities before and after challenge were found in D3 treatment compared with D1 treatment. While in the high soybean group, it is interesting to note that the highest activities of plasma SOD, MPO and lysozyme after bacterial challenge were showed at 0.1% 5′-IMP supplemented treatment. These results were parallel with previous study [8], which reported that dietary administration of inosine monophosphate at levels of 0.2% and 0.4% in juvenile olive flounder (initial body weight: 7.5 ± 0.02 g) significantly increased serum MPO and lysozyme after 14 weeks feeding trial. It has also been demonstrated that dietary nucleotides exhibit the potential immunostimulatory effect by enhancing phagocytic, complement and nitroblue tetrazolium activities of leucocytes in fish [24]. It is reported that inosine could bind directly to adenosine receptors (A_1, A_{2A} and A_3) and motivate intracellular signaling events [25,26]. However, it remains unknown if the same mechanism is manifested in the fish and if the binding will promote the release of lysozyme and MPO in leukocytes. Superoxide dismutase (SOD) catalytically scavenges the superoxide radicals, thus provide a defense against oxygen toxicity [27]. Myeloperoxidase (MPO) and lysozyme played a significant role in killing microorganism, MPO in neutrophils oxidizes chloride ions to strong nonradical oxidant to kill bacteria [28], while lysozyme could



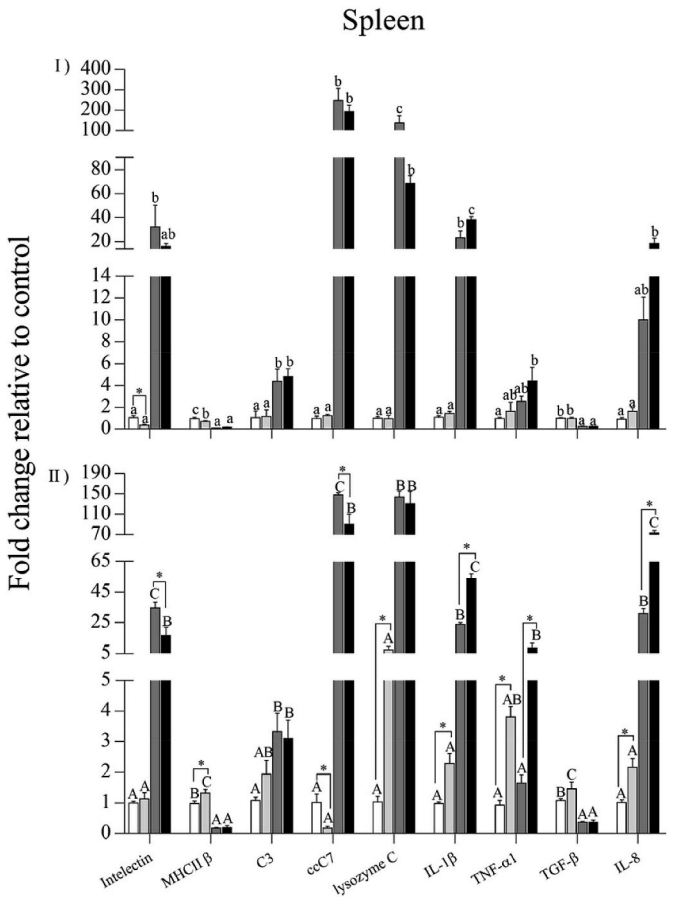


Fig. 4. I) high fishmeal group: □, D1, non-infected; □, D3, non-infected; ■, D1, infected; ■, D3, infected.
II) high soybean meal group: □, D4, non-infected; □, D5 non-infected; ■, D4, infected; ■, D5, infected.
Expression of intelectin, MHC II β, C3, ccC7, lysozyme C, IL-1β, TNF-α1, TGF-β and IL-8 in the liver, spleen and kidney (Fig. 4-a, b and c) at the end of feeding trial and at 7 h post bacterial challenge. Expression was determined by RT-PCR and normalized to β-actin and GAPDH gene expression. The gene expression data for all treatments were compared to the control group (control diet, non-injected) and represented as the mean fold change ± SE (n = 6). Bars with different lower cases means significant changes in high fishmeal group ($P < 0.05$). Bars with different capital letters mean significant changes in high soybean meal group ($P < 0.05$). Bars with * mean significant changes between control treatments and 5′-IMP supplemented treatments ($P < 0.05$).

Fig. 4. (*continued*)

lyse cell wall of Gram-negative bacteria [29]. *Aeromonas hydrophila* applied in current study was Gram-negative bacterium [30]. Therefore, the treatments (D3 and D5) had the highest activities of MPO and lysozyme and possessed the highest cumulative survival rate in high fishmeal group and high soybean meal group respectively after *A. hydrophila* injection. These results indicated that dietary inclusion of 5′-IMP could improve immunity of gibel carp.

For high fishmeal group and high soybean meal group, *A. hydrophila* infection in the present study significantly induced immune-related genes (intelectin, C3, ccC7, IL-1β, TNF-α1, IL-8) mRNA abundance levels of three tested organs (liver, spleen and kidney) in both control and 5′-IMP fed fish. Intelectin, a recently identified galectin, plays a role in innate immune response [31]. In previous studies, intelectin was considered as one of the acute phase response genes in rainbow trout [32,33]. It was also reported that in channel catfish, intelectin 2 was dramatically induced by intraperitoneal injection of *Edwardsiella ictaluri* [34]. Furthermore, Lin et al. found that zebrafish intelectin 3 gene was predominantly expressed in liver and highly upregulated after *Aeromonas salmonicida* infection. This was consistent with results in current study that gene expression of intelectin was more than 400-fold increased in liver of fish through bacterial infection, whereas in spleen and kidney the increased folds of intelectin were far less than those in liver. Complement component C3 and C7 were viewed as integral parts of complement system. C3 is a central component of classical, lectin and alternative pathways [35], while C7 plays a significant role in the assembly of membrane attack complex [36]. Shen et al. [37] reported that significantly increased variations of C7 mRNA levels in grass carp after *Aeromonas hydrophila* infection. IL-1β, TNF-α1 and IL-8, three of proinflammatory cytokines, were involved in host defense against microbial pathogens [38,39]. IL-8, which is a member of CXC chemokine family, produced by several cell types in response to inflammatory stimuli, such as IL-1β [40,41]. Hence gene expressions of IL-8 and IL-1β in tested organs were significantly improved after bacterial infection in current study. Furthermore, the several-fold increased transcription level of IL-1β and TNF-α1 following *Aeromonas hydrophila* infection might be attributed to the existence of aerolysin in this pathogen. The study revealed that aerolysin, a cytotoxic enterotoxin, from *A. hydrophila* evoked the production of TNF-α and up-regulated expression level of IL-1β in murine macrophage cell line RAW264.7 [42]. In agreement with our study, Cao et al. [18] also found that the transcription of genes encoding IL-1β and TNF-α1 were activated by *A. hydrophila* infection.

In this study, we found that changes of transcription level in MHC II β and lysozyme C post bacterial challenge exhibited differently in the three tested organs for both high fishmeal and high soybean groups, MHC II β was significantly down-regulated post challenge in liver and spleen, while it was significantly up-regulated in kidney compared with those at the end of feeding trial. On the contrary, lysozyme C was significantly up-regulated in liver and spleen, whereas it was significantly suppressed in kidney by bacterial infection. MHC II β, one subunit of MHC II, mainly expressed surface of antigen-presenting cells, such as

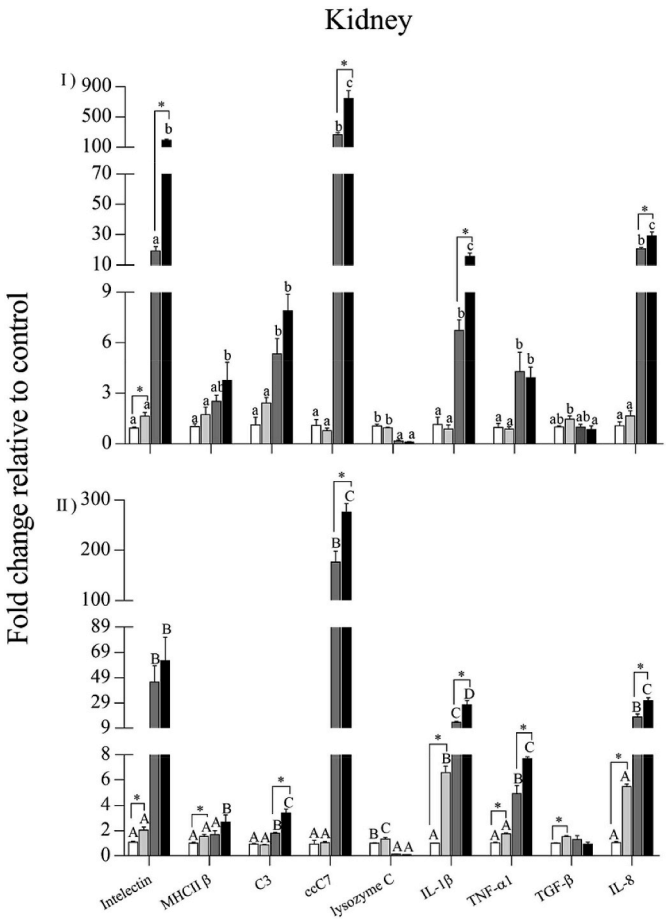


Fig. 4. (*continued*)

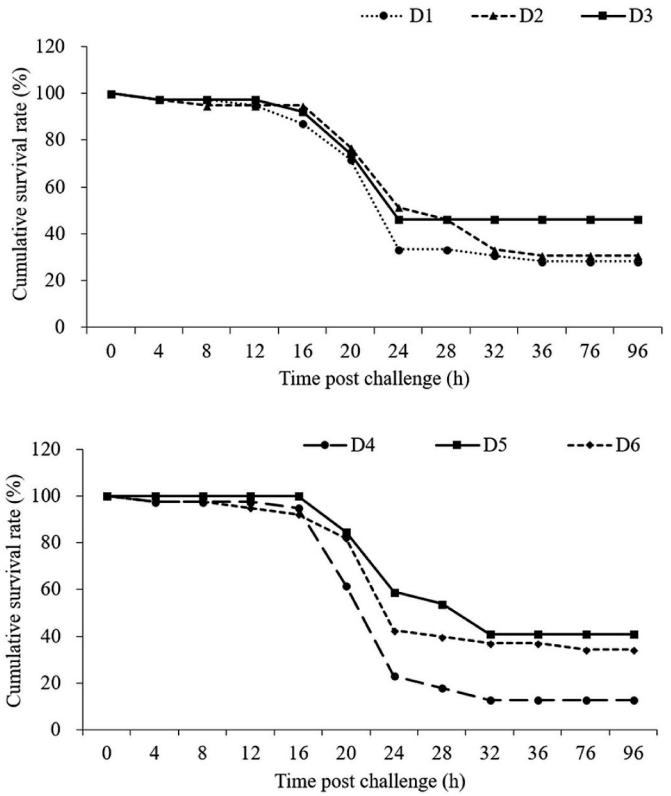


Fig. 5. Effects of dietary administration of 5′-IMP on post-challenge survival of gibel carp from high fishmeal group and high soybean meal group after infection with *A. hydrophila*. The cumulative survival rate of gibel carp were recorded and analyzed. The equation is expressed as survival rate (%) = (final number of fish survivor/initial number of inoculated fish).

monocytes/macrophages and dendritic cells [43]. In Nile tilapia (*Oreochromis niloticus*), a *Streptococcus agalactiae* infection triggered significantly increased expression of MHC II β at 8 h in kidney and spleen, however, this gene was markedly depressed at 24 h, followed by a significantly improved expression at 48 h compared to non-infection group [44]. In addition, the highest expression of MHC II β in liver and blood were at 4 h and 16 h post-infection in spotted halibut, respectively [45]. In this study, the different changes of MHC II β and lysozyme C expression levels post-bacterial infection may be associated with tested time post infection and different tissues. Further studies are required to elucidate the order of the immune organs in response to *A. hydrophila* via intraperitoneal injection and immune related gene changes post infection over time.

Present results indicated that inclusion of 5′-IMP in high soybean meal diets exerted further effects of promoting immunity than counterparts in high fishmeal diets. In high soybean meal group, three pro-inflammatory cytokines (IL-1β, TNF-α1 and IL-8) were significantly up-regulated by dietary 5′-IMP before and after infection in spleen and kidney. However, in high fishmeal group, IL-1β and IL-8 were only significantly up-regulated after infection in spleen and kidney. In addition, it is interesting to note that in high fishmeal group, the gap of the survival rate between D1 treatment (28.20%) and D3 treatment (46.15%) was 17.95% 96 h at post challenge. Whereas the gap of the survival rate between D4 treatment (12.83%) and D5 treatment (41.03%) in high soybean meal group was 28.2%. Accordingly, the number of immune genes significantly affected by dietary 5′-IMP in spleen after infection in high soybean meal group was also higher than that in high fishmeal group. The difference in effect of promoting immunity might be partly attributed to much higher concentration of 5′-IMP in fishmeal (3.5 mg/100 g) than that in soybean meal (0.2 mg/100 g) [46], which was consistent with our results of 5′-IMP contents on dry matter basis in D1(0.06%) and D4 (0.02%). The promoted immunity was also partly attributable to relative high values of methionine and taurine and absence of anti-nutritional factors in fishmeal [47–50]. Simultaneously, these results also demonstrated that 5′-IMP could be supplemented in gibel carp diet as a functional nutrient. Our findings were in agreement with previous study that in juvenile amberjack (*Seriola dumerili*), fishmeal protein was replaced gradually by soybean meal at 25%, 50% and 75% levels, each replacement level was supplemented with or without 0.6% inosine, they found that serum activities of lysozyme, bactericidal and alternative complement pathway were positively influenced by dietary inosine supplementation compared to non-supplemented treatments at each replacement level [51].

In addition, the improved immune gene expression was more significant due to addition of dietary 5′-IMP in high soybean meal group, because of the low 5′-IMP content in high soybean meal diets compare with high fishmeal diets. It is notable that relative expression of TNF-α1 was significantly improved by dietary 5′-IMP in liver, spleen and kidney of gibel carp both before and after bacterial infection, IL-1β and IL-8 mRNA levels were remarkably up-regulated by 5′-IMP in spleen and kidney, in liver and spleen, respectively. J. Roca et al. [52] have demonstrated that TNF-α improved susceptibility of zebrafish against bacterial (*Streptococcus iniae*) infection. Because TNF-α is a potent pro-inflammatory cytokine, which is involved in recruitment and activation of phagocytes during infection [53]. This perfectly explained why fish in D5 have a higher expression level of TNF-α1 in liver, spleen and kidney possessed a higher survival rate following bacterial infection. IL-1β mainly released by monocytes and macrophages plays a vital role in inflammatory response [54]. In *vitro*, it has been proved that pro-inflammatory TNF, IL-1β and IFN-γ increased nitric oxide synthase in

cultured human endothelial cells [55]. It was also illustrated in review that three proinflammatory TNF-α, IL-1β and IL-8 could stimulate inducible nitric oxide synthase [39]. More importantly, the reactive nitrogen intermediates including nitric oxide are known to inhibit pathogens such as bacteria and parasites [56]. Furthermore, it is reported that nitric oxide possesses property to inhibit the bacterial fish pathogen *Aeromonas salmonicida* [57]. These were agreed with results in the current study that fish fed 5′-IMP supplemented diets possessed higher survival rate after *A. hydrophila* challenge test. The increased expression level of these three proinflammatory cytokine by dietary nucleotide in the current study may be bound up with the increased nucleotide pool contributed by exogenous 5′-IMP. It was well documented that activation, overturn and proliferation of leukocyte would increase nucleotide requirement [58–60]. Furthermore, some studies have proved that total leukocyte count of Nile tilapia (*Oreochromis niloticus*) increased significantly from 1st day to 7th day after bacterial infection [61]. It is implied that more nucleotides are needed during infection. Further studies are necessary to elucidate the process via which pathway exogenous nucleotide affect the expression of the proinflammatory cytokine.

In summary, 5′-IMP could be successfully supplemented in gibel carp (*Carassius auratus gibelio*) diet at a dose of 0.2% in fishmeal-based formula and at a dose of 0.1% in soybean meal-based formula to achieve a significantly improved immunopotentiation compared with respective control treatment. Furthermore, more beneficial immune effects are exhibited when 5′-IMP was included as a function ingredient in high soybean meal diet compared with those of high fishmeal diets.

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